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PROTEIN NUTRITION IN GROWTH REGULATION
DURING EARLY DEVELOPMENT

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1. Introduction

The importance of nutrition in relation to the organized rapid growth of embryos has been for some time an essential consideration for the overall understanding of development. Yet today, the study of developmental nutrition has assumed greater importance than in the past. First, the observations that embryos developing in utero are dependent on the nutritional status of the mother and that nutritional inadequacy during development may cause irreversible brain damage, have stressed the importance of developmental nutrition as a major health consideration. Second, extensive studies with bacteria, cell cultures, and postnatal animals have in recent years clearly demonstrated that nutrition can be used as an invaluable tool to expose and investigate regulatory mechanisms. Pertinent examples are the "stringent-relaxed" system in bacteria (Ryan and Borek, 1971), "pleiotypic" regulation in cell cultures (Tomkins, 1972), and the control of protein and RNA "turnover" in the rat (Schimke, 1969, Enwonwu et al., 1971). In so far as many of these types of studies have demonstrated interactions between nutrition and hormones, it would appear that in systems which have poorly developed or nonfunctional endocrines, such as early embryos, nutritional alterations may be a particularly meaningful and useful tool to study mechanisms of regulation.

The objectives of this chapter are to consider the role of nutrition in the regulation of early embryonic growth, and to review the progress that has been made in certain areas of developmental nutrition as well as in the methodologies for providing nutritionally responsive developmental systems which are of potential value in the study of regulatory mechanisms. The selection of topics for this chapter reflect the interests of the author in

the areas of protein and amino acid nutrition during early development and are presented under five major headings. The first section is mainly concerned with attempts to culture chick and mammalian embryos on protein-free chemically defined media while the second section deals with the effects of proteinaceous supplements. The last three sections deal with investigations in which specific proteins and total protein were manipulated to further elucidate the relationship of protein utilization to embryo growth. The first is concerned with a series of three conditions in which the growth of embryos was greater on a mixture of two proteins or protein sources than on an individual protein or protein source. This is followed by a discussion centered on a study in which the quantitative pattern of protein utilization was followed for a single protein, ovalbumin. In the final part, the effect of protein deprivation on the growth and development of early embryos is considered with emphasis on regional differences in sensitivity to this condition and on the relationship of this sensitivity to the state of differentiation of a particular region.

It is hoped that this chapter will provide the reader with some appreciation of the fact that through an understanding of developmental nutrition, it has become possible to culture certain embryos under controlled conditions. Thus, it is now possible to study embryos in a manner previously restricted to bacteria and cells in culture. Embryos in vitro, however, provide a major advantage over bacteria and cell cultures in that analysis can be carried from the organism to the cellular and subcellular levels in seeking mechanisms of regulation. In spite of the advances that have been made, the reader is cautioned of the complexities in studying whole embryos. For example, the elaborate system of extraembryonic membranes which are

involved in the processes of nutrient uptake and transport as well as in nutrient modifications play an important role in the growth and development of an embryo and cannot be ignored. Indeed, in considering protein nutrition, the requirements of the extraembryonic membranes for these processes may be of major importance in determining the ability of a particular protein to support growth and development.

II. The Requirement of Embryos for Nutrient Protein

In so far as proteins are synthesized from mixtures of individual amino acids, it is difficult to understand why early embryos may require intact proteins to provide these amino acids. However, some lines of experimentation suggest that this situation may exist. At first it may appear hard to reconcile the lack of definitive data with the fundamental nature of this question but the problem of isolating embryos free of residual nutrients and the difficulty of providing quantitative biochemical estimates of embryo growth are to a large extent responsible.

A. Chick Embryos

The successful culture of embryos on a protein-free chemically defined medium in which free amino acids are provided as the sole source of nitrogen, would provide the most direct evidence that embryos do not require protein. Spratt (1948), working with explanted chick embryos of definitive primitive streak to early somite stages, provided one of the initial attempts to culture embryos on a chemically defined medium. Although these now classical studies clearly demonstrated a requirement for glucose, the need for amino acids was not apparent. Thus, while amino acids appeared to influence the length attained by embryos, the ten amino acids employed in the defined medium could be replaced by simply providing sufficient levels of a single amino acid, lysine.

This response to a single amino acid suggests that amino acids were not being used as precursors for protein synthesis but contributed to embryo growth in some other manner (possibly by increasing the buffering capacity of the medium).

Hayashi and Herrmann (1959) working with chick embryo explants of 11 to 13 somites provided the first quantitative estimates of embryo growth on a defined medium. During 24 hours of culture, embryo growth as measured by DNA and protein glycine accumulation was not only slight on a defined medium containing amino acids but it also did not differ appreciably from growth on a glucose-saline medium. Additional experiments with explants of 11 to 13 somites have involved attempts to reduce residual nutrients which could interfere with the detection of a growth response to amino acids and to the critical requirement of demonstrating reduced growth when single essential amino acids were omitted from the medium. Although it was not possible to completely eliminate the presence of some residual yolk (as some is undoubtedly contained within the cells of the yolk-sac membrane) it was possible to minimize a contribution from this material by reducing the size of the yolk-sac membrane, thoroughly rinsing the explant prior to culture, and extending the culture period to 48 hours so that the supply of residual nutrient would be exhausted. When these precautions were followed it became possible to demonstrate for the first time that embryos could be grown on a protein-free medium (Klein et al., 1962). However, growth was minimal. Indeed, as improvements in the technique of culturing explanted chick embryos allowed for greater potential growth by extending survival to three days, the inadequacy of chemically defined media became clearly apparent when growth on this medium was compared to that achieved with either whole egg homogenate

medium (the contents of fresh egg combined with an equal volume of chick Ringer's-agar) or in ovo embryos of a comparable age (Klein et al., 1964) (Fig. 1). Nevertheless, the fact that the omission of either leucine or lysine from the chemically defined medium resulted in reduced embryo growth, demonstrated that the explanted chick embryo was highly responsive to nutritional manipulations.

Grau and his collaborators (see Grau, 1968 for review) have worked extensively with the technique of yolk-sac perfusion in attempts to grow embryos on chemically defined media and to investigate amino acid requirements. In so far as this procedure starts with older embryos than have usually been used in explantation (4 days as opposed to 2 days) and allows the embryos with their extraembryonic membranes to be maintained in proper relationship within the egg, embryo growth and to some extent survival has been greater with perfusion than has been obtained with explantation. However, because the egg white is not removed from the egg during yolk-sac perfusion and because of the difficulties associated with the removal of residual yolk by perfusion this technique has some limitations in studies of nutrition. Nonetheless, by careful consideration of these problems as well as such factors as total solute concentration (Grau et al., 1962), sodium, potassium, and calcium levels (Grau et al., 1963), the gaseous environment (Grau et al., 1965a), and particularly the relative concentrations of glucose and amino acids (Austic et al., 1966), it was possible to employ chemically defined media as a perfusion fluid and to study amino acid deficiencies. Tyrosine deficiency resulted in a reduction or absence of retinal pigmentation (Grau et al., 1965b) (Fig. 2) while deficiencies of phenylalanine, methionine, and tyrosine led to reduced embryo growth and degenerative changes in the eyes and central

nervous system (Austin and Grau, 1971). In addition, the omission of lysine and valine from a chemically defined perfusion medium led to reduced embryo survival (Austin et al., 1966). Although it has been possible to demonstrate these amino acid requirements, it should be clearly noted that in studies with yolk-sac perfusion as with explants embryo growth on chemically defined media has been limited relative to control embryos or embryos provided with egg nutrients.

Comparisons of the utilization of radioactively labeled precursors has also been used in an attempt to determine the preferred precursor for protein synthesis. Walter and Mahler (1958) injected ^{35}S -labeled amino acids, peptides, and proteins into the yolk of unincubated eggs and determine the amounts of ^{35}S present in embryo proteins between days five and nine of incubation. Although the amounts of ^{35}S incorporated were comparable for the different precursors, these investigators concluded that proteins rather than amino acids or peptides were the preferred precursors. This was based on the assumption that the greater concentration of protein in the yolk relative to amino acids or peptides would lead to a dilution that was greater for the injected proteins than for either the injected amino acids or peptides. In a second study (Hayashi and Herrmann, 1959) the incorporation of ^{14}C -glycine was compared in explanted chick embryos of 11 to 13 somites cultured under conditions of rapid growth (a medium containing fresh yolk and white) and limited growth (a chemically defined protein-free medium). The protein specific activities of embryos cultured on the limited growth medium were considerably higher than those derived from embryos on rapid growth medium. This apparent discrepancy between embryo growth and protein specific activity was interpreted to indicate that when proteins were provided

in the medium they were preferentially utilized over free amino acids. Although more recent observations have added support to the conclusion reached in the above two studies, the assumptions that were made have been criticized. For example, in reference to the study of Walter and Mahler it has been pointed out (Grau et al., 1962) that soluble proteins when injected into yolk would not necessarily be diluted by the proteins present because yolk proteins are inert relative to soluble proteins. In addition, in a study to be discussed (Hassell and Klein, 1971), a large pool of free amino acids derived from nutrient protein breakdown was detected in the yolk-sac membrane. Thus, in the study of Hayashi and Herrmann the lower protein specific activities of embryos cultured on growth medium could be attributed to the dilution by yolk-sac free amino acids rather than a preferential utilization of protein.

The avian egg yolk does contain free amino acids (Williams et al., 1954; Ducay et al., 1960; Fitzsimmons and Waibel, 1968) as well as proteolytic enzymes (Lineweaver et al., 1948; Emanuelsson, 1955; Ito, 1957) capable of replenishing the pool of free amino acids by degrading yolk proteins. In so far as radioactively labeled amino acids injected into yolk are readily incorporated into embryo proteins, it is difficult to exclude the possibility that the free amino acids in yolk are utilized by the embryo. However, the presence of amino acids and proteolytic enzymes does not necessarily indicate that free amino acids are quantitatively significant contributors to embryo growth or that they are essential nutrients.

B. Mammalian Embryos

The many conflicting reports in the literature make it difficult at present to clearly ascertain if the pre-implantation stages of mammalian

embryos can be successfully cultured on protein-free chemically defined media or alternatively if mammalian embryos at this early stage contain sufficient endogenous nutrients to grow and develop in the absence of an external source of nitrogen. In attempting to evaluate the literature relevant to these questions such problems as variations between and within species, the lack of meaningful quantitation, and the use of small numbers of embryos become strikingly apparent. Daniel (1971) and Brinster (1972) have commented on these difficulties.

Pushotten and Pincus (1961) reported that rabbit embryos of two and eight cells grew and developed for four days on a protein-free chemically defined medium containing amino acids. However, early blastocysts (72 hour embryos) required an additional medium supplement of 10% serum for successful culture. Daniel and Krishnan (1967) working with somewhat older rabbit blastocysts (five days) observed that they expanded to a greater extent for the first 36 hours of culture when the defined medium was supplemented with serum, but by 48 hours of culture expansion was comparable on media with or without serum. In this same paper, ten amino acids appeared essential for expansion while subsequently (Daniel and Olsen, 1968) it was reported that six amino acids were required for the second cleavage division and nine for subsequent divisions. In so far as it was possible to demonstrate specific amino acid requirements and show that serum did not enhance blastocyst expansion over long periods of culture, it would appear that the early rabbit embryo does not require protein. However, in subsequent publications, Daniel and co-workers employed serum supplemented media (Daniel, 1968; Manes and Daniel, 1969) and state (Gulyas et al., 1969) that rabbit blastocysts "grow better" when a chemically defined medium was supplemented with serum.

Kane and Foote have also worked on the nutrition of the early rabbit embryo. They reported (Kane and Foote, 1970a) that embryos progressing from the morula to the blastocyst stage require three and possibly five amino acids in addition to a protein supplement such as bovine serum albumin. Bovine serum albumin without added amino acids was sufficient for embryos to develop from the two and four cell stage to morula. In spite of these observations it should be clearly noted that blastocysts did not form from two or four cell embryos when the medium contained a complete mixture of amino acids but lacked bovine serum albumin (Kane and Foote, 1970b). Finally, an additional worker (Brinster, 1970) has presented data suggesting that single amino acids such as glutamine, proline, or alanine will support the development of the rabbit embryo from two cell to morula when these amino acids are provided as the only metabolizable nutrient in the medium. Thus, from the work of Pushotten and Pincus, Kane and Foote, and the more recent observations from Daniel's group, it would appear that proteins may be essential or at least beneficial for the cultivation of early rabbit embryos to blastocysts. On the other hand, the development of cleavage stages up to morula can be supported by individual amino acids or mixtures of amino acids in the absence of a protein supplement.

Working with mouse embryos Pushotten and Pincus (1961) presented data comparable to that which they had obtained for the rabbit. Two and eight cell stages could be cultured up to morula on a defined medium in the absence of serum but for morula to differentiate into blastocysts serum appeared essential. Brinster (1965) observed that a source of nitrogen was essential for the development of the two cell mouse embryo to the blastocyst stage. This requirement could be met by providing either bovine serum albumin or a mixture

of free amino acids in conjunction with the polymer polyvinylpyrrolidone. In this study, it was not possible to demonstrate an inhibitory response to the omission of single amino acids with the possible exception of cystine but of greater significance, development in the presence of bovine serum albumin was superior to that obtained with a complete amino acid mixture. Two subsequent papers by this investigator were particularly difficult to interpret in view of these observations and the previously discussed studies with mouse and rabbit embryos. First, (Brinster, 1968) it was found that glutathione as the sole source of nitrogen would support the development of the two cell mouse embryo to blastocyst. Second, (Brinster and Thomson, 1966) it was reported that the eight cell embryo would develop into a blastocyst on glutamate, proline, pyruvate or oxalacetate as well as on bovine serum albumin when either one of these nutrients were provided as the sole source of carbon. This would suggest that the eight cell mouse embryo does not require a source of nitrogen. Although it is difficult to understand why the two cell but not the eight cell embryo would have such a requirement, it is particularly interesting to note that the two cell mouse embryo has been successfully cultured to blastocyst in the absence of a nitrogen source (Cholewa and Whitten, 1970). Furthermore, in this latter study it was possible to transfer blastocysts formed in culture into suitably prepared females and obtain a high percentage of successful births. This clearly indicated that the two cell mouse embryo does not even require a source of nitrogen in order to develop into a blastocyst. In support of this, it has been reported (Brinster, 1967) that from the one cell to morula stage, a period of three days of in vivo development, the mouse embryo loses approximately 25% of its protein content. This suggests that endogenous stores of nutrient play an

important role during the early development of this embryo.

In most studies the media used to culture post-implantation stages of mammalian embryos have contained relatively large amounts of serum. However, in one excellent study, quantitative growth measurements were made of one to twelve somite mouse embryos cultured for 22 hours on a defined liquid medium with and without serum supplementation (Clarkson *et al.*, 1969). The growth of embryos (Table I) on the chemically defined medium was inferior to that obtained when this medium was supplemented with 20% rat serum. Levels of either 10% or 100% serum did not support embryo growth as well as the 20% level. Although a similar comparison has not been reported, it should be noted that in his extensive studies New (New, 1966; New, 1967; New and Daniel, 1969) working with post-implantation stages of the rat has extensively used media consisting of 100% serum. Despite the fact that serum contains many constituents which may make important contributions to the physical as well as nutritional qualities of the culture medium, these observations seem to indicate that the proteins of serum serve as important nutrients for embryos at this stage of development.

As previously mentioned, neither the incorporation of radioactive amino acids into embryo proteins nor the presence of amino acids in the immediate environment of the embryo necessarily indicate that free amino acids contribute significantly to the nutritional needs of the embryo. Nevertheless, it should be noted that Mintz (1964) employing autoradiography observed in the cultured mouse embryo an increase in amino acid incorporation from fertilization to the blastocyst stage. This observation was essentially confirmed (Brinster, 1971) for the mouse when quantitative procedures for the analysis of radioactivity were used. During cleavage of the rabbit embryo

in vitro (Manes and Daniel, 1969), amino acid incorporation increased with development when the data were expressed on a per embryo basis but actually decreased when expressed per cell. Incorporation of amino acids into protein by rabbit blastocysts in vitro was shown to be influenced by the presence of proteins from uterine fluid in the culture medium (El-Banna and Daniel, 1972a) and by progesterone (El-Banna and Daniel, 1972b). In the cultured mouse blastocyst, estrogen may also stimulate amino acid incorporation (Smith and Smith, 1971). In regard to this latter point, Weitlauf (1971) has presented some evidence that in the intact mouse, estrogen may indirectly stimulate incorporation by the embryo by acting on the uterus. Finally, Gwatkin (1969) has detected free amino acids in the lumen of the mouse uterus and observed that their level was high the first day after mating as a result of the presence of semen, dropped to the third day after mating, and then gradually increased to the sixth day. It is interesting that the protein content of the lumen followed a similar pattern.

III. The Requirement of Embryos for Specific Nutrient Proteins

As has been noted, protein-free media containing defined mixtures of nutrients differ in many ways from complex media such as those containing serum or the whole egg homogenate medium used for the explanted chick embryo. Obviously, the differences are not limited to the fact that the complex media contain protein. However, the proteins have received more extensive consideration than the other components for both embryo and cell cultures. In general, it appears that proteins as media constituents may serve as a source of amino acids, as protective agents, or as biologically active macromolecules. The distinctions between these three possible functions are

frequently difficult to make and it would seem possible for a single protein to simultaneously contribute to all three activities. Alternatively, a protein may serve different functions depending on the requirements of a particular biological system or experimental condition. For example, the value of serum as an inhibitor of proteolytic enzymes (Wallis et al., 1969) may be limited to the condition of cells in culture.

A. Chick Embryos

The whole egg homogenate medium has supported the growth of chick embryo explants to a greater extent than has been achieved with other media. For this reason, the whole egg medium has been used routinely as a basis for comparison with other nutrient mixtures. The large difference between the whole egg and chemically defined media in total amino acid nitrogen (60 to 80 mg of protein per ml as opposed to 1.69 mg of amino acid per ml) provided the basis for the initial attempts to overcome the growth supporting difference between these media (Klein et al., 1962). When the level of amino acids was increased above 1.69 mg per ml, embryo growth was not improved and the medium appeared toxic. Thus, in order to compare the chemically defined and whole egg media at comparable levels of amino acid nitrogen, the whole egg medium was diluted to 2.5% (Klein, 1968). In this study an attempt was made to maintain medium constituents other than amino acids as similar as possible by supplementing the 2.5% whole egg medium with the chemically defined medium constituents less the amino acids. After 48 hours of culture, embryo growth as measured by protein nitrogen and DNA accumulation was actually slightly lower on the diluted whole egg mixture than on the defined medium. This suggested the possibility that proteins were essential for rapid growth only because they could provide high concentrations of amino acids in a form that

could be tolerated.

In his excellent review, Williams (1967) stated that there was no "real evidence" that the proteins of egg yolk provided more to the developing organism than amino acids. The study in which embryo growth was compared on diluted whole egg and chemically defined media supported this contention. Additional support could be derived from the successful culture of embryos on media containing proteins or protein mixtures of adequate amino acid composition obtained from sources other than yolk. The literature would suggest that this has been achieved in that the in vitro culture of chick embryo has been reported with such diverse media as plasma (Waddington, 1932; Spratt, 1942), sera (Gallera and Nicolet, 1961), embryo extract (Spratt, 1942; Wolff and Simon, 1955), egg white (Spratt, 1947; Reporter and Ebert, 1965), yolk (Spratt, 1947), and mixtures of egg yolk and white (Britt and Herrmann, 1959; Spratt and Haas, 1960). However, it should be clearly noted that the relative ability of these various media to support embryo growth could not be compared because most of these studies lacked quantitative determinations of growth.

When the growth of embryos was quantitatively evaluated, large differences were observed in the response of embryos to various protein sources (Table II) (Klein, 1968). The accumulation of protein nitrogen during a 48 hour culture period was greatest for embryos provided with whole egg homogenate medium (a mixture of yolk and white). This was closely followed in decreasing order by yolk alone (buffered with NaHCO_3), a mixture of horse serum and ovalbumin, and egg white. Ovalbumin alone was intermediate in that embryo growth on this protein was lower than the previously mentioned four sources of protein but considerably greater than the more limited growth

achieved with sub-blastodermal fluid, bovine colostrum, horse serum, or bovine casein. Avian embryo extract, bovine milk, and bovine serum albumin failed to support embryo growth to any extent. (It was interesting to note that the in vitro growth of mammalian embryos did not appear enhanced by embryo extract [Brinster, 1972]). Sera from the horse, hen, rooster, rabbit, and chicken (rooster-hen mixture) were compared both alone and in combination with ovalbumin. The differences between the various sera were generally small and were not consistent when compared with and without ovalbumin. Thus, chicken serum supported the highest embryo protein nitrogen levels when tested alone but horse serum gave the highest values when combined with ovalbumin.

In this study an attempt was made to minimize variations in embryo growth which could be attributed to the non-protein components of the media. The sources of protein were tested at various concentrations, as well as with and without amino acid supplementation in order to overcome differences due to the amino acid composition of the proteins. Five different procedures for providing the low molecular weight supplementation were tested: (1) supplementation directly with the salts of chick Ringer's; (2) water dialysis of the protein source plus supplementation with the components of a chemically defined medium; (3) water dialysis of the protein source plus supplementation by equilibration against whole egg homogenate; (4) water dialysis of the protein source plus supplementation by equilibration against whole egg homogenate plus the components of a chemically defined medium; and (5) water dialysis of the protein source plus supplementation with the components of salts "26" (Grau et al., 1963) and the components of a chemically defined medium. Equilibration against whole egg was accomplished by placing the protein source into a dialysis bag which in turn was placed into a large test

tube containing fresh whole egg homogenate. The tubes were placed on a rocking platform device and allowed to oscillate for five days. A solution of the non-nutritive polysaccharide, Dextran, served as a control for the equilibration process as well as for embryo growth attributable to endogenous nutrients. Equilibrated Dextran supplemented with ovalbumin supported embryo growth to the same extent as ovalbumin equilibrated directly. The data presented in Table II represented the greatest growth achieved with a particular protein source regardless of the supplementation procedure employed. But, it should be noted that all protein sources did not support the highest levels of embryo growth with the same form of supplementation. For example, with whole egg homogenate method 1 gave the greatest embryo growth, with egg white and ovalbumin method 3, and with sera method 4.

If the procedures that were employed to overcome differences in the non-protein components of the media could be considered adequate, the data indicated that proteins derived from yolk or from the egg in general have unique properties to meet the needs of an embryo for rapid growth. Thus, the greatest growth was achieved on media containing yolk followed by media containing either egg white or the major protein of egg white, ovalbumin. These observations may indicate therefore that the proteins of yolk provide some factor or factors other than amino acids to the embryo, and that the proteins of egg white share a common factor(s). Alternatively, these results may indicate that, rather than providing some unknown factor, the proteins of egg are uniquely suited to provide amino acids. For example, egg proteins may be more readily taken up by cells of the embryo than proteins from other sources or they may serve more readily as substrates for the proteolytic enzymes of the embryo.

Several lines of investigation (Rupe and Farmer, 1955; Walter and Mahler, 1958; McIndoe, 1960; Saito and Martin, 1966; Carinci and Manzoli-Guidotti, 1968) have indicated that the chick embryo in ovo relies on the proteins of egg yolk during the first week of incubation. Nevertheless, egg white in combination with yolk was superior to yolk alone. This suggested that egg white may contribute in some non-nutritive manner to the explanted chick embryo such as by providing a more favorable physical substrate than that achieved with yolk alone. The difference observed in the growth response of embryos to ovalbumin and bovine serum albumin should also be noted. Ovalbumin but not bovine serum albumin supported embryo growth in spite of the fact that these two proteins have similar amino acid compositions, molecular weights and isoelectric points. This suggested a high degree of specificity in the nutrient protein requirement of the early chick embryo. On the other hand, the enhanced growth achieved when ovalbumin was combined with serum indicated that egg proteins could be replaced to a limited extent.

Although studies as extensive as those discussed above have not been reported, the following should be noted. Spratt and Haas (1967) observed that the ability of parts of early chick blastoderms to form an embryonic axis (regulative and repair capacities) depended on the ability of the culture medium to support growth. In this study, extract of whole egg was found to be superior to extract of egg white. Grau et al. (1962) perfused four day chick embryos with bovine milk and obtained embryo survivals up to 103 hours. Casein was found to be the active ingredient in milk but these workers felt that this protein facilitated the utilization of endogenous nutrients rather than providing amino acids.

B. Mammalian Embryos

Although, as previously noted, Cholewa and Whitten (1970) reported the culture of pre-implantation stages of mammalian embryos in the absence of protein, these same workers noted that bovine serum albumin had a beneficial effect in that one cell mouse embryos will cleave more readily and the "hatching" of later stages appeared facilitated by this protein.

Furthermore, most culture media used for mammalian embryos have contained some source of protein. Nevertheless, only a few studies have been reported in which various protein sources have been compared and these studies have been limited to non-protein substitutes, sera from various animals, and uterine protein fractions.

In culturing the two cell mouse embryo to the blastocyst stage bovine serum albumin could be replaced by a mixture of amino acids in conjunction with such non-nutritive polymers as Polyvinylpyrrolidone, Acacia, Dextran and Ficoll (Brinster, 1965). This, together with the observation that ova become "sticky" in the absence of protein or a non-nutritive polymer, suggested that serum albumin provided amino acids and in some way acted to maintain the surface of the ovum in a normal state.

Bovine serum was actually found to be superior to rabbit serum in the culture of rabbit embryos from two and four cell stage to the blastocyst stage (Mauer et al., 1970). This was attributed to the difference in osmolarity between these sera. New (1966), on the other hand, achieved superior results with homologous as compared to heterologous sera but his studies involved rat embryos of a later stage of development. Working with static liquid cultures of 11 day rat embryos, rat serum was found to be superior to that of horse, sheep, calf, chick and rabbit in supporting embryo growth and development.

Sera from pregnant, non-pregnant, and male rats gave comparable results. Some heterologous sera were considered toxic. Exposing the toxic sera for 30 minutes to 55-56°C overcame the toxicity to some extent which suggested that complement was inactivated and that differences between sera in this system could have an immunological basis. In spite of pre-heating, it should be noted that New still considered heterologous sera unsatisfactory for embryo culture. In a previous study (New and Stein, 1964) in which mouse and rat embryos were cultured on clots of plasma and embryo extract, it was interesting to note, that development was comparable with embryo extract prepared from 13 day chick embryos or from the entire uterine contents of 17 to 19 day pregnant mice.

Subsequent to the above studies, New (1967) developed an elegant method for culturing rat embryos of 11 to 13 days in an apparatus which would allow the circulation of media. In this system homologous serum has been used exclusively as the culture medium. In attempting to work with younger embryos (7.5 to 8.5 days of pregnancy) extensive development was observed but many embryos did not form a functional circulatory system (New and Daniel, 1969). This appeared due to the failure of the lateral heart primordia to fuse into a single heart. In so far as embryos with double hearts had been experimentally produced by pressure, a similar mechanism was suggested to explain this observation. However, it was recently reported (Steele, 1972) that the use of homologous plasma or "true" serum in place of standard serum allowed the formation of a single heart and significantly increased embryo protein accumulation. Although serum contains considerably more potassium than "true" serum or plasma, the addition of potassium to "true" serum did not lead to the development of embryos with double hearts. Hopefully, future

studies will elucidate the basis for this highly specific effect of nutrient media on this particular developmental process.

The inability to obtain in vitro development of rabbit embryos beyond the early blastocyst stage led Krishnan and Daniel (1967) to examine the effect of uterine proteins on rabbit embryos in culture. Employing analysis by gel filtration, a new protein was observed to appear in the uterine fluid at the time of blastulation. This protein was not detected in the maternal serum, fetal serum or fetal amniotic fluid but was present in the blastocoelic fluid of 6 day embryos. When this protein was added to the culture medium it appeared to enhance blastulation and blastocyst expansion when compared to comparable concentrations of protein from maternal serum. The protein, termed blastokinin, was found to have a molecular weight of approximately 27,000 and contained glucosamine, galactosamine, mannose, galactose, glucose, and fucose but lacked sialic acid (Krishnan and Daniel, 1968). In a more recent publication (El-Banna and Daniel, 1972a) six protein fractions were isolated by gel filtration from the uterine fluid of rabbits of 3 and 5 days of pregnancy and tested as media constituents with 3 day morulae and 5 day blastocysts. Measurements were made of thymidine, uridine, and amino acid incorporation as well as blastocyst expansion. Although the results are presented with considerable reservation, blastokinin stimulated uridine and amino acid incorporation as well as growth while the activities of the other fractions were more restricted. For example, fraction 1 stimulated uridine and amino acid incorporation but not growth or thymidine incorporation. Fraction 3, on the other hand, stimulated growth and thymidine incorporation but not uridine or amino acid incorporation. The authors stressed that their results represent general trends and indeed, quantitative data were not

presented. Nevertheless, it is difficult to understand the discrepancy between incorporation of precursors into macromolecules and growth unless the incorporation of precursors was not related to macromolecular synthesis in this system. Obviously, additional measurements of such factors as precursor pools and the breakdown of RNA and protein must be considered before these interesting observations can be fully appreciated.

It should be noted that Foote and his associates have taken exception to the significance of blastokinins as well as uterine proteins in general. Thus, Kane and Foote (1971) starting with one cell rabbit embryos obtained expanded blastocysts on a defined medium supplemented with only bovine serum albumin. Furthermore, it was reported that it was possible to obtain one full term young from a rabbit embryo transferred into a recipient doe after 88 hours of culture on a medium containing only serum (Mauer et al., 1970).

IV. Nutrient Protein Complementation

A. Ovalbumin and Serum

The first demonstration that a combination of proteins or sources of protein would support a higher level of embryo growth than could be achieved when these materials were individually provided in the medium was made with ovalbumin and serum (Klein, 1968). The combination of these two proteinaceous materials was not fortuitous but was based on the observation that serum and ovalbumin supported different parts of the chick embryo explant system. Thus, serum alone supported the development of a large and highly vascular extraembryonic yolk-sac membrane with a relatively small embryo. On the other hand, with ovalbumin the embryo was large but the membrane was small and poorly vascularized. In combination, serum and ovalbumin supported embryo growth comparable to that achieved on whole egg homogenate medium with a

somewhat larger extraembryonic membrane. The growth values in terms of protein nitrogen content after 48 hours of culture for the embryo and membrane, respectively, in ug per embryo or membrane were for serum (horse) 21.6 and 84.0, ovalbumin 33.2 and 69.9, serum plus ovalbumin 43.6 and 112.5, and whole egg homogenate 49.4 and 90.8. Testing various serum fractions (Fitzsimmons and Klein, 1966) suggested that the active component was associated with the globulins rather than the albumins which might be expected from the observation that bovine serum albumin not only failed to support the growth of the embryo proper but the extraembryonic membrane as well (Klein, 1968). Studies with the protein synthesis inhibitor, puromycin, and the studies to be discussed on ovalbumin utilization, have suggested that the high levels of protein nitrogen in membranes of serum cultured explants were to a large extent the result of nutrient protein uptake rather than a stimulation of protein synthesis. However, the actual extent to which the protein content of the extraembryonic membrane of serum cultured explants represented serum proteins and proteins synthesized by the membrane from amino acids provided by the breakdown of serum proteins has not been determined. Such values have been obtained for ovalbumin and will be discussed in a subsequent section. Although the nutrient media considered above influenced the protein content of the membrane, the relative constancy of membrane DNA content suggested that cell proliferation was not effected.

The differences in the response of explants to serum, ovalbumin and bovine serum albumin should be clearly noted. Serum supported the growth and development of the extraembryonic membrane and not the embryo proper, ovalbumin supported the embryo proper but not the membrane, and bovine serum albumin failed to support the embryo as well as membrane. This suggested

first that the nutritional requirements of the membrane were distinct from those of the embryo proper. Second, it appeared that although proteins could be taken up by the membrane, as with serum, they may lack the capacity to support embryo growth. Third, the process of protein uptake appeared specific to the extent that proteins as similar as bovine serum albumin and ovalbumin could be distinguished by the extraembryonic membrane.

Gross observations of explants cultured on serum and ovalbumin suggested that nutritional factors could also influence the relative growth of embryo regions. This was supported by quantitative measurements of the DNA, RNA, and protein content of isolated embryo regions following 48 hours of culture on horse serum or ovalbumin containing media (Table III) (Klein, 1972). The brain region of explants cultured on ovalbumin contained a higher proportion of the total embryo DNA, RNA and protein than did the brains of embryos cultured on serum. Therefore, the regions composing the trunk (note particularly the DNA of the somites) of serum cultured embryos contained proportionately more of the total embryo DNA, RNA, and protein than did the trunk regions of ovalbumin cultured embryos. Although the mechanism by which these nutrients affected regional growth have not been elucidated, comparisons with explants cultured on various dilutions of whole egg homogenate medium have indicated that the results obtained with serum and ovalbumin were not simply due to differences in overall embryo growth.

B. Ovalbumin and Conalbumin

Two proteins of the avian egg white, ovalbumin and conalbumin, provided a second example of nutrient protein complementation. In the previously discussed studies with ovalbumin, this protein was either obtained from a commercial source or prepared by the classical salt precipitation procedure

(Kekwick and Cannan, 1936). For the study on ovalbumin utilization to be discussed in the following section, ovalbumin was prepared by column chromatography on carboxymethyl cellulose (Rhodes et al., 1958) which provided this protein in greater yields and purity. Purified ovalbumin unlike ovalbumin prepared by salt precipitation would not support embryo growth (Hassell, 1973). An acrylamide gel electrophoretic comparison of the two protein preparations indicated that the ovalbumin prepared by salt precipitation unlike the ovalbumin prepared by chromatography contained the protein conalbumin. By combining various individual egg white protein fractions with ovalbumin, it was possible to demonstrate embryo and membrane growth promoting properties with conalbumin and a few of the other fractions. Conalbumin was selected for additional studies because it could be obtained in large quantities as well as in a highly purified state. The accumulation of protein nitrogen by the embryo and extraembryonic membrane after 48 hours of culture was determined for various combinations of ovalbumin and conalbumin (Table IV). The protein nitrogens of the embryo proper of explants cultured on 100% ovalbumin and 100% conalbumin were low and comparable. A medium in which the protein consisted of 75% ovalbumin and 25% conalbumin supported levels of embryo growth which were high and similar to that previously obtained with serum plus ovalbumin or whole egg homogenate medium. The protein nitrogen accumulated by the membrane, unlike the embryo proper increased with increasing amounts of conalbumin. Thus, the effect of conalbumin on chick embryo explants appeared similar to that obtained with serum. Attempts to determine the basis for the complementation of ovalbumin and conalbumin have not been successful. The iron bound to conalbumin did not appear significant in so far as the removal of iron from conalbumin did not adversely influence

its ability to complement ovalbumin. Furthermore, conalbumin could not be simply replaced by FeSO_4 . Attempts to replace conalbumin with amino acids were also unsuccessful suggesting that conalbumin did not simply provide amino acids which could possibly be deficient in a medium containing only ovalbumin.

C. Lipovitellin and Livetin

A third example of nutrient protein complementation was observed in the course of a comprehensive analysis of the ability of various yolk proteins and yolk protein fractions to support embryo growth and development (Yager and Klein, 1973). In the initial experiments the procedure of Bernardi and Cook (1960) was used to separate fresh yolk into a high density and low density fraction. Employing various procedures to supply the low molecular weight supplement, as previously discussed (Klein, 1968), the high density fraction but not the low density fraction supported embryo growth and development. In fact, during 48 hours of culture embryo growth on the high density fraction was comparable to that achieved with the whole egg homogenate medium. By a sequence of salt dilutions (Bernardi and Cook, 1960) the high density fraction was separated into lipovitellin, livetin, and phosvitin subfractions. Some preparations of lipovitellin supported high levels of embryo growth. However, the variation in response to different preparations of lipovitellin led to the use of a preparatory procedure starting from yolk granules which would provide lipovitellin in a relatively pure state (modification of the procedure of Burley and Cook, 1961). Purified lipovitellin supported considerably less growth than had occasionally been achieved with the crude preparation. Thus, known amounts of the livetin and phosvitin fractions were combined with purified lipovitellin in an attempt to restore

its growth promoting properties to that of the crude lipovitellin preparation or the high density fraction. The relative amounts of livetin and phosvitin used in the media were derived from the reported composition of the high density fraction (Bernardi and Cook, 1960). Lipovitellin in combination with the livetin fraction supported a level of embryo growth which was approximately twice that achieved on lipovitellin alone and comparable to that obtained on the high density fraction (Table V). Phosvitin did not improve embryo growth when added either to lipovitellin alone or to the combined lipovitellin and livetin fractions. It should be clearly noted (Table V) that the protein nitrogen content of the membrane was greatly enhanced when the livetin fraction was combined with lipovitellin.

Two additional observations concerned with the complementation of lipovitellin and the livetin fraction were pertinent. First, conalbumin but neither ovalbumin nor amino acids successfully replaced the livetin fraction when combined with lipovitellin. Second, although the lipovitellin and phosvitin fractions appeared on polyacrylamide gel electrophoresis to be quite pure, the livetin fraction consisted of approximately twelve different proteins. Subfractionation of the livetin fraction by carboxymethyl cellulose provided six fractions of which two were essentially as active as the complete livetin fraction. One of these two "adjacent" fractions consisted of a mixture of proteins while the second appeared to be yolk transferrin according to various characteristics that have been published (Williams, 1962 a and b).

The study by Walker (1967) in which the yolk of four day chick embryos was replaced by various yolk fractions provided an interesting comparison to the above observations and represented the only additional study dealing with the direct effect of yolk fractions on the early embryo. In these studies the

yolk was removed after three days of incubation by the method of yolk-sac perfusion and replaced on the fourth day with a single injection of nutrients. After four additional days of incubation embryos were weighed, their extraembryonic membranes were examined, and survival was recorded. A comparison of the three fractions analyzed in this study with yolk at equal levels of nitrogen indicated that embryo growth on supernatant plasma and the floating fractions was comparable to yolk and somewhat greater than that achieved with the granule fraction. Survival, however, was greatest with the granule fraction, intermediate with the floating fraction, and poorest with the supernatant plasma fraction. The development of the extraembryonic membrane (mainly allantois) and circulatory system provide still an additional sequence of fractions. The supernatant plasma supported membrane development which most closely approximated the yolk controls, the granules were intermediate, while with the floating fraction the extraembryonic membrane was small, poorly developed and anemic. Thus, it would appear that similar to the studies of Yager and Klein (1973) the various yolk fractions do exert different effects on the developing embryo. Furthermore, Walker (1967) indicated, similar to the observations of Yager and Klein (1973), that a combination of fractions complemented each other in supporting embryo growth and development.

Although differences in fractionation procedures do not permit a direct comparison between the studies of Walker, and Yager and Klein, certain similarities and differences should be clearly noted. The most striking similarity between these two studies was observed in the response of the extraembryonic membrane to the livetin fraction. In the studies of Yager with explants, the extraembryonic membrane accumulated a high level of protein

nitrogen and exhibited a well developed circulatory system in response to the livetin fraction. In the yolk replacement studies, the extraembryonic membranes responded favorably to the supernatant plasma but poorly to the floating fraction which was derived from the supernatant plasma by the removal of the livetin fraction. Another similarity between these two studies was the observation that the yolk granules failed to support high levels of growth. A major difference between those studies was in the response of embryos to the floating fraction and the low density fraction. Although these two fractions are not precisely comparable, the floating fraction studied in yolk replacement supported high levels of growth while the low density fraction failed to support the growth of explants. The deleterious effect of the floating fraction on the development of extraembryonic membranes and the circulatory system may provide a basis for this difference in that the less developed circulatory systems of explants may be more sensitive to the components of this fraction than the more advanced circulatory systems of the embryos studied by replacement.

Several attempts have been made to study the preferential utilization of yolk protein fractions by an analysis of changes in yolk during incubation. This approach is severely limited because the large mass of yolk relative to the size of the embryo has made it impossible to detect changes during the first two weeks of incubation, and after two weeks the passage of proteins from the white into the yolk-sac cavity further complicates analysis. Nevertheless, in contrast to the above studies, it was interesting to note that the granules rather than the low density or water soluble fractions were most rapidly and completely utilized between days twelve and eighteen of incubation (Saito et al., 1965; see also Biagi, 1964 a and b). In a second

study (Saito and Martin, 1966) the livetin fraction was followed and the α -livetin (comparable to serum albumin) appeared to be utilized more rapidly and completely than the other components of the livetin fraction. Again, this observation would not have been predicted from the more direct studies discussed above.

D. General Discussion

The several examples of nutrient protein complementation that have been cited have several features in common. Most striking, conalbumin (or ovotransferrin) of egg white and the transferrin of the yolk livetin fraction are both iron binding glycoproteins and appear to differ only in their carbohydrate prosthetic group (Williams, 1962b). These proteins contain the same amount of hexosamine, but conalbumin lacks sialic acid and contains less hexose than does yolk transferrin. As has been noted, both conalbumin and yolk transferrin stimulated the accumulation of protein by the extraembryonic membrane and the development of the circulatory system but neither protein supports a high level of growth for the embryo proper. Whole serum and the yolk supernatant plasma fraction also stimulated membrane development and contained a transferrin fraction. Thus, some form of transferrin may be essential for the development of the extraembryonic membrane and circulatory system. It was therefore of particular interest to note that Nace (1953) suggested that the yolk livetin fraction, which contains yolk transferrin, may serve as serum protein prior to the development of mechanisms by which the embryo can synthesize these proteins. This latter study was based on an immunological comparison of the sera from adult chickens and embryos with egg yolk.

The growth of the embryo proper clearly depends on the extraembryonic

membrane but a well developed membrane did not in itself lead to high levels of embryo growth. Indeed, this disparity between embryo and extraembryonic membrane growth was noted with serum, conalbumin and the yolk livetin and transferrin fractions. A protein such as ovalbumin or lipovitellin appears to be a necessary component in order to achieve favorable embryo growth. The inability to replace ovalbumin with bovine serum albumin suggests that this requirement has some degree of specificity. Although it is possible that these examples of nutrient protein complementation involve a non-protein essential metabolite bound to protein, the diversity of the various proteins which can provide complementation makes this appear improbable. The possibility that the proteins complement each other because of their amino acid composition also appears improbable because it has not been possible to replace a protein with an amino acid mixture. Although numerous possible explanations can be provided for the observed complementation, the following three examples may provide a basis for future study.

First, Ryser (1968) working with monolayer cultures of tumor cells demonstrated that the uptake of macromolecules as well as the ability of these macromolecules to stimulate the uptake of still other macromolecules was favored by high molecular weights and positive charge. However, the observations that homopolymers of D-lysine were more active than L-lysine and that arginine was more active than lysine indicated that there are factors other than charge and molecular weight which contribute to the specificity of macromolecular uptake. In the nutrient protein complementation studies, the higher molecular weight and isoelectric point of conalbumin relative to ovalbumin may indicate that the presence of conalbumin enhanced the uptake

of ovalbumin and therefore increased the total nutrient protein available to the embryo. Second, in a study with sea urchin coelomocytes (Hilgard et al., 1967), prior administration of bovine serum albumin blocked the subsequent uptake of labeled bovine serum albumin but did not appreciably reduce the uptake of bovine γ globulin. Washing the coelomocytes overcame the blockage to albumin uptake observed in those organisms which received a previous injection of albumin. These observations suggested that there were specific receptor sites on the surface of cells for the uptake of proteins. Thus, by providing more than a single nutrient protein to a developing embryo the total uptake of protein could possibly have been increased if the number of receptor sites for any one protein or protein type was limited. Finally, it has been shown that the specificity of glycoprotein uptake by rat hepatocytes was greatly influenced by the carbohydrate prosthetic group (Morell et al., 1971). By linking the glycopeptide of fetuin to bovine serum albumin and enzymatically removing the terminal sialic acid residues the uptake of albumin by hepatocytes was greatly increased (Rogers and Kornfield, 1971). Treating the protein lysozyme in a similar manner also increased the uptake of this protein. However, the inability to increase protein uptake by the use of the γ G immunoglobulin glycopeptide indicated some degree of specificity related to the glycopeptide. In reference to these observations, it was of particular interest that ovalbumin, conalbumin, and transferrin are glycoproteins and contributed to embryo growth. Bovine serum albumin, on the other hand, is not considered to be a glycoprotein (Putnam, 1965) and did not support embryo growth even when combined with other proteins. Thus, the carbohydrate group of a glycoprotein may contribute significantly to its ability to be taken up by cells and therefore to its ability to support growth.

Furthermore, from the studies mentioned above it appears feasible that the carbohydrate group of a protein could influence the distribution of a protein within a developing organism which in turn could act directly on the growth of a particular group of cells or region of an embryo.

The above discussion represents an attempt to provide some basis for the observations that embryos require nutrient proteins, that more than a single protein appears to be essential, that there is some specificity to this requirement, that nutrient proteins can influence growth and development, and finally that these nutrient proteins provide nothing more to the developing embryo than amino acids. Thus, the possible explanations were limited to mechanisms relating nutrient proteins to processes of uptake. However, it should be noted that proteins present in eggs can have a direct influence on development where problems of uptake are clearly not involved. For example, in the Mexican axolotl, an amphibian, a substance possessing various characteristics of a protein appeared absent or deficient in a mutant which was blocked from developing beyond the gastrula stage (Briggs and Cassens, 1966; Briggs and Justus, 1968; Briggs, 1972). When egg cytoplasm from normal axolotls was injected into mutant eggs, development continued beyond gastrulation while the reciprocal injection did not affect development of the normal egg. Although the mechanism by which this substance acts in the amphibian may be quite distinct from nutrient protein complementation in the chick embryo, these observations suggest that the heterogeneity of egg proteins is of significance to developmental processes and may not simply be the fortuitous result of diverse synthesis and non-specific deposition of proteins within the egg cell.

The responsiveness of cells in culture to serum and embryo extract and

to protein fractions derived from these materials continues to be an area of intense study (see review by Temin et al., 1972). Without attempting to review or summarize the extensive literature on cell culture, fractions of serum and embryo extract have been shown to influence such diverse phenomena as proliferation (Holley and Kiernan, 1968; Scher and Todaro, 1971; Paul et al., 1971), migration (Lipton et al., 1971), survival (Paul et al., 1971; Lipton et al., 1971) and the maintenance of the differentiated state (Coon, 1966; Cahn and Cahn, 1966; Spooner and Hilfer, 1971). Although the active components have not been rigorously defined, such observations as the ability to replace serum with insulin (Hershko et al., 1971), the importance of protein conformation in the serum growth promoting response (Tritsch et al., 1970), the selective utilization of serum proteins by different cell lines (Tritsch, 1967), and the ability to relate specific serum protein fractions with proliferation, survival, and migration suggest that proteins may indeed be involved. The observations with embryos discussed in this section suggest that embryos are similar to cells in culture in that they are particularly susceptible to regulation through nutritional factors. In the case of cultured cells, the importance of such factors may simply be related to the unique state in which these cells are placed and therefore may be artifactual (Ellison and Lash, 1971). This latter possibility would appear considerably less applicable to cultures of whole embryos.

V. Quantitative Analysis of Protein Utilization

In the preceding sections the importance of nutrient proteins in developing systems has been stressed. By following the fate of this class of nutrients within an embryo some insights might be gained into the basis of their significance. For some time in both mammalian (Brambell, 1958) and

avian (Schechtman, 1956) species immunological methods have been used to demonstrate the passage of foreign proteins or yolk proteins into the developing embryo. Thus, proteins of yolk have been detected in the embryo proper, area opaca, and area pellucida of the 15 to 17 somite chick embryo (Schechtman, 1947). Antibodies to bacteria pass from the hen to the egg and can be detected in the serum of the newly hatched chick (Buxton, 1952). Bovine γ globulin and serum albumin injected into eggs can be detected in the embryo and embryo serum after eight days of incubation (Schechtman and Knight, 1955). That the passage of proteins is a selective process was suggested by detecting the presence of circulating antibodies in hatched chicks when fowl sera was injected into the yolk-sac at six to eight days of incubation but not when sera from the rabbit, cow, or horse were handled in a similar manner (Brierly and Hemmings, 1956). Employing immunoelectrophoresis, the egg white protein, ovalbumin, was detected in the serum of embryos incubated for sixteen to eighteen days (Gitlin and Kitzes, 1967). In addition to these immunological studies, Bellairs (1963) interpreted her electron microscopic analysis of the yolk-sac membrane of the chick as indicating that yolk is transferred as small particles rather than large globules into the blood of the embryo and particles believed to be of yolk origin were detected in the presumptive neural ectoderm at the head process stage (Bellairs, 1958). Although these observations taken together clearly indicate that proteins can pass from the yolk to the embryo, the methods employed were not quantitative and therefore could not provide an estimate of the importance of protein transfer to embryo growth. In spite of this limitation, it has been generally accepted that proteins as intact macromolecules pass to the embryo in sufficient quantities to significantly contribute to embryo growth.

However, the detection of high levels of proteolytic enzyme activity in the yolk-sac membrane employing either hemoglobin (Emanuelsson, 1955) or casein (Ito, 1957) as substrates as early as four days of incubation appears incompatible with the passage of large quantities of protein through this structure.

The observation that chick embryos could be cultured with some success on a medium containing purified ovalbumin and conalbumin as the sole source of nitrogen, suggested that it would be possible to quantitatively follow the course of nutrient protein utilization (Hassell and Klein, 1971). In the three experiments presented in their report, chick embryos of 11 to 13 somites were either (1) cultured for various periods up to 48 hours on a medium in which the protein consisted of 90% by weight ^{14}C -ovalbumin and 10% unlabeled conalbumin (2) cultured for 24 hours on this same medium with ^{14}C -ovalbumin and then transferred to a medium containing unlabeled ovalbumin and conalbumin, or (3) cultured for 48 hours on a medium containing 10% ^{14}C -conalbumin and 90% unlabeled ovalbumin. The ^{14}C labeled ovalbumin and conalbumin used in these studies were isolated from eggs produced by a hen injected with ^{14}C -labeled amino acids (Hassell et al., 1970). Rabbit antiserum was prepared against ovalbumin but not conalbumin. After the desired culture period the embryo explants were dissected into brain, trunk, and area pellucida, and the area opaca was divided into an inner and outer region. The antibody-antigen precipitation reaction was used to determine the quantity of ovalbumin in a particular region. The amount of radioactivity soluble in 5% trichloroacetic acid provided the amount of ovalbumin present in a region as ovalbumin breakdown products. The quantity of ovalbumin converted to embryonic protein was derived by subtracting the value obtained for intact

ovalbumin from the total radioactivity in protein. Because the specific activity of the ovalbumin provided in the medium was determined, it was possible to convert the radioactivity observed into ug of ovalbumin as intact protein, breakdown products, or as ovalbumin converted to embryo protein.

In view of the large differences in the amounts of tissue contained in the various regions and the apparent presence of considerable quantities of yolk protein associated with the area opaca and pellucida, expressing the results on the basis of the DNA content of a region appeared more reasonable than the use of protein content. After 48 hours of culture the regions of the area opaca contained on the basis of DNA approximately eleven times as much intact ovalbumin as the regions of the embryo proper (Table VI). The area pellucida, however, contained an amount of ovalbumin which was only three times that of the embryo proper. Similar to intact ovalbumin, considerably more ovalbumin as breakdown products was contained in the area opaca than the embryo proper and the area pellucida contained an intermediate level. It was particularly interesting to note that for the embryo proper the amounts of ovalbumin as breakdown products exceeded the levels of intact ovalbumin at all periods examined during the 48 hours culture period. This was also the case for the area opaca during the last 24 hours of culture, however, during the first 6 hours the amounts of ovalbumin as the intact molecule exceeded that of breakdown products. In spite of the large differences between the embryo proper, the area opaca, and area pellucida in the levels of intact ovalbumin and ovalbumin breakdown products, the amounts of ovalbumin converted to newly synthesized proteins (embryonic proteins) were comparable for the various regions.

This experiment provided an overall picture of the distribution of

ovalbumin and products derived from this nutrient protein in the explanted embryo. The high levels of ovalbumin and ovalbumin breakdown products in the area opaca suggested that this was the major site of protein uptake and breakdown. This was in agreement with observations on the uptake of trypan blue and the distribution of acid phosphatase in chick embryos studied at 10 somites (Beck, 1965). In a study using colloidal gold and carmine particles (Bellairs and New, 1962) the most active region of uptake was found to be at the edge of the area opaca rather than the entire area opaca as had been suggested from the comparison of the inner and outer region of the area opaca in the ovalbumin studies. However, the embryos used in the gold and carmine study were only at the primitive streak stage and the materials used can not be considered comparable to soluble proteins. In view of the many immunological studies previously discussed, it was particularly striking to find a very low level of intact ovalbumin in the embryo proper. It was also striking to note the difference between the large pools of nutrient protein and protein breakdown products in the area opaca relative to the small pools of these materials in the embryo proper. Clearly, the area opaca and embryo proper are not in direct equilibrium with regard to these materials and therefore, the area opaca can not be considered a passive structure in nutrient transport.

The first experiment, although providing an overall view of nutrient protein utilization, did not permit an evaluation of the sequence of steps in this process. In the second experiment an attempt was made to approach this question. Embryo explants were cultured for 24 hours on a medium containing ^{14}C -ovalbumin and then transferred to a medium containing unlabeled ovalbumin. During the second 24 hour period the amounts of intact ovalbumin and ovalbumin

breakdown products per ug of DNA remained low and constant in the brain and trunk regions of the embryo proper. After an initial drop during the first 3 hours following transfer, the area pellucida followed a pattern similar to the embryo proper for the changes in ovalbumin and ovalbumin breakdown products. The ovalbumin pattern in the area opaca was, however, quite distinct from these other regions. The level of intact ovalbumin drop precipitously during the first 3 to 9 hours after transfer and then continued to drop off at a gradual rate to a very low level. On the other hand, the level of ovalbumin breakdown products remained at a high level during the transfer period and showed a gradual decline. The maintenance of a large pool of ovalbumin breakdown products in the area opaca while the levels of intact ovalbumin rapidly decreased, would suggest that the breakdown pool was maintained through the proteolysis of ovalbumin in this region. In view of this large pool of breakdown products in the area opaca and the low level of intact ovalbumin in the embryo proper, it would appear that the transfer of intact protein from the medium to the embryo did not play a significant role in the nutrition of the embryo. This statement is in agreement with the electron microscopic study of the yolk-sac by Lambson (1970) in which a structural basis could not be established for the passage of protein through the cells of the yolk-sac and into the circulatory system of the embryo.

These observations therefore suggested that nutrient proteins are mainly taken up by the area opaca of the yolk-sac and that these nutrients are broken down to constituent amino acids in this region. In view of the low levels of amino acids (ovalbumin breakdown products) in the embryo proper and the large pool of these nutrients in the area opaca, it would also appear that free amino acids as such are not transported in appreciable quantities

to the embryo. Although a gradual release of free amino acids from the area opaca coupled to a rapid utilization by the embryo can not be excluded, the synthesis of proteins by the membrane from nutrient protein followed by the transfer of these newly synthesized proteins to the embryo may provide a plausible alternative. In this regard, minces of the yolk-sac from eggs incubated for 16 to 18 days have been shown to synthesize several serum proteins (Gitlin and Kitzes, 1967) and radioactivity was lost from the proteins of the yolk-sac when embryos of 11 to 13 somites were exposed for a short period to labeled amino acids and subsequently cultured on growth medium (Klein et al., 1971).

Although the successful culture of chick embryos on a medium containing purified proteins made it feasible to attempt a study of nutrient protein utilization, it should be clearly noted that the proteins used, ovalbumin and conalbumin, are proteins of egg white. As previously mentioned, the proteins of egg yolk are used exclusively during the period of development employed in this study. Thus, the proteins of yolk could conceivably be utilized in a manner quantitatively distinct from ovalbumin and the studies with labeled conalbumin to be considered makes this a distinct possibility. A second important limitation of the ovalbumin studies was that they were restricted to embryos of 11 to 13 somites cultured for 48 hours (approximately from day 2 through 4 of incubation). The manner in which proteins are utilized may differ considerably both prior to as well as following the developmental period studied. For example, electron micrographs have shown that the number of yolk drops in the cells of the area pellucida decrease rapidly between the primitive streak and 10 to 16 somite stage (Bellairs, 1958). This suggested (Bellairs, 1963) that the area pellucida plays an important role in protein

uptake prior to the establishment of a functional circulatory system when the area opaca assumes the major function.

In the studies of ovalbumin utilization that have been discussed, an attempt was made to relate the amounts of ovalbumin present as intact ovalbumin plus ovalbumin converted to embryonic proteins from radioactivity determinations, to the actual increase in protein content during the 48 hour culture period. In the total explant (membrane plus embryo) it was possible to account for only 57% of the protein as being derived from ovalbumin. For this reason, explants were cultured for 48 hours on a medium in which the protein consisted of 10% by weight ^{14}C -conalbumin and 90% unlabeled ovalbumin. The amounts of conalbumin as intact protein and as that converted to embryonic protein were not distinguished because antiserum to conalbumin was not prepared. Nevertheless, the balance or 43% of the protein accumulated by the explant could be precisely accounted for as being derived from conalbumin (Table VII). Thus, in spite of the fact that the medium contained nine times as much ovalbumin as conalbumin, the difference in the percentage of these proteins utilized came to only 14%. This clearly indicated that conalbumin relative to ovalbumin was preferentially taken up and utilized. Selectivity in uptake by the yolk-sac was previously suggested (Brierly and Hemming, 1956) for the passage of antibodies from the yolk to the circulatory system but such studies unlike the above could be criticized for not distinguishing between selective uptake as opposed to the possibility of selective degradation following uptake.

Studies comparable to these carried out with the chick embryo have not been reported for mammalian embryos. However, such studies are anticipated (New, 1971) and should provide considerable insight into the manner by which

these embryos utilize nutrient protein as well as provide some basis for the specificity of their requirements.

VI. Protein Deprivation During Early Development

Through the study of nutritional deficiencies, the functional significance of many nutritional factors such as minerals, vitamins and amino acids have been clearly defined. In studies dealing with the protein requirement of embryos and its significance to growth and development, a similar approach could provide considerable insight. In addition to this particular problem, embryos starved of protein may provide a unique research tool for use on various other developmental problems. This possibility is borne out by the many publications that have appeared in recent years in which protein and amino acid deprivation has been used to successfully investigate regulatory mechanisms of macromolecular synthesis and breakdown in whole animals as well as in cultures of eukaryotic and prokaryotic cells.

In mammalian embryos, maternal protein starvation at the beginning of gestation results in a failure to maintain pregnancy. For example, providing rats with a protein free diet for only the first ten days of pregnancy resulted in 62% of the positively mated females failing to litter (Zamenhof et al., 1971). Such striking effects from protein starvation during the time when the quantity of protein required by the embryo is minute, indicated that the nutritional factors are not acting directly on the embryo. Indeed, the difficulty of maintaining pregnancy during protein starvation has been traced to deficiencies in the production of various maternal hormones. Thus, it would appear extremely difficult to study the direct effect of protein starvation on the metabolism of early mammalian embryos in utero.

As procedures are perfected for the in vitro culture of mammalian embryo, particularly post-implantation stages, the study of protein deprivation in these embryos may become feasible.

The chick embryo can be cultured in vitro at a relatively early stage of development and is nutritionally responsive. For studies of protein starvation with these embryos, a medium was sought which would meet both the physical requirement and those nutritional requirements other than protein. Dextran, equilibrated against whole egg, appeared to meet these criteria. As previously discussed in this chapter (III, A.), equilibrated Dextran was used as a non-nutritive control for those studies in which proteinaceous materials were placed into dialysis bags and allowed to equilibrate against whole egg homogenate. Equilibrated Dextran supplemented with ovalbumin supported embryo growth to a level comparable to that achieved when ovalbumin was directly equilibrated. Embryos cultured on Dextran alone survived for 48 hours and exhibited various aspects of morphological differentiation (Fig. 3), in spite of slight increases in DNA, RNA and protein content. Egg equilibrated Dextran has therefore been used as a protein starvation medium. Chick embryo explants cultured on dialysis tubing placed on top of whole egg homogenate medium appeared comparable to those cultured on Dextran and such preparations have also been used to study starvation but dialysis tubing has created some manipulatory problems associated with transferring embryos to new media.

A preliminary series of experiments with chick embryo explants cultured on Dextran medium, suggested that all regions of the embryos were not affected to the same extent by protein starvation. In order to quantitate this response, procedures were developed which would allow the dissection of embryos

into brain, neural tube, somite, heart, and extraembryonic membrane (area opaca plus area pellucida) in a reproducible manner so that the quantities of DNA, RNA and protein in these regions could be determined. Relative to embryo explants cultured on whole egg homogenate medium, in those cultured on protein starvation medium the accumulation of macromolecules in the brain region was most restricted and the heart least.

As an initial approach to an analysis of the basis for the differences between embryo regions in their apparent sensitivity to protein starvation the breakdown of protein was studied (Klein *et al.*, 1971). Chick embryos of 11 to 13 somites were exposed to ^{14}C -labeled amino acids for three hours in buffered chick Ringer's salt solution. Explants were next cultured for six hours on semi-solid medium to reduce the level of free ^{14}C -amino acids and to allow protein labeling to increase. They were then transferred to either whole egg homogenate growth medium or protein starvation medium. Explants were cultured for various periods up to forty-eight hours, dissected, and regions were analyzed for protein radioactivity and the radioactivity soluble in 5% trichloroacetic acid. Protein and DNA contents were also determined.

In one experiment, when explants were exposed to ^{14}C -valine and cultured on Dextran medium there was a loss of 30% of the radioactivity from the protein of the brain region and a 17% loss from the neural tube regions after thirty-six hours of culture (Table VII). During this same period, the protein content of the brain increased by 19% and the neural tube by 53%. The somites increased slightly in radioactivity and the protein content of this region more than doubled. The heart region also showed an increase in radioactivity and protein but of a greater magnitude than that of the somites. In a similar experiment, a loss of radioactivity from the somites did occur while a loss from the heart region was not detected. Thus, the regions could be placed in

the following sequence for decreasing loss of radioactivity and increasing accumulation of protein: brain, neural tube, somites, and heart. Although there was a relationship between loss of radioactivity and an increase in protein, these parameters were not quantitatively comparable. Indeed, the loss of radioactivity in the presence of an actual increase in protein as observed in the brain and neural tube would suggest that protein turnover occurred under these conditions of growth limitation. The extraembryonic membrane was quite distinct from the embryo proper in that this region lost comparable amounts of protein radioactivity and protein. Furthermore, when embryo explants were cultured on whole egg homogenate growth medium following exposure to labeled amino acids, the extraembryonic membrane was the only region to show a loss of radioactivity. These observations add support to the possibility that the membrane synthesizes proteins which are transported to the embryo proper. It should be noted that under starvation conditions the same regional pattern of protein breakdown or turnover was observed with different radioactive amino acids, the presence or absence of ^{12}C -amino acids in the culture medium, the use of growth or starvation media during the six hour pre-experiment culture period, and the use of either Dextran or dialysis tubing for protein starvation cultivation.

Although regional differences in protein breakdown were detected, differences between regions in protein synthetic capacities could have contributed to this difference. For example, protein breakdown for the various regions could be similar but reutilization of these breakdown products through protein synthesis could be adversely effected by protein starvation in certain regions. Thus an attempt was made to directly measure the effect of protein starvation on regional protein synthesis. Embryo explants that had been cultured on either growth or protein starvation medium for periods up to

thirty-six hours were exposed for ninety minutes to ^{14}C -valine in chick Ringer's solution. When the specific activities of the protein in the various regions were corrected for the radioactivities in the 5% trichloroacetic acid soluble material of a particular region, the differences in regional synthetic rates were not related to regional differences in protein breakdown. After twenty-four hours of culture under conditions of protein deprivation, the synthetic capacity of the heart was actually lower than the brain and by thirty-six hours these two regions were comparable. Furthermore, the regional differences although generally small, were similar in embryos of explants cultured under growth and starvation conditions. It should be clearly noted, however, that only the radioactivity of the 5% trichloroacetic acid was used to correct for possible regional differences in amino acid precursor specific activity. This was justified by the observation that during culture of pre-labeled explants on growth or starvation media the radioactivity in the trichloroacetic acid soluble fraction remained fairly constant. These estimates of protein synthesis could possibly have been improved by a correction for the actual specific activity of the amino acid in the acid soluble fraction. However, these values would not necessarily represent the most immediate precursor for protein synthesis. It would appear that the determination of the specific activity of the amino acid attached to transfer RNA would provide a more accurate estimation of the precursor dilution factor. Such an approach has been used (Henshaw *et al.*, 1971) in a system in which the amounts of tissue available were not severely limited. In small embryos such determinations may be difficult. However, the elegant and sensitive method of Regier and Kafatos (1971) for the determination of amino acid specific activities would suggest that this approach is feasible with small amounts of tissue.

Employing ^{14}C -uridine and following the breakdown and synthesis of RNA

in experiments similar to those described above gave results comparable to those obtained for protein (Klein et al., 1973). The loss of radioactivity from subcellular fractions has also been determined in pre-labeled explants cultured under conditions of protein deprivation (Hickey and Klein, 1973). As might be expected, from the observations on total RNA, most of the radioactivity lost from the sensitive regions (brain and neural tube) was derived from the ribosomal fraction.

In addition to these biochemical studies, some histological and electron micrographical observations have been made (Sawyer, 1973). These observations have concurred with the biochemical findings in that the cells of the developing nervous systems were found to be particularly sensitive to protein starvation. In growing embryos the cells surrounding the neural tube elongate perpendicular to the lumen of the neural tube. However, in protein starved embryos these cells became rounded and as culture was continued degenerative changes occurred. It was of interest to note that the cells of the nervous system of embryos cultured in the presence of colchicine also lost their perpendicular orientation relative to the neural tube and rounded up (Karfunkel, 1972). In addition, when embryos were perfused with media deficient in phenylalanine or tyrosine degenerative changes were observed in the cells of the developing nervous system (Austic and Grau, 1971).

The extreme sensitivity of the brain region of the early chick embryo to protein deprivation represents a striking departure from observations that have been made in older mammals. In the rat studies of dietary protein restriction during pregnancy (Zeman, 1967), nutrient limitation by ligation of a uterine artery (Oh and Guy, 1971), and dietary malnutrition in the newborn (Winick and Noble, 1966; Winick et al., 1968) have all shown that the brain was one of the most resistant organs of a developing animal. In the young mouse

the proteins of the brain show a lower turnover rate than those of the liver (Lajtha et al., 1957; Lajtha, 1959). In the adult rat, the brain and heart were observed to be the most resistant organs to protein starvation as measured by protein depletion, the liver the most sensitive and the muscle to be intermediate between these extremes (Munro, 1964). Thus, it would appear that the brain may pass from a period of high sensitivity to protein starvation as shown in the early chick embryo to a period of relative resistance. Winick and Noble (1966) have been able to relate periods of rapid DNA accumulation by an organ to malnutrition sensitivity. Although all regions of the chick embryo rapidly accumulate DNA, the brain at the stage studied in explants accumulated DNA at a greater rate than the other regions.

In the above studies, a loss of radioactivity from embryo regions was not detected with either protein or RNA labeling when explants were cultured on growth media (Klein et al., 1971; Klein et al., 1973; Hickey and Klein, 1973). This was in agreement with the studies of Herrmann and Marchok (1963) in which the loss of radioactivity was only detected when the growth of chick embryo explants was severely repressed either by adding the amino acid analog fluorophenylalanine to the culture medium or by reducing the size of the extraembryonic membrane. These authors, therefore, concluded that the breakdown of protein was of negligible significance during rapid growth. These studies by Herrmann and Marchok were, however, of relatively short duration. Furthermore, it should be clearly noted that in one experiment reported with protein starvation (Klein et al., 1971) a loss of 32% of the radioactivity of the brain was observed while the protein of the brain more than doubled over a forty-eight hour culture period. In addition, the possible absence of protein breakdown during rapid embryo growth would suggest the conservation of all proteins synthesized by an embryo. This would appear incompatible with present

concepts of differentiation which indicate that this process involves a change in the cellular protein inventory. Thus, the inability to detect protein breakdown during rapid growth may simply reflect the limitations of the methods that have been employed.

In reference to differentiation and protein breakdown, the state of differentiation of a particular embryo region may actually be directly related to its capacity to break down protein. Thus, in the protein starvation studies which were initiated at the 11 to 13 somite stage, the heart which does not exhibit protein breakdown had started to pulsate at the 9 somite stage (Hamilton, 1952), myosin (Holtzer et al., 1957) and striated cells (Przybilski and Blumberg, 1966) first appeared after fifty to sixty hours of incubation or ten to twenty hours after the start of the explant experiments, and a protein specific for the brain (Friedman and Wenger, 1970) did not appear to accumulate rapidly until four to six days of incubation or after the completion of the explant studies.

Although the data presented can not support or exclude the possibility of protein and RNA turnover during rapid embryo growth, it would appear that in embryos starved of protein the extensive breakdown of protein and RNA may provide precursors for the synthesis of new species of proteins and RNA which would allow differentiation to continue in the absence of a net increase in these macromolecules. In this regard, it is of interest that Schinke (1969) suggested that protein breakdown during starvation may provide precursors which allow the organism to adapt to changes in the environment. In support of this, the induction of β galactosidase in bacteria cultured on minimal medium was inhibited by the addition of various protease inhibitors to the cultured medium (Goldberg, 1971).

Finally, the studies on protein starvation in the early chick embryo have provided some insights into the role of protein nutrition and have posed several pertinent questions. For example, it would be particularly important to determine if all regions of the embryo are starved to the same extent and therefore, the regional differences observed reflect actual differences in response to starvation or alternatively that nutrients are transferred selectively to the regions which appear less sensitive. In a recent study with young rats (Dallman and Spirito, 1972), data were presented which suggested that the resistance of the brain to a low protein diet was in part due to the utilization by the brain of amino acids derived from the breakdown of proteins in other regions of the body. In spite of the uncertainty of this question in the early embryo, it does appear that protein can regulate cellular activity. In addition to the observations on protein and RNA breakdown, the ribosomal RNA per cell (DNA) can be affected by protein nutrition. In chick embryo explants cultured under conditions of rapid growth the ribosomal RNA per DNA in the brain and neural tube approximately doubles during thirty-six of culture while this value does not change appreciably in the somites or heart region (Hickey and Klein, 1973). During a similar culture period on protein starvation medium the level of ribosomal RNA per DNA remained essentially constant in all regions including the brain and neural tube. Thus, similar to studies in various other systems (Munro, 1968) protein nutrition and the availability of amino acids may have a direct effect on the level of ribosomes within a cell which ultimately may play a central role in growth regulation during early embryonic development.

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LEGENDS TO FIGURES

- Fig. 1 Comparison of the protein nitrogen content of chick embryos incubated in ovo under air, and cultured in vitro on whole egg homogenate and chemically defined media under 75% air + 25% O₂ for 0 to 24 hours and 95% O₂ + 5% CO₂ for 24 to 48 and 48 to 72 hours. Zero time was 40 hours of preincubation in ovo. From Klein et al. (1964).
- Fig. 2 Gross appearance of tyrosine-deficient and normal embryos. The embryo on the left is a normal 6½-day-old control. At the right is a typical embryo of the same age that has been nourished with the defined medium beginning at 4 days. The middle embryo received the defined medium without tyrosine. From Grau et al. (1965).
- Fig. 3 Photographs of live explants taken at 0 time (11-13 somites) (a) and after 36 hours of culture (b-d). (a) 11-13 somite explant prepared for experimentation; (b) equilibrated Dextran medium (lines in background from lens paper), (c) dialysis tubing on whole egg homogenate medium, (d) 50% whole egg homogenate growth medium. From Klein et al. (1971).

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TABLE I
The Growth of Mouse Embryos on Various Nutrient Media^a

Culture medium	DNA (ug/embryo)	RNA (ug/embryo)	Protein (ug/embryo)
CD ^b	3.43 ^d ± 0.34	8.3 ± 0.36	38.6 ± 2.07
CD + 10% S ^c	4.37 ± 0.23	8.1 ± 0.25	40.6 ± 3.10
CD + 20% S	5.69 ± 0.30	11.58 ± 0.39	59.8 ± 1.86
100% S	3.71 ± 0.17	8.0 ± 0.56	45.0 ± 1.35

^a Summarized from Tables 1, 2, 3, and 4B in Clarkson et al. (1969).

^b CD = chemically defined medium, Waymouth's MB 752/1.

^c S = rat serum.

^d mean ± standard error.

TABLE II
Protein Nitrogen Content of Chick Embryos Cultured for
48 Hours on Various Sources of Protein^a

Source of protein	Protein nitrogen (ug/embryo)
Whole egg homogenate	49.4 $\pm$ 0.4 ^b
Yolk (buffered)	44.6 $\pm$ 1.6
Sub-blastodermal fluid	24.3 $\pm$ 0.4
Egg white	40.1 $\pm$ 2.0
Embryo extract	11.4 $\pm$ 0.6
Milk	16.7 $\pm$ 1.1
Casein	21.1 $\pm$ 1.5
Colostrum	22.9 $\pm$ 1.6
Horse serum	21.6 $\pm$ 0.6
Horse serum + ovalbumin	43.6 $\pm$ 3.6
Ovalbumin	33.2 $\pm$ 1.6
Bovine serum albumin	13.4 $\pm$ 2.0
Dextran	13.3 $\pm$ 0.9

^aSummarized from Table 6 in Klein (1968).

^bmean $\pm$ standard error.

TABLE III

The Regional Content and Percentage Distribution of DNA, RNA, and Protein
in Chick Embryos Cultured for 48 Hours on Various Media^a

Region	Medium	DNA		RNA		Protein	
		(ug/region)	(%)	(ug/region)	(%)	(ug/region)	(%)
Brain	Ovalbumin	4.0 ± 0.3^b	43 ^c	15.4 ± 0.6	39	85.3 ± 5.0	36
	Serum	1.3 ± 0.1	32	4.8 ± 0.5	35	56.2 ± 2.5	28
Neural tube	Ovalbumin	1.9 ± 0.2	20	8.1 ± 0.1	21	42.8 ± 2.8	18
	Serum	0.9 ± 0.0	22	3.0 ± 0.2	22	40.1 ± 3.8	20
Somites	Ovalbumin	2.1 ± 0.1	23	7.7 ± 0.3	20	45.7 ± 2.5	20
	Serum	1.4 ± 0.0	34	3.5 ± 0.4	25	44.6 ± 2.0	22
Heart	Ovalbumin	0.4 ± 0.1	4	3.2 ± 0.3	8	21.7 ± 2.3	9
	Serum	0.2 ± 0.0	5	1.5 ± 0.1	11	24.1 ± 2.5	12
Limb buds	Ovalbumin	0.9 ± 0.1	10	4.9 ± 0.4	12	38.7 ± 3.7	17
	Serum	0.3 ± 0.0	7	1.1 ± 0.2	8	37.7 ± 4.2	19
Total	Ovalbumin	9.3	100	39.3	100	234.2	100
	Serum	4.1	100	13.9	101	202.7	101

^aFrom Klein (1972).

^bMean $\pm$ standard error.

^cPercentage distribution is based on the sum of the regions being equal to 100%.

TABLE IV
Protein Nitrogen Content of Embryos and Extraembryonic
Membranes of Chick Embryos Cultured on Various Amounts
of Ovalbumin and Conalbumin for 48 Hours^a

Amount in the medium		Protein nitrogen	
Ovalbumin	Conalbumin	Embryo	Membrane
(%)	(%)	(ug/embryo)	(ug/membrane)
100	0	19.2 ± 1.1 ^b	48.9 ± 5.1
99	1	25.6 ± 2.8	52.8 ± 5.1
95	5	26.1 ± 2.1	53.1 ± 6.6
90	10	33.7 ± 2.5	61.9 ± 7.7
75	25	40.6 ± 3.0	71.3 ± 6.9
0	100	19.5 ± 1.5	80.9 ± 14.6

^aFrom Hassell (1973).

^bmean ± standard error.

TABLE V
Protein Nitrogen Content of Embryos and Extraembryonic
Membranes of Chick Embryos Cultured on Various
Media for 48 Hours^a

Medium	Protein nitrogen	
	Embryo (ug/embryo)	Membrane (ug/membrane)
High density fraction	47.6 $\pm$ 1.5 ^b	82.7 $\pm$ 2.1
High density fraction + amino acids	43.0 $\pm$ 1.0	87.5 $\pm$ 5.0
Lipovitellin	24.6 $\pm$ 1.2	51.1 $\pm$ 2.6
Lipovitellin + amino acids	27.1 $\pm$ 0.7	61.0 $\pm$ 3.2
66% Lipovitellin + 34% livetin	44.8 $\pm$ 3.2	84.7 $\pm$ 3.9
85% Lipovitellin + 15% phosvitin	24.2 $\pm$ 2.3	53.6 $\pm$ 2.8
51% Lipovitellin + 34% livetin + 15% phosvitin	38.2 $\pm$ 2.1	72.3 $\pm$ 5.1

^a Summarized from Table 7 in Yager and Klein (1973).

^b mean $\pm$ standard error.

TABLE VI

The Distribution of Ovalbumin as Intact Ovalbumin, Ovalbumin as Breakdown Products, and as Ovalbumin Converted to Embryonic Proteins After the Culture Chick Embryos for 48 Hours on a Medium Containing ^{14}C -Ovalbumin^a

Region	Intact (ug ovalbumin/DNA)	Breakdown products (ug ovalbumin/DNA)	Converted to embryonic proteins (ug ovalbumin/DNA)
Embryo			
Brain	0.136	0.427	2.35
Trunk	0.161	0.324	2.29
Area opaca			
Outer region	1.71	2.76	5.03
Inner region	1.55	2.20	3.15
Area pellucida	0.372	0.756	3.02

^aSummarized from Fig. 3a, b, c in Hassell and Klein (1971).

TABLE VII

Contribution of Ovalbumin and Conalbumin to Chick Embryo Protein Content
 After 48 Hours of Culture on Media Containing ^{14}C -Ovalbumin
 or ^{14}C -Conalbumin^a

Region	Ovalbumin ^b Intact + converted to embryonic protein (ug/region)	Conalbumin ^b Intact + converted to embryonic protein (ug/region)	Protein increment ^c 48 hours - zero time (ug/region)
Embryo			
Brain	14.4	14.0	62
Trunk	24.5	18.7	109
Area opaca			
Outer region	51.3	31.8	0
Inner region	23.4	16.4	0
Area pellucida	16.9	19.0	57
Total	130.5	99.9	228

^aSummarized from Table 1 in Hassell and Klein (1971).

^bcalculated from radioactivity.

^ccalculated from protein nitrogen.

TABLE VIII

Protein and Protein Radioactivity of Starved Explants^{a, b}

Region	Zero time		36 Hours		% of zero time	
	Protein	Cpm	Protein	Cpm	Protein	Cpm
	(ug/region)	(cpm/region)	(ug/region)	(cpm/region)	(%)	(%)
Brain	20.1 ^c ± 1.4 ^d	1162 ± 85	24.0 ± 2.4	813 ± 69	119	70
Neural tube	9.7 ± 1.7	594 ± 76	14.8 ± 0.5	492 ± 38	153	83
Sonites	9.6 ± 1.5	597 ± 59	21.2 ± 1.5	675 ± 47	221	113
Heart	4.5 ± 0.9	202 ± 20	15.5 ± 3.9	300 ± 47	344	149
Membrane	269 ± 18	9832 ± 874	154 ± 8.7	5558 ± 509	57	57

^aTable 1 in Klein *et al.* (1971).^bExplants were exposed to valine-¹⁴C for 3 hours, cultured for 6 hours on equilibrated Dextran medium, and then transferred at 0 time to equilibrated Dextran medium containing 112 ug of valine-¹²C per milliliter.^cEach value represents the mean of 4 determinations.^dStandard error.