FREE AMINO ACID LEVELS IN THE YOLKS OF TUMOUR-BEARING EMBRYONATED EGGS

by

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Thesis

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TABLE OF CONTENTS

page

EXPERIMENTAL

INTRODUCTION

1. THE EGG

The fertilized hen's egg consists of shell, shell membrane, albumen and yolk. A diagrammatic representation of the fertilized hen's egg and its constituents is given on page 2. From this it may be seen that the yolk floats in the albumen with a whitish disc, the blastoderm, on its upper surface. It is from the blastoderm that the embryo and all its membranes are formed. The vitelline membrane, which is a transparent membrane, encloses the fluid mass of the yolk and the blastoderm.

(a) The Shell and Shell Membrane

The shell is made up of three layers, the inner mammillary layer, the intermediate spongy layer and the surface cuticle. The two inner layers are composed mainly of crystalline salts while the outer cuticle is a very thin continuous layer of protein containing scattered globules of fat (1). The entire shell is permeable to gases and thus allows embryonic respiration and evaporation of water to occur (2).

The shell membrane consists of two layers, a thick outer layer next to the shell and a thinner one next to the albumen. They are generally thought to be composed of

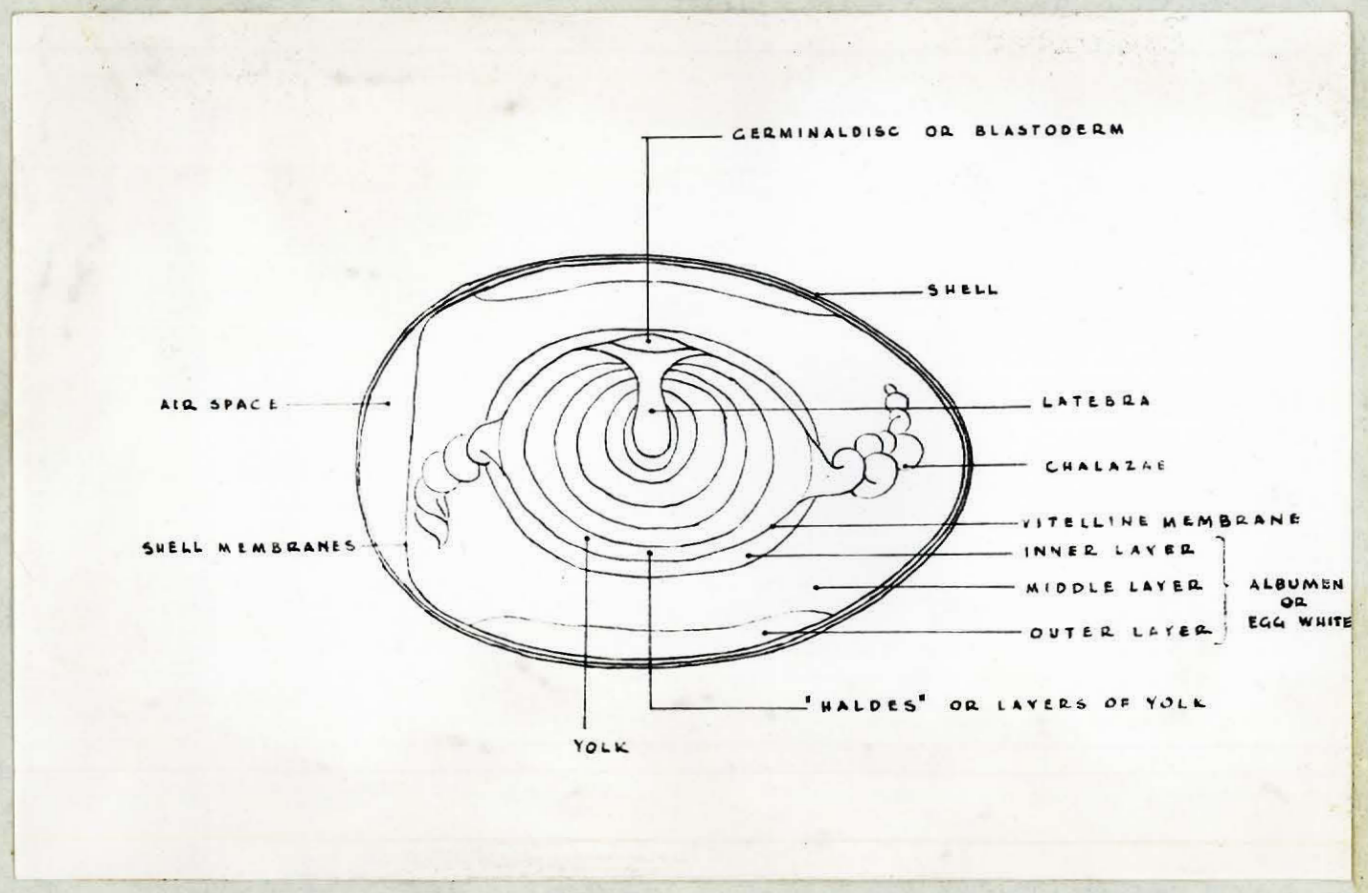


Diagram 1. Diagrammatic representation of a fertile unincubated egg.

keratin fibres matted together. It was shown by Calvery (3) that the fibres are made up of exceptionally pure and typical keratin. Mucin fibres have also been detected in them (4). The fibres of the inner layer are of the same size and cross one another in all directions. Those of the outer layer are variable in size, the mesh is looser and there is a star-like condensation of fibres at certain points which are embedded in the calcite of the shell. This union serves to strengthen the shell which would otherwise be brittle and fragile. At the blunt end of the egg the two layers are separated and form a chamber containing air that enters after the egg is laid (5).

(b) The Albumen

The albumen or egg-white consists of three distinct layers, a thin but dense inner layer, a thick layer and a thin outer layer (6). The inner layer is next to the vitelline membrane which surrounds the yolk and is prolonged in the form of two spirally coiled opalescent cords towards the blunt and narrow ends of the egg respectively. These cords are called the chalazae.

There are four well established proteins in the egg-white. They are two albumins, ovalbumen (75%) and conalbumen (3%) and two glycoproteins, ovomucin (7%) and ovomucoid (13%) (7). A fifth protein, ovoglobulin, still

remains a matter of dispute, since it is difficult to isolate free from ovalbumen and ovomucin and must be present in minute quantities if at all (8). According to Lillie (5a) about 2% of the total egg-white protein is ovoglobulin.

The general composition of the albumen is as follows: (9)

	Average weight in grams	% of total
Total	32.9	100.0
Water	28.9	87.9
Solids	4.0	12.1
Total Organic Matter	3.8	11.5
Protein	3.5	10.6
Lipids	trace	--
Carbohydrates	0.3	0.9
Inorganic Matter	0.2	0.6

The exact nature of the carbohydrates in the glycoproteins is obscure, but they are polysaccharides and are believed to contain mannose in various combinations with glucosamine or galactose (5b).

Koga (10), who was the first to study the enzymes of the egg-white extensively, showed the presence of lipases, proteases and lecithinase in the egg-white. The presence of esterases has also been reported (11).

Egg-white also contains lysozyme. This is a bacteriolytic protein of low molecular weight with the properties

of an enzyme, and has been crystallized (12). The egg-white contributes, therefore, not only to the nutrition of the embryo, but serves as a protective envelope shielding the embryo both from physical injury and from bacterial contamination.

(c) The Vitelline Membrane

The vitelline membrane of the hen's egg is made up of a network of keratin fibres about 25 microns in thickness (7a). It separates two very different systems, the egg-white and the yolk, and the normal development of the embryo is dependent to some extent upon the efficient separation of these systems.

On one side is the egg-white which consists of about 85% water and 15% protein. On the other side of the membrane is the yolk which consists of about 50% water, 33% fat and 16% protein, and a little carbohydrate. The weight of the ash of the white is approximately half that of the yolk. As a result of these differences in composition, there is a difference in the osmotic pressure of about 1.5 atmospheres (7b).

The membrane is permeable to water since it swells and bursts in a hypotonic salt solution, remains unchanged in an isotonic solution and shrinks in a slightly hypertonic solution. In a 10% NaCl solution instead of shrinking it swells awing to the penetration of the saline and the

consequent osmotic pressure due to the dissolving of the vitelline in the saline (13).

It has been shown by Orru (14) that the vitelline membrane is permeable to electrolytes, since water in which yolks were immersed gained in electrical conductivity. In spite of the permeability of the vitelline membrane to electrolytes, the pH of the yolk varies from 4.5 to 6.0 whereas the pH of the egg-white is between 8.6 and 9.6.

The permeability of the vitelline membrane varies with the age of the egg. However, after a long period of storage, an equilibrium is reached. Substances such as vitamin B_1 (15) and copper, found only in the yolk of a fresh egg, and zinc, found only in the white, are equally distributed after some months of storage.

Straub & Hoogerduyn (17) concluded that the "living" vitelline membrane tends either to encourage the exit of water from the yolk and to impede its entry, or to encourage the entry of salts and impede their exit. An apparatus was devised by Needham (18) to investigate the osmotic properties of the isolated vitelline membrane, and it was found that when placed between two salt solutions of different strengths, the membrane offers no resistance to rapid equilibration. Equilibration is slower with yolk and white on either side than with salt solutions, though not as slow as in the intact egg. It is probable that the physical structure of the yolk and white has some feature which retards the attainment of

equilibrium and which is not wholly destroyed by mixing each phase on its removal from the egg. The slow attainment of equilibrium in the intact egg is probably due to the cooperation of the two phases with the membrane. The destruction of lipoprotein complexes has been put forward as an explanation for the more rapid equilibration between yolk and white following their removal from the egg (19).

The average bursting strength of the vitelline membrane at laying is about 4500 dynes/sq. cm. (20). After 40 hours incubation of a fertile egg, the vitelline membrane becomes so weak over the germinal area that its bursting strength can no longer be measured. With the growth of the yolk-sac the vitelline membrane disappears.

(d) The Yolk

The yolk of the fertilized egg is made up of concentric layers of white and yellow yolk. The yellow yolk is secreted in layers around a central core of "white" or milky yolk in the ovary of the hen (21). These layers give the appearance of "haloes" in the finished egg and can often be seen in eggs coagulated by boiling. The white yolk in the centre has a flask-like shape, the latebra, with the neck leading up to the surface of the yolk underneath the germinal disc and then continuing in a very thin layer all around the exterior of the yolk underneath the vitelline

membrane. The white yolk is thus the first source of nourishment for the embryo (22).

Balfour and Foster (23) described the yolk as consisting of tiny spheres 25 to 100 microns in diameter for the yellow yolk and 4 to 75 microns in diameter for the white yolk. Within the spheres there are minute highly refractile granules, probably lipoprotein in nature.

(i) Proteins

The principal proteins of the yolk are the phosphoproteins which are present as lipoproteins (24).

Two lipoproteins have been identified and their protein components have been called vitellin and vitellenin. Another group of proteins and/or degradative products of phosphoproteins have been isolated and called vitellinic acid and phosvitin. A third group, the water soluble proteins, have been collectively termed livetin (25).

The lipoprotein complex, lipovitellin, was first prepared by Osborne and Campbell (26) who found that it contains 15 to 30% of lipid which they believed to be lecithin. No further study was made of the lipovitellin until Chargaff in 1942 (27) repeated the extraction as described by Osborne and Campbell. He found that the lipovitellin complex contained 18% bound lipid which was composed of both lecithin and cephalin.

A second lipoprotein, lipovitellenin, was described by Alderton and Fevolt (28) in 1945. This fraction contains 36

to 41% combined phospholipid which is mainly lecithin. The proteins of the two lipoproteins, vitellin and vitellenin, differ only in their phosphorus content, the vitellin containing about 1% phosphorus and the vitellenin containing about 0.3% phosphorus. Their amino acid distribution is the same since they both contain: alanine, arginine, aspartic acid, cystine, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophane, tyrosine and valine. Thus of all of the common amino acids, only hydroxyproline, di-iodotyrosine and cysteine have not been found in vitellin (29)(30)(31)(32).

The phosphorus in the vitellin is attached to the protein through serine by a serine-phosphoric acid linkage (33)(34)(35). However, the distribution of the phosphorus and serine in the vitellin molecule has not yet been established.

Probable degradation products of vitellin which are high in serine and phosphorus content have been reported. Levene and Alsberg (36) isolated one degradation product and called it vitellenic acid. Another group recently reported the extraction of a very similar substance which was called phosvitin (37). It was found by Posternak and Posternak (33)(34)(35) that there is an increase of proteoses when the yolk is allowed to autolyse. It was suggested that this might be of biological importance in phosphorus transfer from raw materials to embryonic nuclein and bone phosphate.

A third group of proteins found in egg-yolk is livetin, a pseudoglobulin containing only 0.07% phosphorus. It is water soluble and makes up about 20% of the total yolk proteins. Although livetin was clearly recognized by earlier workers, Plimmer (38) was the first to separate livetin from vitellin. The vitellin was prepared by diluting an ether extracted solution of egg yolk in 10% NaCl with a large volume of water and filtering off the precipitated protein. There remained a relatively large amount of protein in the filtrate. It was precipitated by boiling with acetic acid and the soluble protein which was thus coagulated was called livetin.

A study of the amino acid constituents of this protein fraction was made (39) and the amino acids present were found to be similar to those of the vitellin fraction. When the livetin fraction was investigated electrophoretically, it was found that there are at least three components to this water soluble protein (40). The livetin fraction of the yolk proteins apparently contains the majority of the enzymes of the egg. Lineweaver et al. (41) found esterase, amylase, peptidase, phosphatase and catalase activity in the livetin fraction of the egg yolk.

Another protein, vitellomucoid, which contains about 10% carbohydrate, is present in small amounts in the yolk (42).

(ii) Carbohydrates

The egg contains relatively small quantities of carbohydrate. The total amount averages about 0.5 grams, or one percent of the total weight of the egg (43)(44). About 75% is contained in the albumen and the remainder is in the yolk. The carbohydrates in the yolk are found both in the free form, mainly as glucose and as protein-bound polysaccharides of the mannose-glucosamine type (45)(39)(46).

(iii) Lipids

The egg-yolk is extremely rich in neutral fats, phosphatides and sterols. An egg of average weight (55 gms.), with a yolk of about 20 grams, contains in the yolk 5.58 grams of neutral fat and 1.28 grams of phosphatides of which 0.68 grams is lecithin (47)(48).

It was found by Rae (49) that in yolk lecithin, the β-form of glycerophosphoric acid predominates (i.e. the fatty acids are arranged symmetrically about the choline-phosphate radical), in contrast to brain lecithin, in which the α-form predominates and liver lecithin, which is an equal mixture of the two. The fatty acids of the yolk lecithin are mainly isopalmitic, oleic, linoleic, clupanodonic and 9:10-hexadecenoic, with some stearic and palmitic (50)(51). The fatty acids in the neutral fats

are mainly oleic, palmitic and stearic (52). Yolk from the egg of the hen and many other birds contains 1.75% cholesterol (53).

(iv) Inorganic Constituents

The egg is similar to other animal cells in its inorganic constituents. The ash from the whole hen's egg contains Al, Ba, B, Ca, Cu, Fe, Mg, Mn, P, K, Ru, Si, Na, Sr, Ti and V, boron and manganese being found in the yolk only (54). It should, however, be noted that potassium is found in higher concentrations than sodium in both the yolk and the white. This is one of the characteristics of the egg cell (6a). The yolk also contains a very high percentage of phosphorus, most of which is in organic combination (55).

The iron of the yolk is absorbed in some way onto the proteins or lipoprotein complexes in inorganic form. Most of the iron is found in an iron-containing moiety "haemativin" in the vitellin fraction. Jukes and Kay (56) pointed out a possible relationship of this compound to hemoglobin formation since there is a sharp decrease in the vitellin of the egg yolk and a simultaneous increase in the haemoglobin of the chick embryo at about the fourteenth day of incubation.

(v) Free Amino Acids

One of the first to investigate the amount of free amino acids in the yolk of the hen's egg was Tomita (57). He found that free amino nitrogen makes up 18 mgs. percent of the wet weight of the yolk. Other investigators have reported higher values, the variation being due to different methods of extraction. Thus, Cook (58) found 92 mgs. percent and Aggazzotti (59) 58 mgs. percent of amino nitrogen.

With the development of paper chromatography (60) and ion-exchange chromatography (61), it has been possible to study the free amino acids in biological preparation both qualitatively and quantitatively. The limiting factor for accurate estimations of the amino acids has thus become the quantitative extraction of the material. William et al. made use of these recent advances and were able to identify and estimate the amount of threonine, serine, glutamic acid, proline, glycine, alanine, cysteine, valine, isoleucine, leucine, tyrosine, phenylalanine, lysine, arginine and methionine in the yolk of the hen's egg. From their results, the total free amino acid nitrogen may be calculated to be 630 uM per 10 grams wet weight, or 0.15 mg. per gram dry weight of yolk.

2. GENERAL DEVELOPMENT OF THE CHICK EMBRYO

The fertilized egg, when laid by the hen, contains a whitish disc about 3.5 mm. in diameter known as the

blastoderm (62). The embryo proper arises within a clear area in the centre of the blastoderm called the area pelucida. The remainder of the blastoderm is extraembryonic and from it arises the embryonic membranes, the amnion, the chorion and the yolk-sac.

During the first four days of development the blastoderm spreads very rapidly and by the fourth day has given rise to blood vessels which cover the greater portion of the yolk (area vasculosa). By this time, also, the embryo is fairly well developed. The wing and leg buds are recognizable (63), the head is well defined and the pigmentation in the eye is visible (64).

The next visible change occurs at about the sixth day when the appendages become clearly visible. The wing is bent at the elbow and the second digit is distinctly longer than the others, which are separated by shallow grooves. The toes stand out as ridges separated by distinct grooves and have indications of webs between them. The auditory sense organs are visible and the neck between the "collar" and mandibular process has lengthened. The beak is well advanced in development. Towards the seventh day, feather germs begin to develop on either side of the spinal cord at the brachial level and at the level of the legs. The egg tooth, which is used only during the time of hatching to crack the shell, is slightly protruding.

The embryo is further advanced by the end of the tenth

day. Both the wings and legs are long and flight feathers are conspicuous. The nostril has narrowed to a slit and the beak is continuing its development. The nictitating membrane covers most of the eye and the lower lid has grown upward to the level of the cornea.

By the end of the fourteenth day the embryo resembles the hatched chick. Most of the body is covered with feathers. The eyelids are well formed and the opening between them is reduced to a thin crescent. The beak and toes are now well developed. Except for the increase in size and the final formation of the beak and toes, there is no other visible change in the development of the chick until it hatches on the twenty-first day. At this time the yolk-sac is retracted into the body where it continues to supply nutrients until the chick can digest the food from the outside world on the second or third day.

3. CHANGES IN THE YOLK DURING EMBRYONIC DEVELOPMENT

(a) The Yolk-sac

According to Needham:

> "The temporary structures used by the embryos of birds during development are three in number, the yolk-sac through which the absorption of nutrient materials from yolk and white is carried on, the amnion which encloses the embryo in its 'private pond', and the allantois which receives the excreta from the cloaca and allows the exchange of respiratory gases through its blood vessels closely applied to the inner shell membrane" (7c).

The yolk-sac is the earliest membrane to form and can be recognized on the second or third day as essentially a continuation of the intestine. In its growth it eventually encloses the entire yolk (65). Its surface area increases very rapidly up to the sixth day, decreases gradually from that time until the eleventh day, and then diminishes at a more accelerated rate (66). The apparent shrinkage of the yolk-sac is correlated with the decrease in the volume of its contents (67)(68).

The yolk-sac reaches a maximum wet weight of about 3.5 grams on the fifteenth day of incubation, after which its weight falls to about 2.5 grams at hatching (69). There is no direct relationship, therefore, between the area of the yolk-sac membrane and its weight. Weight is still increasing throughout the time that surface area is decreasing, a fact which is explained by the thickening and folding of the inner wall of the membrane and also by the inclusion of absorbed yolk substances within the yolk-sac cells and blood vessels (65).

The chief functions of the yolk-sac are the formation of blood, the absorption of nutritive materials from the enclosed yolk and the transport of the absorbed nutrients to the embryo. The capillary network in the yolk is formed during the period of self-differentiation before circulation begins. The main arteries and veins are formed only after the blood starts to flow in the area vasculosa at the 16-somite stage (70). The larger arteries develop from the portions of the network through which the blood

passes most rapidly. The vascular layer comprises the greatest part of the thickness of the yolk-sac membrane. The blood vessels protrude into the endoderm of the wall, which is thus thrust into the mass of the yolk underlying it. The radiating folds and ridges produced in this manner become pronounced after the ninth day and constitute an excellent absorptive surface (65a).

The endodermal cells of the yolk-sac secrete enzymes into the yolk. These enzymes act upon the yolk, which is an emulsion of fat suspended in a colloidal phosphoprotein. There are lipases which act upon the fat and proteases which act upon the proteins. The liquified material is then absorbed by the cells where digestion is continued (71).

At the beginning of incubation the yolk is arranged in the form of spheres, each surrounded by a semipermeable membrane. According to Grodzinski, lipases secreted by the yolk-sac dissolve the membranes of the yolk spheres and transform the contained fats from glycerides to phosphatides (72). The proteases act upon the continuous phase of the yolk emulsion. The presence of lipases and proteases was shown by several investigators at the beginning of this century (73)(74). The activity of the yolk proteases, which is low in the yolk-sac at the beginning, is believed to reach a maximum on the tenth day, the time of maximal catabolism (75). Lipolytic activity is low until the fifth day and reaches a maximum

at about the 16th day. Goldstein et al. (76)(77) have shown that the kathepsin activity of the yolk and yolk-sac increases up to the 15th day and then remains constant. This picture so much resembles that of the growth of the yolk-sac that the latter is probably the largest factor in it.

Needham has shown that the yolk-sac absorbs the yolk at a very high rate during the first five days of incubation (78). For instance, it takes up more than three times its own weight on the second day. However, most of the material absorbed during the early period is utilized for the growth of the yolk-sac itself. The rate of absorption falls on the 6th day and from then on stays constant. The amount of yolk taken up per day after this does not exceed the weight of the yolk-sac membrane, which at this time requires only a small portion of the absorbed material for its own metabolism.

(b) The Yolk Proteins and Amino Acids

At the beginning of development the hen's egg contains about six or seven types of proteins and a number of smaller nitrogenous molecules. At the end it consists of another set of proteins, mostly the avian body and serum proteins, a further collection of simpler nitrogenous substances, and a quantity of incombustible products of protein breakdown. Since the yolk is the main source of nutrient for the embryo, there are corresponding changes in the yolk.

Sznerovna (79) studied the changes that took place in

the white, yolk and embryo during development. She measured the protein nitrogen of both the egg-white and the yolk at the beginning of incubation and again near the end of the incubation. She found no intrinsic change in them during development, but in the embryo protein the mono-amino-acids decreased and the di-amino acids increased.

Riddle (80) showed that the level of yolk proteins rises, during the last five or six days of incubation, when estimated as a percentage of the dry solids in the yolk. This is due to a preference on the part of the embryo for lipoids and fats, the concentration of which decreases in a correspondingly rapid manner. For five or six days before hatching, the absorption intensity for protein falls rapidly and that for fatty substances rises (6b). Onoe (81) showed that while vitellin and livetin are absorbed by the yolk-sac at about the same rate, vitellomucoid is absorbed at a more rapid rate during the second half of incubation.

The changes in individual amino acids in the protein during incubation have been investigated (82)(83). Sendju noted a fall in tyrosine, tryptophane and cystine, but no change in histidine, arginine and lysine. Some of these results were confirmed by Calvery who, however, could find no change in tryptophane and noted a definite fall in lysine content.

Many investigators have estimated the free amino acid

nitrogen of the egg-yolk at various stages of embryonic development. Among the first to study the changes in the free amino acid levels were Tomita (57) and Nakamura (84). They showed a rise in the free amino acids in the yolk from about 5 mgm. percent wet weight Before incubation to approximately 11 mgm. percent wet weight on the second day of incubation. After that there was a slight increase in the amino nitrogen to about 15 mgm. percent wet weight. Unfortunately, neither of them took into account the varying water content of the white and yolk and their values are therefore difficult to assess. As the water content of the white is decreasing, the yolk increases its water content during the first ten days of incubation, so that the values reported by Tomita are probably too low. Aggazzotti (59) took the changing water content of the yolk and of the white into consideration. He found that the free amino acid nitrogen showed practically no change during development when related to dry weight in the yolk. There was approximately 1 mgm. free amino nitrogen per gram dry yolk.

There is certainly no '<u>a priori</u>' reason for expecting big changes, for the transfer of amino acid molecules from egg to embryo might vary greatly in intensity without involving a difference in absolute amount or concentration of the intermediary substances (6c). More recent work (85) has shown that the amino acids increase until the ninth day but then decrease until the twelfth day. Further changes were not investigated by these authors.

4. TUMOUR TRANSPLANTATION TO THE CHICK EMBRYO

The fertilized egg is an excellent medium in which to study the effects of a tumour upon a host. Except for the exchange of gases, the fertilized egg is a closed system and thus the metabolism of the embryo can be investigated under fairly standard conditions. Further, one preparation of a tumour can be injected into many eggs at the same or different stages of development. Since the only requirement for the maintenance of the chick embryos is a constant temperature incubator, there is no problem of feeding and cleaning as is found with the usual laboratory animals.

It was shown by Rous and Murphy (86) that tumours could be transplanted to the chorio-allantoic membrane of the chick embryo. They used minced tumour and a filterable agent of Rous sarcoma. The following year Murphy (87) reported that mouse and rat tumours could be maintained on the chorio-allantois of the embryo by continuous passage from egg to egg. This is possible because the developing chick embryo has no defence against the growth of heterologous tissues and it will supply blood vessels and connective tissue for the growth of the tumours (88)(89)(90). It is also possible to obtain a pure strain of tumour cells after several egg passages, since the tumour cells by this time may have outgrown their original stroma (91).

After Taylor *et al.* (92) succeeded, in 1942, in transplanting mouse and rat tumours into the yolk-sac of the chick embryo, heterologous tumour transplantation has been

used extensively. Tumours from chickens, ducks, mice, rats, rabbits, guinea pigs and man have now been successfully transplanted to the fertile egg through the chorio-allantoic membrane, yolk-sac, intra-embryonic and intravenous routes (91). It is thus possible with this technique to obtain data in much less time and more convenience than is possible with the use of the intact animals. The cultivation of tumours in eggs also makes possible the rapid screening of possible chemotherapeutic agents with reference to both normal and tumour tissue. The tumour and the embryo share a common blood supply but do not interfere physically with each other's growth and development, so that it is also possible to obtain information on the tumour-host relationship.

5. AMINO ACID INCORPORATION INTO PROTEINS

(a) In Normal Tissues

The study of amino acid incorporation into proteins was made possible when Schoenheimer and Rittenberg (93) showed that N^{15} labelled amino acids, administered to animals, were incorporated into tissue proteins. Since these early experiments, the incorporation of isotopically labelled amino acids into body proteins has been used as an indication of the biosynthesis of proteins.

The utilization of free amino acids instead of peptides in the synthesis of proteins has been shown recently. The direct incorporation of free amino acids into proteins has

been verified by the study of enzyme synthesis in yeasts (94), bacteria (95), pancreas slices (96) and by the study of the synthesis of milk proteins (97).

Halvorson and Spiegelman (98) investigated the effect of the composition of the free amino acid pool of yeast on induced synthesis of enzyme. They found that when they induced synthesis of the enzyme maltozymase of the resting cells suspended in a nitrogen free medium, there was a net decrease in the concentration of internal free amino acids.

Other methods used in the study of free amino acid utilization in yeast have been those of nitrogen starvation and replenishment procedures (99), and the inhibition of enzyme synthesis by interference with the utilization of the free amino acids.

Enzyme synthesis studies with bacteria (95)(100) as well as those with yeast lead to the conclusion that the primary pathway of induced enzyme formation involves synthesis of new enzyme from free amino acids.

The synthesis of other proteins from free amino acids has been investigated. Askonas et al. (101)(102)(103) injected radioactive amino acids intravenously and found that the free amino acids of the blood represented the major precursors of the milk proteins. The radioactivity of the free amino acids and of the milk proteins were the same, thus showing that the only source of amino acids for the synthesis of the milk protein was from the amino acid

pool. The specific activity of the radioactive amino acid, wherever it occured in the protein chain, was exactly the same. This proved that in all parts of the protein molecule the injected free amino acid was used, and that preformed peptides or other precursors were not used to any significant extent.

The aldolase and phosphorylase in the rabbit muscle have been shown to be synthesized from free amino acid by radioisotope technique (104). Free amino acids are also incorporated into the ferritin of rat liver (105).

Conclusive evidence that free amino acids are the sole source of proteins has been put forth by Monod et al. (106). They cultured bacteria in the presence of radioactive substrate, which was thus incorporated into the proteins of the organisms. The bacteria were then transferred to non-radioactive medium and enzyme synthesis was induced. The induced enzyme contained no radioactivity and hence could not have been formed from the pre-existing proteins.

The synthesis of various proteins from free amino acids has been demonstrated by several investigators under entirely different experimental conditions. Yeast, bacterial and muscle enzymes, milk proteins and ferritin all seem to be derived from the free amino acid pool. If this conclusion is justified in each case, it seems reasonable to assume that all protein is synthesized from the free amino acid pool.

(b) In Tumours

Although there does not seem to be any difference between the amino acid composition of normal and tumour tissues (107)(108), there is evidence which indicates a very active amino acid metabolism and synthesis of protein in tumours. There seems to be a one-way passage of proteins or protein-precursors from the metabolic pool to the growing tumour, and this has led to the use of the term "nitrogen trap" with reference to the tumour (109). It has been shown (110) that the tumour grows by deriving amino acids for protein synthesis from the breakdown of the proteins of normal tissues and utilizing them for its own growth.

There is a variation in the rate of amino acid incorporation in normal and in tumour tissues. Schemin and Rittenberg (111) found that although the tumour and liver picked up the radioactive glycine in the rat at the same rate, the liver excreted the amino acid in about half the time that the tumour tissue excreted it. On the other hand, the muscle and skin excreted the glycine-N^{15} at an even slower rate than the tumour. More recent work (112) has shown that the incorporation of glycine-2-C^{14} into the liver is more rapid than the incorporation into the proteins of tumour and kidney of the rat. The incorporation of tyrosine was also investigated (113), and it was found that although the

final specific activity of the intestinal mucosa, kidney and tumour was the same, the rate of incorporation of tyrosine into the tumour was the slowest.

Zamecnik et al. (114) were the first to study the incorporation of amino acids into tumour slices. They showed that alanine-1-C^{14} and glycine-1-C^{14} were incorporated into rat hepatomas to a greater extent than into normal liver. The discrepancy between rates of incorporation into the tumour and liver, in in vitro and in vivo experiments has been explained by Zamecnik et al. (115). They claim that although the tumour has a greater capacity to incorporate amino acids into proteins than liver, the lower rate of incorporation which is observed in the intact animal is caused by a relatively poor blood supply to the tumour. It was shown by Winnick (116) that amino acids are incorporated into peptide bonds, since no radioactivity was released on treatment of the protein with ninhydrin until after hydrolysis.

Most of the energy required for the formation of peptide bonds from the amino acids is derived from that liberated during the oxidation mediated by the functioning of the citric acid cycle (117). This is done by the storage and transfer of energy through the medium of compounds with high energy phosphate bonds, particularly adenosine triphosphate. The presence of this cycle in tumours has been demonstrated by Weinhouse S. et al. (118). Further evidence for the presence of this cycle was given by Kit and Greenberg (119),

who showed that acetate-1-C^{14} was partly oxidized to CO_2 by mouse lymphosarcoma cells and normal spleen cells and that some of the C^{14} from the acetate was found in the cell proteins. They also found that these processes and respiration were inhibited by fluoroacetate. Kit and Greenberg (119) also showed that oxidation of labelled glycine, leucine and alanine to $C^{14}O_2$ occurred in mouse lymphosarcoma, the oxidations probably involving the citric acid cycle.

The source of the necessary energy in other tissues has been shown to be the operation of the tricarboxylic acid cycle and it is probable that this is also true of mammalian tumours with the avian egg as the host.

6. THE THESIS PROBLEM

It was observed, while transplanting tumours from egg to egg, that the chick embryo with a tumour in the yolk-sac or on the chorio-allantoic membrane was smaller than the normal eighteen day old embryo. Since both the tumour and the embryo are actively growing tissues, it seemed possible that the tumour was competing with the embryo for the available nutrients coming from the yolk.

The proteins of the yolk are hydrolyzed to free amino acids in the yolk itself. The liberated amino acids are then absorbed by the yolk-sac, pass into the blood stream and are thus made available for growth of

the embryo. If there is a greater demand on the free amino acids coming from the yolk in the presence of a tumour, it is likely that there will be a decrease in the free amino acid concentration in the yolks of tumour-bearing eggs. A study was therefore undertaken to determine the amino acid levels in the yolks of embryonated tumour-bearing eggs.

MATERIALS AND METHODS

1. MATERIALS

The chemicals used were all of reagent grade and all solutions were made up with distilled water. The tumour used in the present studies was the mouse sarcoma 37 which was routinely carried in this laboratory. It was maintained in a heterologous strain of male Swiss white mice by transplantation from mouse to mouse. The fertilized white-shelled eggs were all obtained from the same variety of hens (White Leghorn) and were purchased in 30 dozen lots per week from Stevenson's Poultry Farm, Ottawa, Ont.

2. METHODS

(a) Tumour Transplantation

(i) Storage of the Eggs

The eggs were shipped by express and on receipt were put in storage at 20°C. until required (usually one to three days after arrival). It was found that it is possible to store the eggs for one to two weeks at 12°C. to 20°C. without affecting their viability. The embryo in the egg develops to about the thirty-two cell stage while the egg is still in the oviduct of the hen. When the egg is laid, the egg cools and development ceases until the egg is placed in an environment where there is optimum temperature (39-40°C.) and optimum humidity (82%). It is for this reason that it is possible to

store the eggs for some time without ill effects. Six days before the eggs were needed for tumour transplantation onto the chorio-allantoic membrane, they were placed into an incubator in which the temperature and humidity were kept constant at the optimum values.

(ii) Candling

On the sixth day of incubation the eggs were candled. This was done in darkened room by shining a light from within a blackened box through the eggs. The shells of the eggs are translucent enough to permit the blood vessels of the living embryo to be seen when the eggs are held in the light.

In the living embryo the blood vessels form a network lying against the shell membrane occupying a space of about 4 cms. in diameter and this network can be seen through the shell candling. If the embryo is dead however, the blood vessels do not appear as a neat network of red vessels, but as a brownish mass floating within the egg. In an unfertilized egg, there are no blood vessels or brownish flecks and the yolk can be clearly seen. In this way, it is possible to sort out the non-embryonated eggs and eggs with dead embryos from the eggs which contain living embryos. The six day old embryonated eggs were then used for tumour transplantation.

(iii) Preparation of Tumour for Transplantation

The egg is an excellent cultural medium for bacteria. For this reason all possible precautions must be taken to prevent bacterial contamination while preparing the tumour for transplantation. The tumour is prepared in a box (see photograph p. 32) which has been washed with 70% alcohol and irradiated with an ultra-violet lamp. Rubber gloves are worn and the arms are protected by rubber sleeves which are attached to the box. All instruments and Petri dishes are sterilized by heating in a hot air oven at 180°C. for 2 hours. The saline used to make the tissue suspension is sterilized in an autoclave for 20 minutes at 120°C. at 15 lbs. pressure. By the use of this sterile technique bacterial contamination is avoided.

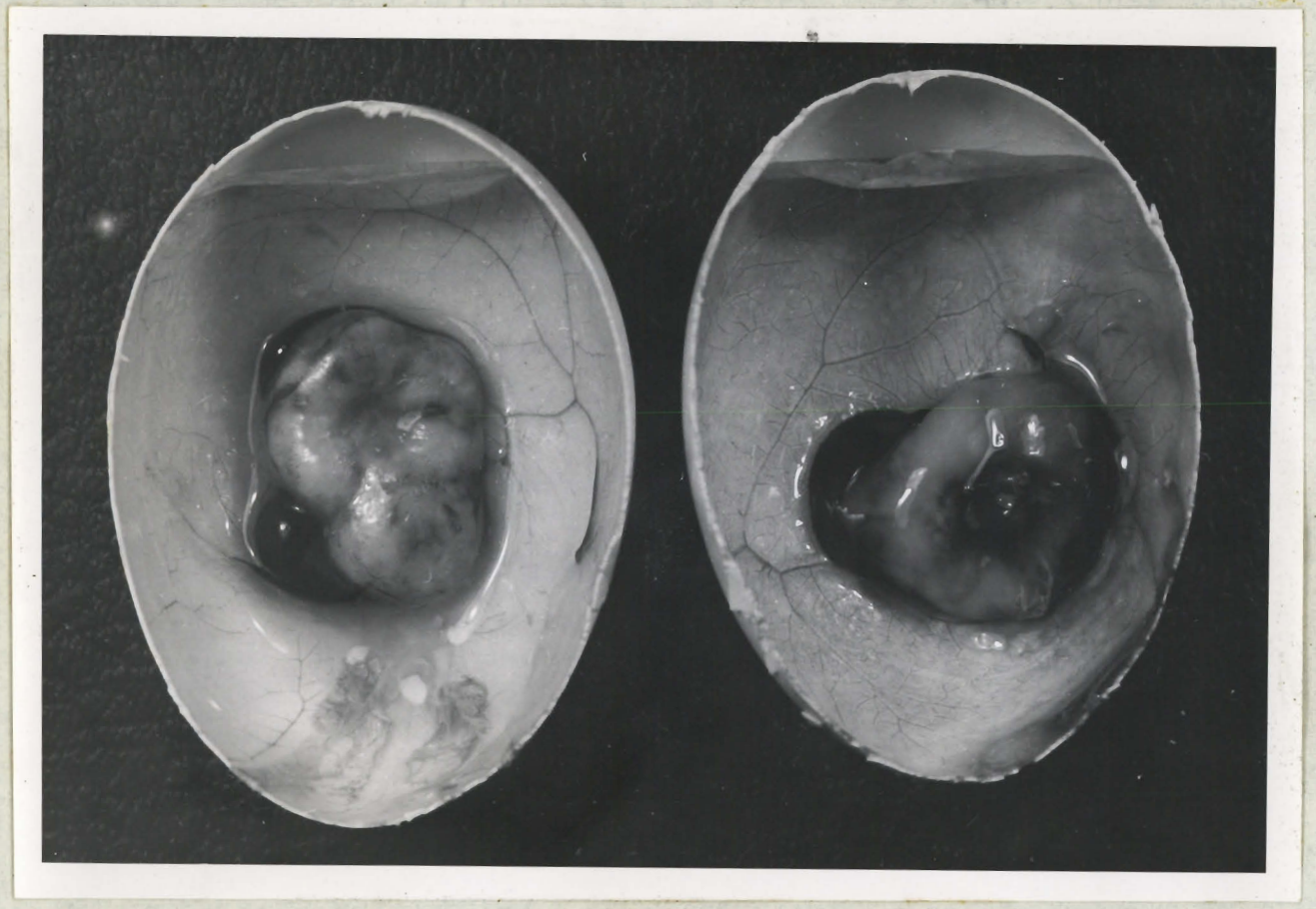
Mouse sarcoma 37 was transplanted onto the chorio-allantois of the six day old embryo in the following manner. The tumour for injection into the eggs was obtained either from a mouse or from an egg. When a mouse was used as a source of tumour, the animal was decapitated, the fur washed first with 70% alcohol and then 1% alcoholic potassium iodide and finally placed in the sterile box. Removal of the tumour was then carried out. The sarcoma 37 was removed from the mouse by cutting along the midline on the ventral surface, taking care not to cut the peritoneal wall. The skin was then cut laterally on either side of the tumour and gently

Photograph of the box in which tumour transplantation is performed.

pulled back so that the tumour, which is enclosed in a capsule of connective tissue, was exposed. The tumour was removed with forceps and scissors, if possible in one piece, and placed in a sterile Petri dish where it was finely minced with scissors.

When the tumour was considered to be well minced, it was placed into a 5 ml. syringe through the top of the barrel and its volume was measured. Approximately 2 ml. of 0.85% sterile saline was drawn up into the barrel with a No. 19 needle. The tumour was well mixed by rolling the barrel between the hands and then it was injected into a 20 ml. rubber capped vial. Sufficient saline was added to dilute the tumour 1:4. The tumour suspension was further homogenized by drawing it into the barrel and then squirting it into the vial. This was repeated several times until the homogenate passed freely through the needle. The tumour was then ready for transplantation.

The tumour for transplantation may also be harvested from either the chorio-allantoic membrane (see photograph p. 34) or the yolk-sac of the 18-day old embryo. The egg containing the tumour was placed in an egg cup in the sterile box and the shell over the air space was painted with iodine. The painted shell was then gently removed with forceps. With another pair of forceps, the shell membrane was removed and the chorio-allantois was exposed.



Photograph of a tumour on the chorio-allantoic membrane. The egg was cut in half longitudinally and all of the contents, except the tumour and the membrane, were removed.

This was pierced, and the embryo with the yolk-sac was carefully removed. The tumour was located on the chorio-allantoic membrane or inside the yolk-sac around the sinus terminalis (see diagram p. 36), depending upon the site of injection. The tumour was removed and placed into a sterile Petri dish where it was treated in the same manner as the tumour from the mouse.

(iv) Method of Transplantation

It is possible to cultivate tumour tissue at two main sites in the egg, the chorio-allantoic membrane of the six-day old embryo and the yolk-sac of the four-day old embryo. Since the objective of the experiments was to study the amino acid levels in the yolk, the presence of a tumour, which is difficult to separate from the yolk and from the yolk-sac, would give rise to complications. For this reason the tumour was grown on the chorio-allantoic membrane, where it was separated from the yolk and yolk-sac and the only contact between the two was via the blood vessels.

Before the tumour suspension was prepared, the size and position of the area vasculosa of the six day old embryos were determined by candling the eggs. Tumour tissue, when placed into a foreign environment, requires a blood supply immediately, in order to obtain the nutrients essential for growth. For this reason, the tumour tissue which is to grow

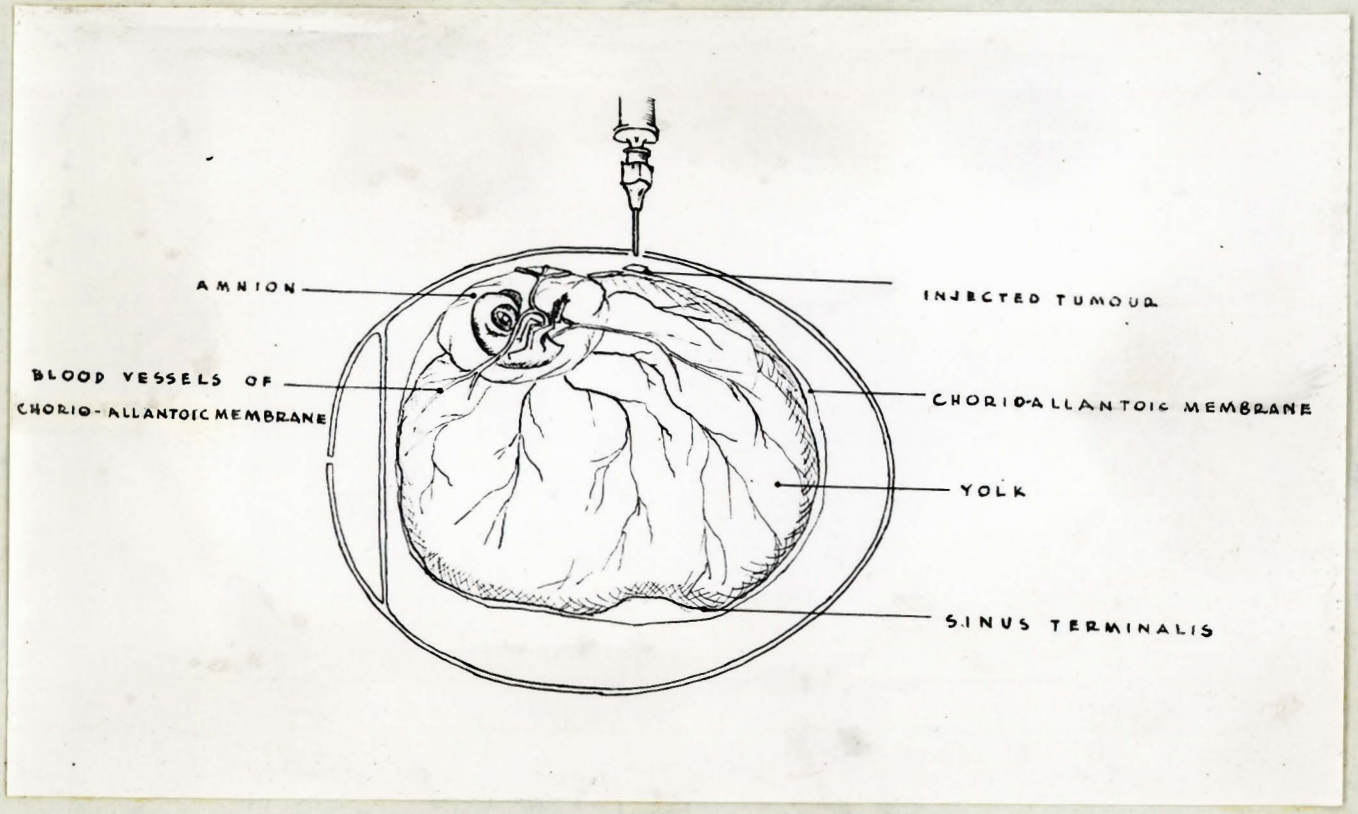


Diagram 2. Diagrammatic representation of tumour transplantation on the chorio-allantoic membrane.

on the chorio-allantoic membrane must be placed on the blood vessels of the area vasculosa of the embryo. The area vasculosa must therefore be made easily accessible and this is accomplished by turning the eggs on their sides, so that the area vasculosa floats on top. The shell and shell membrane need then only be pierced in order to allow the tumour to be placed directly on the blood vessels. For this reason the eggs were kept on their sides in the incubator during the preparation of the tumour suspension.

One dozen eggs on a tray was then placed in the sterile box and the shell over the air space and over the area vasculosa of each egg was painted with iodine. Holes were made in the shell over the air space and over the area vasculosa with a metal pointed rod. Each was punctured in two places to relieve the pressure produced by the volume of the injection.

The tumour suspension was drawn up into a 1 ml. syringe and the standard No. 19 needle was replaced with a blunt-tipped No. 18 needle. The needle was gently placed into the hole, piercing the shell membrane above the area vasculosa and 0.1 ml. of the homogenate was placed onto the chorio-allantoic membrane (see diagram p. 36). When the dozen eggs had been injected, the tray was removed from the sterile box, the holes were closed with cellulose tape and finally the eggs were placed back into the incubator. This procedure

was repeated until as many eggs as were required had been injected. Daily candling of the eggs was carried out to check for eggs containing dead embryos. These eggs were discarded. Twelve days later, when the embryos were eighteen days old, the eggs were opened and the amino acid determinations were made on the yolk.

(b) Extraction of Amino Acids from Yolk

Free amino acids have been extracted from eggs by several workers using various methods (59)(57). More recently the free amino acids of the yolks of embryonated eggs were extracted by homogenizing the several yolks together with a Waring Blendor and then precipitating the proteins of the yolks with cold 15% trichloroacetic acid V/V (120). The amino acids were found in the filtrate when the yolk precipitate was removed by filtration.

Another method was used by Williams et al. (85). These authors precipitated the proteins of individual yolks with 150 ml. cold acetone to which was added 3 ml. N HCl. The acetone solution was separated by filtration and the acetone removed by passing a current of air over the solution. The dried residue was made up to an appropriate volume with citrate buffer. The concentration of amino acids was determined on this extract. These methods were not considered to be adequate for quantitative extraction of the free amino

acids and the following modified procedure was therefore adopted.

Individual yolk-sacs and yolks from eighteen-day old embryos were used. The yolk-sac was removed by carefully cutting it away from the intestine and from the chorio-allantoic membrane. If the yolk-sac was broken during this procedure, it was discarded, since the complete yolk was needed for each extraction. The yolk-sac was placed in a beaker of cold distilled water and rinsed free of egg-white and uric acid. It was then dried with tissue paper and weighed. The yolk and yolk-sac were then homogenized in the Waring Blendor for 30 seconds and an aliquot of about 0.1 gm. was removed by means of a stirring rod. The aliquot was then placed on a pre-weighed aluminum planchet for water-content determination. This was done by weighing the planchet and yolk before and after drying to constant weight in a hot air oven at 100°C. overnight.

The homogenized yolk was precipitated according to the method of Williams et al. (85) by addition of 150 ml. cold acetone containing 3 ml. N HCl. The pH of the mixture was about 2.5. The mixture was further homogenized in the Waring Blendor and the yolk was then allowed to stand overnight in the acetone. Due to the water content of the yolk the final concentration of acetone was about 80%. The following morning the acetone was filtered off and the precipitate

was washed with 80% acetone. The filtrate and washings were collected and the acetone was removed by heating in a water bath at 60°C. under vacuum.

There remained in the flask a thick yellow emulsion of fats and water which contained the amino acids. The fat was removed by washing the extract several times with ether until the ether washings were colourless. At this stage the aqueous layer, which contained all the amino acids, had become clear. The last traces of ether were removed from the aqueous layer by passing a current of air over the solution. The solution was then made up to 100 ml. with distilled water and the determinations were made on this solution.

(c) Amino Nitrogen Determination

The Van Slyke Manometric Blood Gas Apparatus was first used for the determination of the gases in blood (121)(122). Since that time it has been applied to many microchemical determinations such as the measurement of the amino nitrogen in the aliphatic α-amino acids. The primary amino nitrogen reacts with nitrous acid to yield nitrogen according to the equation:

$$RNH_2 + HNO_2 \longrightarrow ROH + H_2O + N_2$$

The nitrogen from other nitrogenous groups in the amino

acids is not measured by the method because only the α-amino groups react quantitatively in 3 to 4 minutes at room temperature (123), while NH_2 groups in other types of substances react much more slowly. About 25% of any ammonia which may be present will react in the time required for complete reaction of α-amino acids (124) and only 6 to 7% of the urea present (125).

The α-amino groups in the aliphatic acids may be determined in the presence of many other forms of nitrogen. Therefore, the guanidine groups of guanidine itself, creatine or arginine do not react at all, nor does the nitrogen in the imidazole ring of histidine, the indole ring of tryptophane or the pyrollidine ring of proline and oxyproline (123).

The reaction was carried out by degasing the glacial acetic and the amine together under Torricellian vacuum in the apparatus (123). The sodium nitrite was then added without allowing air from the atmosphere to enter the vessel. The solutions were then mixed by shaking under vacuum and the nitrogen from the amine and the nitric oxide formed by spontaneous decomposition of the nitrous acid were collected. The collected gases were washed in alkaline potassium permanganate in a Hempel pipette to absorb the nitric oxide. The pressure of the nitrogen at a constant volume was then measured and from this the nitrogen content was calculated in the usual way (see example p. 60).

EXPERIMENTAL

1. INTRODUCTION

The complete extraction of the amino acids is essential if their level in the yolk is to be determined accurately. Although excellent methods such as chromatography are available for the determination of amino acids in aqueous solution, these methods may only be applied to biological materials after quantitative extraction. In the presence of proteins, it is important to ensure that the method of extraction, though thorough, does not lead to hydrolysis of any of the protein material that is present.

As discussed previously, White *et al.* (120) homogenized yolks from normal and tumour-bearing eggs in the Waring Blendor, precipitated the proteins with cold 15% trichloroacetic acid V/V and compared the glutamic acid levels in the filtrates.

Williams *et al.* (85) precipitated the proteins of individual yolks with 150 ml. of cold acetone containing 3 ml. of N HCl. The filtrates were evaporated under a current of air and the dried residues taken up in citrate buffer at pH 3.4. After centrifugation, the soluble extracts were chromatographed both on paper and in an ion-exchange column for the identification and estimation of the amino acids.

The methods employed by the above authors were used

initially in attempts to obtain complete extraction of the free amino acids from yolk. Both 15% trichloroacetic acid and acetone were found to precipitate the yolk proteins completely, but it was found that after re-extraction of these precipitates with acetone, a considerable amount of amino acids was present in the acetone extracts. Further extraction of the residue with acetone yielded no further amino acid nitrogen. A modification of Williams' technique based on this observation was therefore adopted.

Since a measure only of the total quantity and not of the individual amino acids was desired, the Van Slyke manometric technique was used to determine the amino acid nitrogen in the yolk extracts.

2. RECOVERY OF GLYCINE AT VARIOUS CONCENTRATIONS

Solutions of glycine at various concentrations were prepared and the amount of nitrogen in each solution was recovered in the Van Slyke Manometric Apparatus to determine the accuracy of the method. The amino nitrogen was liberated with nitrous acid and measured manometrically as described in the previous section. Sample calculation is given on the following page.

Sample Calculations

5 ml. water for water blank determination at 20° to 25°C.;
Gas volume exerted by water = 2 ml.

Gas + water = 100 mm. Hg.
water = 98
Water blank = 2 mm. Hg.

Sample solution contained 3.5 mg. glycine or 0.6 mg. N in 5 ml. distilled water.
Pressure exerted by gas from sample and water at 25°C.;
Gas volume = 2 ml.

Gas + water = 498 mm. Hg.
water = 98
Therefore gas = 400 mm. Hg.
- Water blank = 2
398 mm. Hg.

x factor to give mg. amino N in sample. 0.001506
0.599388 mg. N

Percent error $\frac{0.0006}{0.6000} \times 100 = 0.1\%$ Error

Therefore 0.6 mg. nitrogen was measured in a sample of glycine which contained 0.6 mg. nitrogen.

2. Sample Calculations

5 ml. water for water blank determination at 24°C.;
Gas volume exerted by water = 0.5 ml.

Gas + water = 189 mm. Hg.
water = 174
Water blank = 15 mm. Hg.

Sample solution contained 0.0067 mg. glycine or 0.00125 mg. N in 5 ml. distilled water. This was obtained by serial dilution.
Pressure exerted by gas from sample and water at 25°C.;
Gas volume = 0.5 ml.

Gas + water = 192 mm. Hg.
water = 174
Therefore gas = 18 mm. Hg.
- Water blank = 15
3 mm. Hg.

x factor to give mg. amino N in sample. 0.000378
0.001128 mg. N

Percent error $\frac{0.00004}{0.00117} \times 100 = 3.0\%$ Error

Therefore 0.00113 mg. nitrogen was measured in a sample of glycine which contained 0.00117 mg. nitrogen.

TABLE 1

The Recovery of Glycine Nitrogen

Mg. N Contained in Sample	Mg. N Measured
0.6	0.599
0.3	0.299
0.15	0.151
0.075	0.0749
0.0375	0.0379
0.01875	0.01865
0.00937	0.00939
0.00468	0.00465
0.00234	0.00236
0.00117	0.00113

Summary

The use of the Van Slyke Manometric Apparatus is an easy and accurate method for the estimation of free ∝-amino acid nitrogen.

2 (b) Recovery of Glycine After Extraction Procedure

It was important to see whether or not the extraction procedure would effect the recovery of amino acids in

aqueous solution. Glycine and alanine were the two amino acids used.

Several 10 ml. solutions of varying concentrations of alanine, glycine and alanine plus glycine were treated with acetone and ether as described for the extraction of free amino acids from egg-yolk. The final volume was made up to 100 ml. and 0.5 ml. aliquots were used for each determination.

TABLE II

Recovery of Glycine and Alanine After Extraction Procedure

Mg. N Contained in 0.5 ml. Sample		Mg. N Recovered
Alanine	0.157	0.156
	0.0785	0.078
	0.0392	0.039
Glycine	0.224	0.223
	0.112	0.112
	0.056	0.057
Alanine plus Glycine	0.381	0.381
	0.1905	0.190
	0.0952	0.095
	0.0476	0.0475

Summary

The amino acids are not destroyed in any way during the extraction procedure, nor does one amino acid interfere with the measurement of another. In all cases it was possible to determine quantitatively the amino acids present in aqueous solutions.

3. COMPARISON OF EXTRACTION PROCEDURES

Method 1

Individual egg-yolks from eighteen-day old embryos were extracted according to the method of Williams et al. (85). After removal of aliquots for the determination of water, acetone containing N HCl was added and the mixture was stirred briefly with a glass rod. The extracted amino acids were dissolved in distilled water instead of citrate buffer at pH 3.4. The citrate buffer is the desired solvent for ion-exchange chromatography. However, since only the total amino acid nitrogen was to be measured, distilled water was considered to be an adequate solvent for these experiments. Aliquots of the aqueous solution were used for the nitrogen determinations.

The yolk precipitate was then re-extracted by homogenizing in the Waring Blendor in 150 ml. of 80% acetone containing 3 ml. N HCl. The mixture was allowed to stand overnight at room temperature and the following morning the

acetone was filtered off and the precipitate was washed with 80% acetone. The extract and washings were treated in the manner already described (see p. 39).

To check for complete extraction of the amino acids, the twice-extracted yolk precipitate was once more extracted with acetone and treated as above.

Method 2

A variation of Williams' technique was made by homogenizing the acetone precipitated egg-yolk in the Waring Blendor. The acetone was filtered off immediately and the aqueous solution was then prepared for the nitrogen estimation.

The precipitate was again extracted by leaving it in the acetone overnight and the amino nitrogen in the aqueous solution was determined.

To ensure complete extraction the procedure was again repeated on the twice-extracted egg-yolk and the amino nitrogen was measured in the aqueous solution.

Method 3

A different technique (120) for the extraction of amino acids was also tested. The yolk proteins were precipitated with trichloroacetic acid as described earlier and the aqueous solution was used for the free amino acid nitrogen determination. The precipitate was further homogenized

with 80% acetone in the Waring Blendor and again extracted. Finally re-extraction of the yolk was made to ensure completeness of the procedure and the nitrogen was determined in the final solution.

Method 4

A second modification of Williams' technique was made in which the homogenized yolk was left in acetone overnight to ensure complete extraction of the free amino acids. The precipitate was again extracted with acetone and the nitrogen was estimated in both extracts.

The amino acid nitrogen values for the different methods of extraction are given in Table III on the following pages.

Summary

It may be noted that all four methods of extraction were effective in removing the free amino acids from the egg-yolks. However, a comparison of the results obtained for the extracts within each method shows that only after homogenizing in the Waring Blendor and allowing the precipitated yolk to remain in acetone overnight was the major portion of the free amino acids liberated. The fact that large discrepancies are evident in the average total weights of amino nitrogen obtained is in agreement with the observations of other workers (126) (85) (127)(128) who observed that wide variations occur in the amino acid content of different eggs. An attempt to demonstrate this and more validly to compare the extraction methods

TABLE III

Comparison between Different Methods of Extracting Amino Acids from Yolk

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group A * Method 1					
Extract 1 Yolk stirred with acetone; then filtered	6.3	43.0	3.6	2.11	0.58
Extract 2 Ppt. homogenized with acetone; left overnight				71.8	19.8
Extract 3 Same as Extract 2				0.02	negligible
Total mg. N/Yolk				73.9	
% extracted on Extract 1				2.9%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group B * Method 1					
Extract 1 Yolk stirred with acetone; then filtered	10.5	44.0	5.88	4.11	0.68
Extract 2 Ppt. homogenized with acetone; left overnight				123.7	21.0
Extract 3 Same as Extract 2				.005	
Total mg. N/Yolk				127.8	
% extracted on Extract 1				3.2%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet.wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group A * Method 2					
Extract 1 Yolk homogenized with acetone; then filtered	9.1	41.5	5.3	33.7	6.35
Extract 2 Ppt. homogenized with acetone; left overmight				63.6	12.0
Extract 3 Same as Extract 2				0	0
Total mg. N/Yolk				97.3	
% extracted on Extract 1				34.6%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group B * Method 2					
Extract 1 Yolk homogenized with acetone; then filtered	10.6	68.5	3.2	30.1	9.55
Extract 2 Ppt. homogenized with acetone; left overnight				54.3	17.2
Extract 3 Same as Extract 2				0.008	negligible
Total mg. N/Yolk				84.4	
% extracted on Extract 1				35.6%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group A * Method 3					
Extract 1 Yolk stirred with TCA; then filtered	14.9	61.5	5.75	4.5	.785
Extract 2 Ppt. homogenized with acetone; left overnight				176.2	30.6
Extract 3 Same as Extract 2				0	0
Total mg. N/Yolk				180.7	
% Extracted on Extract 1				2.5%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group B * Method 3					
Extract 1 Yolk stirred with TCA; then filtered	11.0	48.5	5.3	4.53	.86
Extract 2 Ppt. homogenized with acetone; left overnight				162.9	30.8
Extract 3 Same as Extract 2				0	0
Total mg. N/Yolk				169.4	
% Extracted on Extract 1				2.7%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group A * Method 4					
Extract 1 Yolk homogenized with acetone; left overnight	8.3	46.7	4.4	83.2	18.9
Extract 2 Ppt. homogenized with acetone; left overnight				0.004	negligible
Total mg. N/Yolk				83.2	
% Extracted on Extract 1				100%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

Table continued on following page.

TABLE III (continued)

	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group B * Method 4					
Extract 1 Yolk homogenized with acetone; left overnight	11.6	46.5	6.3	145.1	23.1
Extract 2 Ppt. homogenized with acetone; left overnight				0	0
Total mg. N/Yolk				145.1	
% Extracted on Extract 1				100%	

* The values given within each group represent an average of the determinations on five egg-yolks obtained from the same batch of eggs.

by subjecting aliquots of the same egg-yolk to the various extraction procedures was made by the writer. Unfortunately, it was found that homogeneous aliquots of the yolk and yolk-sac could not be obtained.

Hydrolysis of proteins to free amino acids does not occur when the yolk proteins are left in the acetone overnight. This is proved by the fact that amino acid nitrogen cannot be measured in the second extract after extracting the homogenized yolk precipitate with acetone.

Since Method 4, in which the yolk was immediately homogenized and extracted overnight in acetone, gave the most rapid and satisfactory results without prior treatments, it was adopted for further studies on the free amino acid contents in the yolks of embryonated tumour-bearing eggs.

4. DETERMINATION OF FREE AMINO NITROGEN IN EGG-YOLK

(a) Sample Calculation

2 ml. of the aqueous solution of egg extract was diluted to 20 ml. and 5 ml. of the diluted solution was used for each determination.

Yolk wet wt.	% Water	Dry wt. of Yolk
11.4 gms.	48.5	5.97 gms.

Pressure exerted by gas from sample and water at 23°C.;
Gas volume = 2.0 ml.

Gas+water =	497 mm. Hg.
water =	114
Therefore gas =	383 mm. Hg.
- Water blank =	2
	381 mm. Hg.
x factor to give mg. amino	0.001516
N. in sample.	0.577596 mg. N

5 ml. or 0.5 ml. original solution contains 0.577 mg. N.
100 ml or 1 yolk contains 200 x .577 mg. N = 115.5 mg. N.
1 gm. dry yolk contains 19.4 mg. N.

(b) Recovery of Glycine from Egg-Yolk

The possible interference of other substances in the yolk with the extraction of the amino acids was tested. A known amount of an amino acid was added to the yolk and the total amino acids present in the yolk were determined by the methods previously described. An increase in amino nitrogen equal to the amount added was expected.

To each egg-yolk of unfertile eggs was added 44.8 mg. N or 239.7 mg. glycine in 5 ml. distilled water before the yolk was precipitated with acetone. The amino acids were then extracted in the usual manner and the total amino acid nitrogen estimated in the Van Slyke Manometric Apparatus. The values are given in Tables IV and V.

TABLE IV

Data On Normal Unfertilized Eggs

Yolk wet wt.	% Water	Dry Wt.	Mg. N/Yolk	Mg. N/gm. dry Yolk
20.15	46.5	10.8	114.0	10.3
20.8	45.7	9.5	121.3	10.75
18.6	48.05	9.65	96.6	10.0
19.5	46.6	10.4	110.5	10.6
18.6	49.5	9.4	115.65	12.3
19.1	48.6	9.8	120.5	12.3
19.3	49.9	9.65	117.5	12.3
18.9	49.7	9.5	115.5	12.2
20.1	50.15	10.0	124.9	12.5
Average				
19.4	47.8	9.7	114.6	11.4

TABLE V

Data On Normal Unfertilized Eggs With Glycine Added

Yolk wet wt.	% Water	Dry Wt.	Mg. N/Yolk	Mg. N/gm. Dry Yolk	Mg. N added/gm. Dry Yolk
18.5	46.7	9.85	149.65	15.2	4.6
17.95	51.3	9.0	141.10	15.6	5.0
20.0	45.0	11.0	162.4	14.8	4.1
19.35	48.01	10.05	152.1	15.1	4.5
19.8	47.6	10.4	172.65	16.8	4.3
19.0	50.2	9.45	168.7	17.8	4.8
18.6	49.8	9.35	162.35	17.4	4.8
18.0	47.5	9.45	165.1	17.5	4.75
19.2	50.5	9.5	170.0	17.9	4.75
		Average			
18.8	48.1	10.9	160.1	16.3	4.6

Actual Average Values:

(1) Normal yolk contains 110.0 mg. N

(2) Yolk plus glycine contains 160.1 mg. N

(3) Glycine added per yolk 44.8 mg. N

Theoretical amount of nitrogen in yolk = (1) + (3) = 154.8 mg. N

This is in close agreement with the actual value (2) = 160.1 mg. N

Actual Average Values:

(4)	Normal yolk contains	11.4 mg. N/gm. dry yolk
(5)	Yolk plus glycine contains	16.3 mg. N/gm. dry yolk
(6)	Glycine added	4.6 mg. N/gm. dry yolk

Theoretical amount of nitrogen in yolk - (4) + (6) = 16.0

This is in close agreement with the actual value (5) = 16.3

Summary

The glycine added to the egg-yolk may be recovered completely and there is no interference with the recovery from the other substances in the yolk.

5. MEASUREMENT OF POSSIBLE INTERFERING SUBSTANCES

It is possible to liberate 6-9% nitrogen from the total urea present and 25% nitrogen from the total ammonia present in the Van Slyke Manometric Apparatus during the reaction period for α -amino acids (124) (125). If urea and ammonia are present in the yolk it would not be possible to estimate the amino nitrogen without also measuring some nitrogen from urea and ammonia. In view of this fact it was necessary to estimate the amount of urea and ammonia measured in the Van Slyke Manometric Apparatus and also to measure the amounts of these substances in the yolk. Whether or not uric acid, the main excretory product of the chick, may be measured in the Van Slyke apparatus was also investigated.

(a) i Uric Acid Estimation In Aqueous Solution

5 ml. sample contained 1.68 mg. uric acid or o.6 mg. N.
Amount of nitrogen measured = 0.0 mg.

ii Uric Acid Estimation In Egg-Yolk Extract

0.3 mg. N in 0.84 mg. uric acid was added to an aliquot of egg-yolk extract and the nitrogen was measured.

Aliquot contained	0.32 mg. N
Aliquot plus uric acid contained	0.319 mg. N

Therefore uric acid cannot be measured in the Van Slyke Manometric Apparatus. For this reason it was not considered necessary to estimate the amount of uric acid in the yolk nor does it interfere with the complete extraction of the amino acids.

(b) i Urea Estimation In Aqueous Solution

5 ml. sample contained 1.07 mg. urea or 0.5 mg. N.
Amount of nitrogen measured = 0.043 mg. N.

$$\text{Percent measured} \quad \frac{0.04}{0.5} \times 100 = 9.1\%$$

Therefore 9.1% of the total urea is estimated in the Van Slyke Manometric Apparatus under the conditions of amino nitrogen determination.

ii Urea Estimation In Egg-Yolk

The urea in the aqueous yolk extract was measured according to the method of Krebs and Henseleit (129).

TABLE VI

Urea Estimation In Egg-Yolk

Addition to Basic Medium	Mg. urea added	Mg. urea recovered	Mg. urea attributed to added egg-yolk extract
Blank Water and acetate buffer	0.0	0.053	--
Urea	0.214 mg.	0.211	--
Yolk extract and urea	0.214	0.279	0.012
Yolk extract	0.0	0.048	--

It can be seen from Table VI that there is no significant amount of urea in the extract. Therefore the methods employed do not reveal the presence of urea in the yolk extract.

(c) i Ammonia Estimation In Aqueous Solution

5 ml. sample contained 0.49 mg. NH_4Cl or 0.125 mg. N.

Amount of nitrogen measured = 0.042 mg.

Percent measured $\frac{0.042}{0.125} \times 100 = 34.5\%$

It is thus possible to measure 34.5% of the total ammonia in aqueous solution in the Van Slyke Manometric Apparatus.

ii Ammonia Estimation In Egg-Yolk Extract

The micro-technique of Braganca, Quastel and Schucher (130) using the Warburg Manometric Apparatus was employed for the determination of ammonia in aqueous extracts of the yolk.

TABLE VII

Ammonia Estimation In Egg-Yolk

Addition to Basic Medium	uM NH_3 added	uM NH_3 recovered	uM NH_3 attributed added egg-yolk extract
Water Blank	0.0	0.5	--
NH_4Cl	5.5	6.0	--
Yolk extract	0.0	0.4	--
NH_4Cl and Yolk extract	5.5	5.7	--

It can be seen from Table VII that there is no significant amount of ammonia in the egg-yolk extract. The ammonia liberated from amides can also be estimated in this manner. Since there is no appreciable liberation of ammonia, it can be concluded that there are no amides in the extract.

Summary

Nine per cent of urea nitrogen and 34.5% of ammonia nitrogen present in aqueous solution can be measured in the Van Slyke Apparatus under the conditions of amino nitrogen determination.

There is no ammonia or urea measured in egg-yolk extract; uric acid if present cannot be measured by the techniques used in these experiments.

6. EFFECT OF TUMOUR ON THE FREE AMINO ACIDS IN THE YOLKS OF EMBRYONATED EGGS

Free amino acids can be incorporated into tumour (119). When a tumour is growing in an embryonated egg it is likely that the free amino acids in the yolk may be utilized in this manner. To investigate this possibility, the free amino acid levels were measured in yolks of eggs containing 18-day old embryos with 12-day old tumours on the chorio-allantoic membrane and eggs containing normal 18-day old embryos. Eighteen-day old eggs were chosen for these studies for two reasons. The tumour is well developed in size after 12 days of growth and thus exerts its maximum effect at this stage.

Secondly, the yolk-sac has not yet started to be retracted into the abdomen of the embryo as is the case in older eggs.

Tables VIII and IX show the amino acid levels in the yolks of 20 embryonated non-tumour-bearing eggs and 20 embryonated tumour-bearing eggs.

TABLE VIII

Data on Normal 18-Day Old Embryonated Eggs

Embryo Wt.	Yolk wet wt.	% Water	Dry wt.	Mg. N/yolk	Mg. N/gm. dry yolk
Group A					
25.2 gm.	10.6 gm.	42.1	6.0 gm.	120.2 mg.	19.9 mg.
26.5	11.5	40.6	6.8	146.8	21.7
23.4	8.3	48.6	4.25	86.5	20.4
24.3	8.3	43.1	4.7	101.4	21.5
25.0	13.4	50.4	6.6	154.2	23.2
Group B					
24.1	10.5	62.8	3.9	78.3	22.5
22.6	9.1	41.9	5.3	100.4	25.6
23.9	5.15	53.6	5.15	74.0	14.3
25.1	12.3	48.5	6.3	78.8	12.4
25.9	8.5	47.1	4.5	6.0	1.3
24.0	11.0	46.1	5.9	24.2	4.1

Table continued on following page.

TABLE VIII (continued)

Embryo Wt.	Yolk wet wt.	% Water	Dry wt.	Mg. N/yolk	Mg. N/gm. dry yolk
Group C					
26.9 gm.	12.8 gm.	47.1	6.8 gm.	112.8 mg.	18.1 mg.
27.1	11.7	47.2	6.2	87.6	14.2
25.9	12.7	51.0	6.2	221.1	35.6
23.1	11.4	48.5	6.0	115.5	19.3
24.8	10.3	45.9	5.6	242.15	43.5
Group D					
26.1	10.6	44.95	5.8	94.2	16.1
23.2	12.2	48.4	6.3	101.0	16.0
22.5	10.2	46.0	5.5	92.2	16.8
25.0	12.6	44.10	7.0	110.1	15.6
Average					
24.8 gm.	10.65 gm.	48.4	5.7 gm.	107.4 mg.	19.1 mg.

TABLE IX

Data on 18-Day Old Embryonated Eggs with Tumour om Chorio-allantoic Membrane

Embryo wt.gms.	Tumour wet wt. mgs.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group A						
19.0	250	9.0	46.6	4.8	50.2	10.5
18.9	456	12.8	48.5	6.6	47.1	7.15
18.1	398	13.5	51.4	6.6	61.5	9.4
16.1	1115	8.2	44.1	3.8	10.9	2.9
17.6	300	8.6	50.3	4.3	47.4	11.1
Group B						
17.9	295	10.6	45.15	5.9	45.0	7.6
16.6	1210	9.8	42.3	5.8	8.6	1.5
Yolk broken in one egg						
19.1	571	12.2	50.2	6.1	64.8	10.65
18.5	653	10.9	42.0	6.3	21.1	3.3
17.0	1256	12.5	46.8	6.65	1.5	0.25
Group C						
17.5	779	11.4	47.7	6.0	36.6	6.13
16.9	1361	11.4	43.2	6.4	5.1	0.79
18.1	478	12.7	47.0	6.7	81.9	12.1
17.2	501	8.3	47.2	4.4	49.8	11.3
16.1	1016	13.4	43.2	6.40	18.9	2.95

Table continued on following page

TABLE IX (continued)

Embryo wt.gms.	Tumour wet wt. mgs.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
Group D						
18.1	801	13.1	49.6	6.6	27.4	4.15
17.9	778	15.0	48.2	7.8	40.95	5.3
17.8	221	11.8	47.5	5.6	81.0	14.3
15.7	698	12.6	48.2	6.5	33.5	5.2
		Average				
17.7	683	10.9	46.8	5.9	37.3	6.1

When the two tables are compared it can be seen that the average free amino acid level in the yolks of tumour-bearing eggs is considerably lower than that of the yolks of non-tumour-bearing eggs. A more detailed examination of the data in Table IX reveals further that the total amino acid concentrations in the yolks of tumour-bearing eggs appear to be inversely proportional to the wet weight of the tumour. This relationship between tumour weight and amino acid nitrogen in the yolk is illustrated in Figure 1 (see p. 73).

Results

It can be seen that the chick embryos with a tumour on the chorio-allantoic membrane weigh 7 gms. less than the normal embryos. A relationship could not be found between the difference in weight of the chicks and the wet weight of the tumour. There seems to be a slight decrease in average water content of the yolk of a tumour-bearing egg which may or may not be significant since there is quite a variation in the water content from egg to egg. The level of the free amino nitrogen decreases in the presence of a tumour. This decrease is inversely proportional to the size of the tumour.

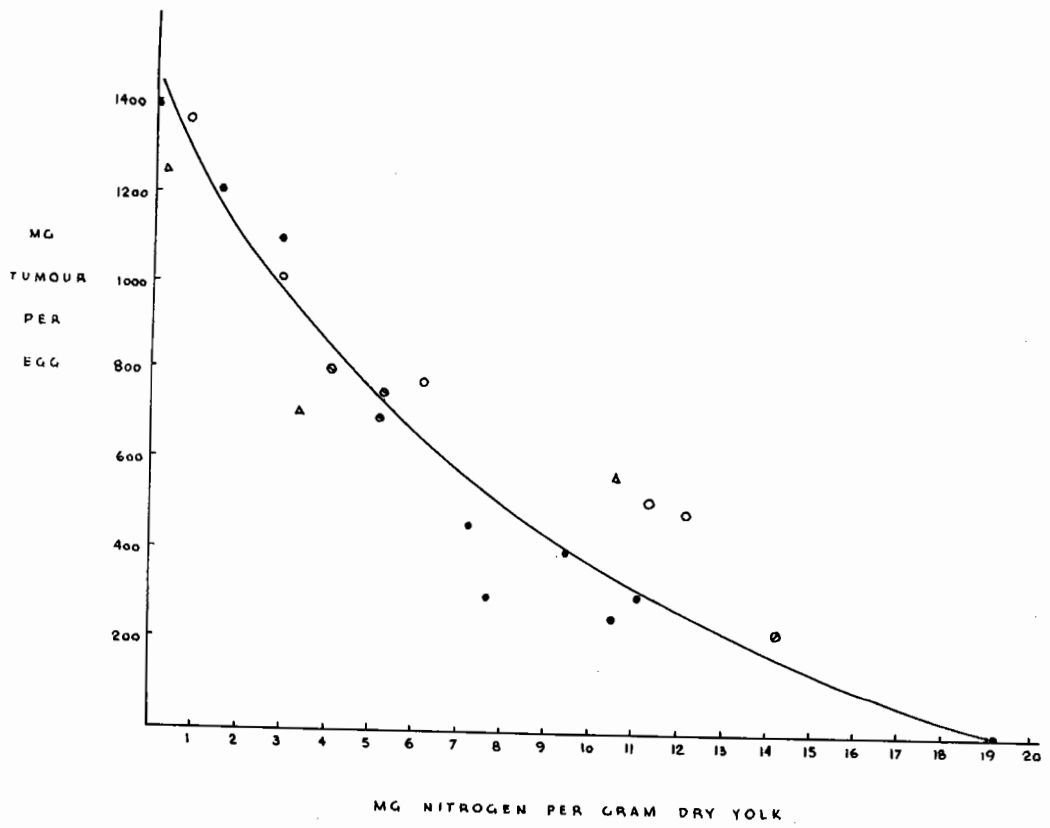


Figure 1. Relationship between size of tumour and amino nitrogen concentration in the yolk of a tumour-bearing egg.

- • Group A
- ▲ Group B
- o Group C
- ⊖ Group D

7. EFFECT OF GLYCINE UPON THE FREE AMINO ACIDS OF THE YOLK OF THE TUMOUR-BEARING EMBRYONATED EGG.

Since the free amino acids in the yolk are decreased in the presence of a tumour, it may be possible that the addition of an amino acid to the yolk during the growth of the tumour may reduce this tendency. The tumour might utilize the added amino acid and thus leave the yolk amino acids for the growth of the chick embryo. Glycine was the amino acid added to alleviate the demands on the yolk amino acids. It was added in sufficient amounts to be detected above the other amino acid nitrogen in the Van Slyke apparatus.

The yolks of 5-day old fertilized eggs were injected with 95.8 mg. glycine or 17.9 mg. N in 0.4 ml. distilled water. The tumour was placed onto the chorio-allantoic membrane one day later. The amino acids were extracted in the usual way on the 18th day. The effect of glycine upon normal fertilized eggs was investigated first and the results are given in Table X.

TABLE X

Data on Normal 18-Day Old Embryonated Eggs with Glycine Added

Embryo wt. gms.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk	Mg. N added/gm. dry yolk
Normal						
22.7	11.0	39.7	6.65	79.3	12.0	
21.7	10.8	43.0	6.1	78.5	12.75	
20.0	8.3	40.0	5.0	72.8	14.6	
22.3	9.1	44.0	5.1	77.0	15.1	
21.8	11.4	43.2	5.7	76.3	13.4	
Average						
21.7	10.1	41.9	5.7	76.8	13.6	
Glycine Added						
19.0	11.5	45.45	6.3	98.3	15.5	2.85
19.4	12.0	47.4	6.3	95.6	15.1	2.8
18.3	11.6	48.2	6.0	93.5	15.55	3.0
15.8	11.2	46.9	5.9	94.8	15.9	3.0
16.3	11.3	41.3	6.6	94.6	14.3	2.7
Average						
17.7	11.5	45.8	6.2	95.3	15.3	2.8

Actual Average Values:

(1)	Normal yolk contains	76.8 mg. N
(2)	Yolk plus glycine contains	95.3 mg. N
(3)	Glycine added per yolk	17.9 mg. N

Theoretical amount of nitrogen in yolk = (1) + (3) = 94.7 mg. N
This is in close agreement with the actual value (2) = 95.3 mg. N

Actual Average Values:

(4)	Normal yolk contains	13.6 mg. N/gm. dry yolk
(5)	Yolk plus glycine contains	15.3 mg. N/gm. dry yolk
(6)	Glycine added per yolk	2.8 mg. N/gm. dry yolk

Theoretical amount of nitrogen in yolk = (4) + (6) = 16.4 mg. N/gm. dry yolk. This is in close agreement with the actual value (5) = 15.3 mg. N/gm. dry yolk.

Results

The results given in Table X show that the addition of glycine causes a decrease in the weight of the chick embryo and that the added glycine nitrogen is quantitatively recovered from the normal yolk.

7' (b) Recovery of Glycine from Egg-Yolks of Tumour-bearing Embryonated Eggs.

Glycine was injected into normal and tumour-bearing eggs and the results are given in Tables XI and XII.

TABLE XI

Data on Normal 18-Day Old Embryonated Eggs with Glycine Added

Embryo wt.gms.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
28.0	14.6	47.1	7.7	118.7	14.1
30.2	12.5	48.0	6.5	91.6	14.1
29.0	13.3	48.8	6.8	102.1	15.0
25.7	8.3	46.7	4.4	83.2	18.9
26.1	10.4	48.8	5.3	100.3	18.8
26.8	9.6	49.3	4.85	97.8	20.2
27.1	10.9	47.2	5.75	91.7	16.0
26.5	11.3	49.1	5.75	89.8	15.6
Average					
27.4	11.3	48.1	5.9	96.3	16.5

Table continued on following page.

TABLE XI (continued)

Embryo wt.gms.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk	Mg. N added/gm. dry yolk
22.8	9.9	44.1	5.5	109.6	19.9	3.2
23.0	12.4	48.5	6.4	115.9	18.1	2.8
19.2	10.8	44.1	6.05	112.3	18.6	2.9
21.5	11.8	46.5	6.3	118.2	18.6	2.8
23.2	11.6	50.75	5.7	108.5	18.7	3.1
22.4	10.9	46.7	5.8	113.3	19.5	3.0
21.0	12.1	49.5	6.1	112.2	18.3	2.9
21.8	11.5	50.2	5.7	117.8	20.3	3.1
Average						
21.8	11.3	47.3	5.9	113.4	19.0	2.9

TABLE XII

Data on 18-Day Old Tumour-bearing Embryonated Eggs with Glycine Added

Embryo wt.gms.	Tumour wet wt. mgs.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk
17.2	515	10.8	47.7	5.65	28.2	5.0
18.1	261	8.0	39.9	4.80	50.1	10.4
19.2	438	9.3	50.1	4.65	40.0	8.6
18.6	849	12.5	49.4	6.3	31.8	5.05
16.5	1002	13.1	48.7	6.7	22.8	3.4
19.1	660	12.4	49.3	6.3	35.2	5.6
18.7	345	9.3	47.6	4.8	40.4	8.4
18.5	565	8.5	48.3	4.4	33.4	7.6
17.9	950	9.3	47.6	4.9	17.2	3.5
17.5	245	9.2	45.3	5.05	63.7	12.6
Average 17.8	580	11.5	46.6	5.3	36.3	7.2

Table continued on following page.

TABLE XII (continued)

Embryo wt.gms.	Tumour wet wt. mgs.	Yolk wet wt. gms.	% Water	Dry wt. gms.	Mg. N/Yolk	Mg. N/gm. dry yolk	Mg. N added /gm. dry yolk
15.9	993	15.0	76.0	3.6	15.8	4.4	4.95
12.6	548	11.1	44.5	6.15	44.7	7.1	2.9
17.2	522	19.6	56.4	8.55	76.9	9.0	2.1
16.3	909	11.2	49.2	6.8	30.6	4.5	2.6
18.1	301	12.1	48.7	6.2	76.9	12.4	2.9
17.5	502	10.7	51.6	5.2	60.4	12.0	3.4
18.2	718	12.1	49.8	6.05	39.0	6.45	2.95
17.7	827	10.1	50.2	5.05	35.8	7.1	3.5
16.9	381	11.1	48.7	5.7	61.0	10.7	3.1
18.1	213	12.0	49.1	5.9	86.7	14.7	3.0
Average 16.1	591	12.5	52.4	5.9	52.8	8.8	3.1

Actual Average Values:

(1) Normal yolk contains 96.3 mg. N

(2) Yolk plus glycine contains 113.4 mg. N

(3) Glycine added per yolk 17.9 mg. N

Theoretical amount of nitrogem in yolk = (1) + (3) = 114.2 mg. N

This is in close agreement with actual value (2) = 113.4 mg. N

Actual Average Values:

(4) Normal yolk contains 16.5 mg. N/gm. dry yolk

(5) Yolk plus glycine contains 19.0 mg. N/gm. dry yolk

(6) Glycine added per yolk 2.9 mg. N/gm. dry yolk

Theoretical amount of nitrogen in yolk = (4) + (6) = 19.4 mg. N/gm. This is in close agreement with actual value (5) = 19.0 mg. N/gm. dry yolk.

Actual Average Values:

(1) Yolk of tumour-bearing egg contains 36.3 mg. N

(2) Yolk plus glycine contains 52.8 mg. N

(3) Glycine added per yolk 17.9 mg. N

Theoretical amount of nitrogen in yolk = (1) + (3) = 54.2 mg. N

This does not agree with the actual value (2) = 52.8 mg. N, therefore glycine is not completely recovered from the yolk in the presence of a tumour.

Actual Average Values:

(4) Yolk of tumour-bearing egg contains 7.2 mg. N/gm. dry yolk

(5) Yolk plus glycine contains 8.8 mg. N/gm..dry yolk

(6) Glycine added per yolk 3.1 mg. N/gm. dry yolk

Theoretical amount of nitrogen in yolk = (4) + (6) = 10.3 mg. N/gm. dry yolk. This does not agree with the actual value (5) = 8.8 mg. N/gm. dry yolk, therefore glycine is not completely recovered from the yolk in the presence of a tumour.

Results

The results in Tables XI and XII indicate that injected glycine in the yolk is utilized in the presence of a tumour. A comparison of the figures reveals that the amount of free nitrogen in the yolk from the injected glycine is inversely proportional to the weight of the tumour. This relationship between the tumour weight and the amino acid nitrogen in the yolk is illustrated in Figure 2 (see page 83). Another curve was plotted which represents the theoretical amount of amino acid nitrogen which might be present in the yolk of a tumour-bearing egg in the presence of added and unutilized glycine.

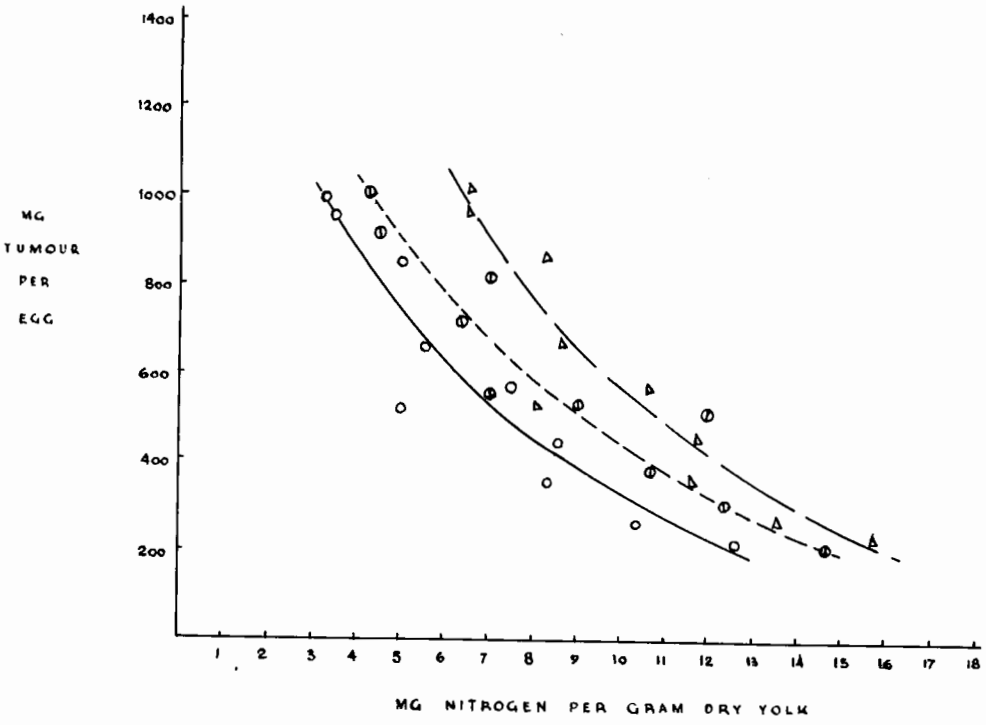


Figure 2. Relationship between size of tumour and amino acid nitrogen concentration after the injection of glycine.

o	Curve A	Endogenous free amino nitrogen in the presence of a tumour
⊘	Curve B	Endogenous free amino nitrogen plus glycine nitrogen in the presence of a tumour
Δ	Curve C	Theoretical amount of amino nitrogen in the presence of a a tumour if the glycine nitrogen is not utilized (Curve A 3.1 mg. glycine N).

Results

It can be seen from this graph that the amount of injected glycine nitrogen found in the yolk increases with a decrease in the size of the tumour. Curves B and C approach one another and would probably meet when the tumour is too small to have an effect on the utilization of the amino acids in the yolk.

The average weight of 18-day old embryos was approximately 3.0 gms. lower following an injection of glycine into the yolks on the 5th day of incubation than the average weight of normal 18-day old embryos. In the presence of a tumour the average weight of the 18-day old embryos following the injection of glycine into the yolks on the 5th day of incubation was about 1.7 gm. lower than the average weight of uninjected tumour-bearing controls.

8. IDENTIFICATION OF AMINO ACIDS IN EGG-YOLK.

Attempts to identify the amino acids in the egg-yolk were made. The normal yolk extract was concentrated 2.5 times so that there was approximately 0.125 uM amino nitrogen in each 0.01 ml. used for each spot. The chromatograms were run in two dimensions in two different sets of solvents. Propanol, acetic acid, water and m-cresol, phenol, borate buffer (131) was used for one set of solvents and propanol, acetic acid, water and phenol, water and 8-hydroxyquinoline (132) was used as the second set of solvents. Unfortunately,

the salts in the yolk were not removed and the interference from these was so great that identification of the amino acids was impossible in their presence.

DISCUSSION

It was observed that the growth of the chick embryo was inhibited by the presence of a tumour on either the chorio-allantoic membrane or in the yolk-sac. It was supposed, therefore, that there is competition between the two rapidly growing tissues, the tumour and the chick embryo, for the available nutrients in the yolk. Among the nutrients which may be affected by the presence of a tumour are the free amino acids and thus a study of the levels of these substances was undertaken.

Several investigators have studied the amino acid concentration in egg-yolks of normal fertilized eggs. Tomita obtained approximately 0.4 mg. amino nitrogen per gm. dry yolk, Aggazzotti obtained 1 mg. per gm. dry yolk, Cook obtained 2 mg. per gm. dry yolk and more recently Williams *et al*. found 0.15 mg. nitrogen per gm. dry yolk. None of these values are in close agreement with the 19 mg. per gm. dry yolk reported in the experiments of this thesis.

From the results of the control experiments it seems likely that in the past the amino acids were not completely extracted. The total amino nitrogen extracted was approximately the same for Method 1, using acetone and Method 3, using trichloroacetic acid. These methods were found to extract only 3% of the total amino nitrogen extracted by Method 4. By this method the precipitated proteins were

homogenized in the Waring Blendor and left in the acetone overnight. When the yolk precipitated with acetone or trichloroacetic acid was re-extracted with acetone and HCl, the yield of amino nitrogen accounted for the difference obtained by the two methods of extraction.

It was found that more amino acids could be extracted if the acetone and precipitated yolk were homogenized in the Waring Blendor. This innovation increased the efficiency of the extraction by about 35%. The remainder of the amino acids could be obtained when the protein precipitate was re-extracted overnight with acetone. Complete extraction occurred only when the homogenized yolk extract was allowed to stand in the acetone solution overnight.

One might suspect that the increase in the amino acids extracted by leaving the mixture overnight is due to hydrolysis of the proteins. This is not so, however, because after re-extracting the precipitate with acetone, amino nitrogen could not be detected in the extract. If hydrolysis had taken place, it would have been possible to measure amino nitrogen in the final extract. The fact that no amino nitrogen could be detected indicates that complete extraction of the amino acids had occurred.

A second possible reason for the discrepancy in the results is that the small peptides were not precipitated by the acetone. The α-amino nitrogen from the terminal

groups of the peptides may thus have been measured in the Van Slyke Manometric Apparatus. It was found, however, that acetone, in the proportion used to precipitate the proteins in Method 4, also precipitated glycyl-glycyl-glycine, glycyl-glycine and glutathione. It is, therefore, fairly certain that all the nitrogen was derived from free amino acids and little, if any, from peptides.

It is essential for the complete extraction of amino acids from egg-yolks that the precipitated protein be well homogenized in order to prevent trapping of the free amino acids in the precipitate. It is also necessary to allow the mixture to stand for some time so that complete extraction can take place.

Once the proper method for the extraction of amino acids was established, it was then possible to study the effect of the tumour upon the amino acid concentration of the yolk of an eighteen-day embryonated egg. Several changes occur in the egg when a tumour is transplanted into it. The most obvious change manifests itself in the size of the embryo. The embryos which have a tumour on the chorio-allantoic membrane weigh about 7 gms. less than the normal eighteen-day old 25 gm. embryos. It thus becomes obvious that the tumour is in some way interfering with the growth of the embryo. One of the possible ways by which the tumour can inhibit the embryonic growth is by competing

with the embryo for the available nutrients. Since most of the nutrients are found in the yolk, the tumour would probably derive much of its nourishment from the yolk. The studies on the amino acid levels in the yolk showed this to be true.

The total amino nitrogen in the yolk of a tumour-bearing embryonated egg was found to be approximately 6.1 mg. per gm. dry yolk. That of the normal egg was found to be approximately 19.1 mg. per gm. dry yolk. It was also shown that the size of the tumour bore a definite relationship to the amount of free amino nitrogen in the yolk. The larger the tumour, the less amino nitrogen was found in the yolk. This is not surprising since the larger the tumour, the greater would be its demand upon the yolk for the available free amino acids present. There is the other possibility that the demand upon the yolk nutrients is negligible and the building blocks for the tumour are obtained from the chick embryo itself without affecting the yolk. This does not seem to be the case, since there is a diminution of amino acids in the yolk in tumour-bearing eggs. It would be interesting to devise a method whereby the source of the amino acids in the tumour could be investigated.

Another interesting question arises from this - do the amino acids from the yolk pass directly to the tumour via the blood stream in the yolk-sac and chorio-allantoic

membrane, or do the amino acids enter the embryo first and then indirectly reach the tumour? Whichever pathway the amino acids take, they are probably incorporated into the tumour proteins as free amino acids without necessarily first being formed into peptides. This is the most probable way in which proteins are synthesized according to Askonas, Spiegelman and other investigators.

The fall in the level of amino acids in the yolk in the presence of a tumour brings up another question - is there a decrease in all of the free amino acids; is there a decrease in some and not in others giving an overall effect of diminution of amino acids; or is there an increase in one or more amino acids and a greater decrease in others giving a net loss of amino acids? The last explanation may be the most acceptable one because White et al. found an increase in glutamic acid of the yolk in tumour-bearing eggs. There was a corresponding decrease of glutamine.

An attempt was made to identify the amino acids by paper chromatography and then obtain their approximate concentrations by the comparison of the intensity of the colours of the spots. This was not successful, however, and thus only the total values of amino nitrogen are reported in this thesis.

There may or may not be a decrease in the water content of the yolk in the presence of a tumour. There

was a decrease in water content in one set of experiments and an increase in another set. One would expect a decrease since water is needed for the growth of the tumour. The water required for tumour growth may, however, come from the albumen of the egg.

Since there was a disappearance in the amino acids of the yolk in the presence of a tumour, the possibility that the addition of an amino acid to the yolk might replenish the lost amino acids needed for embryonic development was investigated. A saturated solution of glycine was injected into the yolk one day before the injection of the tumour. The concentration of the glycine had to be high so that it could be detected above the other amino acids. It was possible to recover all of the added amino nitrogen in the normal unfertilized eggs and also in the fertilized eggs containing eighteen-day old embryos.

At first glance it seems surprising that all of the added amino nitrogen should be recovered after being in the yolk for two weeks (from the 5th to the 18th day of development). The reason for this may be as follows: the fertilized egg is a closed biological system where all conditions are optimal for embyonic development. The addition of substances will not improve conditions but will instead interfere with development. The glycine which was added was not required for embryonic growth since there was an optimum

concentration of nutrients in the yolk in the beginning. The glycine was therefore not utilized but remained in the yolk. Whether or not the glycine remained as such or was converted to another amino acid cannot be stated. The total amino nitrogen remained the same, however.

It was found that the addition of glycine inhibited the growth of the chick embryo. This is probably due to the high concentration of the amino acid used since any substance, if used in a high enough concentration, will be toxic.

The picture is not the same if there is a tumour present in the egg. The extra amino nitrogen from the added glycine could not be recovered completely. Again the size of the tumour seemed to play a part in the recovery of the amino nitrogen. Although the number of eggs used in this study was not sufficient to prove that a relationship exists between the size of the tumour and the removal from the yolk of added glycine, the values from the hen eggs used were plotted and the curve obtained seemed to indicate that the larger the tumour in the egg, the smaller the amount of glycine nitrogen that could be recovered (see figure 2).

Another curve was plotted which represents the theoretical amount of amino nitrogen which might be present in the yolk of a tumour-bearing egg in the presence of added

and unutilized glycine. The comparision of the two curves indicates that the utilization of added glycine increases with an increase in the size of the tumour. Therefore, as the tumour size decreases, the amount of detectable glycine nitrogen increases until a point is reached at which no glycine nitrogen is utilized and, hence, all of it can be recovered.

One would expect that the addition of glycine would make more amino acids available for the development of the embryo in the presence of a tumour. This does not seem to be the case since the weight of the chick embryo may even be further decreased by the addition of the glycine as indicated by the following data (taken from tables VIII, IX, X, XII)

	Average Weight
Normal embryo	24.8 gms.
Normal embryo + glycine	21.9 gms.
Tumour-bearing embryo	17.7 gms.
Tumour-bearing embryo + glycine	16.8 gms.

The loss in the weight of the embryo caused by the addition of glycine is not as great in the tumour-bearing egg as in the normal egg. If the glycine added to the egg has a toxic effect upon the growth of the embryo, it is possible that the presence of a tumour alleviates this condition by utilizing this extra glycine and thus reducing its concentration.

SUMMARY AND CONCLUSIONS

1. Acetone was found to be an efficient solvent for the complete extraction of amino acids from egg-yolks provided that the yolk precipitate was well homogenized and allowed to stand in the acetone overnight. Hydrolysis of proteins did not take place under these conditions.
2. One amino acid did not interfere with the recovery of another amino acid during the extraction procedure.
3. Neither urea nor ammonia was found in the yolk extracts of 18-day old embryonated eggs.
4. The average weight of the 18-day old chick embryo in the presence of a tumour was less than the average weight of the normal embryo.
5. The free amino nitrogen in the yolks of tumour-bearing eggs was substantially less than that in non-tumour-bearing eggs. The concentration of the total free amino acids was shown to be inversely proportional to the weight of the tumour.
6. Glycine, injected into the yolk on the fifth day of incubation, inhibited the growth of the normal chick embryo even though the added glycine was completely recovered from the yolk after the second week of incubation.

7. Injected glycine was partly removed from the yolk in the presence of a tumour. The amount of glycine removed was shown to be directly proportional to the weight of the tumour.

BIBLIOGRAPHY.

1. Herzog, R.O., Chem. Abstract, 1925, 19, 2679, Kolloid Zeitschr., 1925 Special Number.

2. Huffner G., Arch. f. Anat. u. Physiol. (Mullers) 1892, 476.

3. Calvery, H.O., J. Biol. Chem., 1933, 100, 183.

4. Moran, T., Hale, H.P., J. Exp. Biol., 1936, 13, 35.

5. Lillie's Development of the Chick - H. L. Hamilton, New York, Henry Holt & Co., Third Edition 1952, 23.

5a. ibid., p. 28.

5b. ibid., p. 27.

6. Needham, J., Chemical Embryology, Cambridge University Press, 1931, 265.

6a. ibid., p. 302.

6b. ibid., p. 1072.

6c. ibid., p. 1068.

7. Needham, J., Biochemistry & Morphogenisis, p. 8, Cambridge University Press 1950.

7a. ibid., p. 11.

7b. ibid., p. 11.

7c. ibid., p. 66.

8. Hektoen, L., Cole, A.G., J. Infectious Dis., 1928, 42, 1.

9. The Avian Egg by Romanoff A. L. and Romanoff A.J. - J. Wiley & Son, p. 23.

10. Koga, T., Biochem. Z., 1923, 141, 430.

11. Ammon, R., Schutte, E., Biochem. Z., 1935, 275, 216.

12. Abraham, Robinson, J.R., Nature, 1937, 140, 24.

13. Osborne, W.A., Kincaid, H.E., Biochem. J., 1914, 8, 28.

14. Orru, A., Boll. Soc. Ital. Biol. Sper., 1933, 8, 286.

15. Chick, H., Copping, A.M., Roscoe, M.H., Biochem. J., 1931, 24, 1748.

16. Wolff, L.K., Lancet, 1932, 223, 617.

17. Straub, J., Hoogerduyn, M.J.J., Recueil des Trav. Chim. des Pays-Bas, 1929, 48, 49.

18. Needham, J., J. Exp. Biol., 1931, 8, 330.

19. Hale, H.P., Hardy, W.B., Proc. Roy. Soc. B. (London), 1933, 112, 473.

20. Moran, T., J. Exp. Biol., 1936, 13, 41.

21. Ridder, O., J. Morph., 1911, 22, 455.

22. Spohn, A.A., Riddle, O., Amer. J. Physiol., 1916, 41, 397.

23. Balfour, E.M., Foster, Sim M., Elements of Embryology 1877, MacMillan, cited in C. E.

24. Dumas, J.B., Cohours, L., Ann. de Chim. et de Phys. 1842, Ser. 3, 422, cited in C. E.

25. Fevolt, H.L., Advances in Protein Chemistry, Academic Press Inc., New York 1951, VI, 239.

26. Osborne, T.B., Campbell, C.F., J. Am. Chem. Soc., 1900, 22, 413.

27. Chargaff, E., J. Biol. Chem. 1942, 142, 491.

28. Alderton, G., Fevolt, H.L., Arch. Biochem., 1945, 8, 415.

29. Osborne, T.B., Jones, D.B., Amer. J. Physiol., 1909, 24, 153.

30. Levene, P.A., Alsberg, C.L., J. Biol. Chem., 1906, 2, 127.

31. Calvery, H.O., White, A., J. Biol. Chem., 1932, 94, 635.

32. Lewis, J.C., Snell, N.S., Hirschnan, D.J., Fraenkel-Conrat, H., J. Biol. Chem., 1950, 186, 23.

33. Posternak, S., Posternak, Compt. rend. soc. biol., 184, 306, 909.

34. ibid., 1615, 185, 1909.

35. ibid., 1928, 187, 313.

36. Levene, P.A., Alsberg, C.L., J. Biol. Chem., 1906, 2, 127.

37. Mecham & Olcott, J. Am. Chem. Soc., 1950, 71, 3670.

38. Plimmer, R.H.A., J. Chem. Soc., 1908, 93, 1500.

39. Jukes, T.H., J. Biol. Chem., 1933, 103, 425.

40. Shepard, C.S., Hottle, C.A., J. Biol. Chem. 1949, 179, 349.

41. Lineweaver, H., Morris, H.J., Kline, L., Bean, R.S., Arch. Biochem., 1947, 16, 443.

42. Onoe, T., J. Biochem. (Tokyo), 1936, 24, 1.

43. Needham, J. Brit. J. Exp. Biol., 1927, 5, 6.

44. Donhoffer, C., B. J., 1933, 27, 806.

45. Grankel, S., Jellinek, C., Biochem. Z., 1927, 185, 392.

46. Jukes, T.H., J. B. C., 1939, 129, 225.

47. Lintzel, W., Ber. u. d. ges. Physiol. u. Pharmacol. (Maly's), 1932, 65, 530.

48. Bornmann, J. H., Chem. Abstracts, 1932, 26, 779.

49. Rae, J.J., Biochem. J., 1934, 28, 152.

50. Riemerscheider, R.W., Ellis, N.R., Titus, H.W., J. B. C., 1938, 126, 255.

51. Sueyoski, Y., Furukobo, T., J. Biochem., 1931, 13, 155, 177.

52. Paladino, R., Biochem. Z., 1909, 17, 356.

53. Miyamori, S., C. A., 1937, 31, 7098.

54. Drea, W.F., J. Nutrition, 1935, 10, 351.

55. Plimmer, R.H.A., Scott, F.H., J. Physiol. 1909, 38, 247.

56. Jukes, T.H., Kay, H.D., J. Nutrition, 1932, 5, 81.

57. Tomita, M., Biochem. Z., 1921, 116, 12.

58. Cook, J., United States Dept. of Agriculture, Bureaux of Chemistry, Bulletin No. 115, 1908.

59. Aggazzotti, A., Arch. di Sci. Biol., 1919, 1, 120.

60. Consden, R., Gordon, A.H., Martin, A.J.P., Biochem. J., 1944, 38, 224.

61. Moore, S., Stein, W.H., J. B. C., 1951, 192, 663.

62. Spratt, N.T. Jr., J. Exp. Zool., 1946, 103, 259.

63. Saunders, J.W. Jr., J. Exp. Zool., 1948, 108, 363.

64. Ucke, F., Arch. F. Mikr. Anat., 1891, 38, 24.

65. Romanoff, A.L., Annals of the New York Academy of Sciences, 1952, 55, Art. 2, 288.

65a. ibid., p. 291.

66. Grodzinski, Z., Bull. intern. acad. polon. sci. ser. B., 1934, II, 415.

67. Romanoff, A.L., Anat. Rec., 1943, 86, 143.

68. Romanof, A.L., Romanoff, A.J., Anat. Rec., 1933, 55, 271.

69. Byerly, T.C., J. Exp. Biol., 1932, 9, 15.

70. Hughes, A.F.W., J. Anat., 1935, 70, 76.

71. Konopacka, B., Arch. Biol., 1933, 44, 251.

72. Grodzinski, Z., Bull. intern. acad. polon. sci. ser., 1947, B II, 169.

73. Wohlegmuth, J., Z. of physiol. Chem., 1905, 44, 540.

74. Tallarico, D., Arch. di Farmacol. Sper., 1908, 7, 535.

75. Remotti, E., Ricerche Morfol., 1927, 7, 199.

76. Goldstein, B., Enzymologia, 1938, 2, 193.

77. Goldstein, B., Gingburg, M., Enzymologia, 1937, 1, 369.

78. Needham, J., Proc. Roy. Soc. B. (London), 1933, 113, 429.

79. Sznerovna, E., Trav. du Lab. Physiol. de l'Institut Nencki, Varsovie, 1921, 1, No. 3 Chem. Abstract.

80. Riddle, O., Amer. J. Physiol., 1916, 41, 409.

81. Onoe, T., J. Biochem. (Tokyo), 1936, 24, 9.

82. Sendju, J. Biochem. (Japan), 1927, 7, 175.

83. Calvery, H.O., J. Biol. Chem., 1929, 83, 649.

84. Nakamura, Y., Z. F. Physiol. Chem., 1928, 177, 34.

85. Williams, M.A., Da Costa, W.A., Neisman, L.H., Marshall, L.M., Nature, 1954, 173, 490.

86. Rous, P., Murphy, J.B., J. Amer. Med. Assoc. 1911, 56, 741.

87. Murphy, J.B., J. Amer. Med. Assoc., 1912, 59, 874.

88. ibid., J. Exp. Med., 1913, 17, 482.

89. ibid., 1914, 19, 181.

90. ibid., 1914, 19, 513.

91. Karnofsky, D.A., Ridgeway, L.P., Patterson, P.A., Annals of the New York Academy of Sciences, 1952, 55, Art. 2, 327.

92. Taylor, A., Carmichael, N., Norris, T., Cancer Research, 1948, 8, 264.

93. Schoenheimer, R., Rittenberg, D., Physiol. Revs., 1940, 20, 218.

94. Halvorson, O., Spiegelman, F., J. Bact., 1952, 64, 207.

95. Rotman, Spiegelman, F., J. Bact., 1954, 68, 419.

96. Hokin, L.E., J. Biochem., 1951, 50, 216.

97. Askonas, B.A., Campbell, P.N., Biochim. et Biophys., 1953, Acta II, 304.

98. Halvorson, O., Spiegelman, F., J. Bact., 1952, 64, 207.

99. ibid., J. Bact., 1953, 65, 496.

100. Monod, J., Pappenheimer, A.M., Cohen-Bazire, C., Biochim. & Biophys., 1952, Acta 9, 648.

101. Askonas, B.A., Campbell, P.N., Biochim. & Biophys., 1953, Acta II, 304.

102. Work, T.S., Campbell, P.N., Askonas, B.A., 2nd International Congress of Biochem., 1952, 50.

103. Barry, J.M., J. B. C., 1952, 195, 795.

104. Heinberg, M., Valeck, S.F., Federation Proc., 1954, 13, 227.

105. Lotfield, R.B., Harris, A., J.B.C., 1956, 219, 151.

106. Monod, J., Cohen, M., Internat. Cong. Microbiol. Symposium, 4, Rome 1953.

107. Zamecnik, P.C., Cancer Research, 1949, 9, 612.

108. Sauberlich, H.E., Baumann, C.A., Cancer Research, 1951, 11, 67.

109. Mider, C.B., Cancer Research, 1951, 11, 821.

110. LePage, G.A., Potter, V.R., Busch, H., Heidelberger C., Hurlbert, R.B., Cancer Research 1952, 12, 153.

111. Schemin, D., Rittenberg, D., J. Biol.,Chem., 1944, 153, 401.

112. Tyner, E.P., Heidelberger, C., LePage, G. A., Cancer Research, 1952, 12, 158.

113. Winnick, T., Friedberg, F., Greenberg, D.M., J. Biol. Chem., 1948, 173, 189.

114. Zamecnik, P.C., Frantz, I.D., Lotfield, R.B., Stephenson, M.L., J. Biol. Chem., 1948, 175, 299.

115. Zamecnik, P.C., Lotfield, R.B., Stephenson, M.L., Steele, J.M., Cancer Research, 1951, 11, 592.

116. Winnick, T., Arch. Biochem., 1954, 27, 65.

117. Johnson, M.J., Energy Relations in Metabolic Reactions in H. A. Hardy (ed.) Respiratory Enzymes p.262, Minneapolis, Burgess Pub. Co. 1949.

118. Weinhouse S., Millington, R., Wenner, C.E., J. Amer. Chem. Soc., 1950, 72, 4332.

119. Kit, S., Greenberg, D.M., Cancer Research, 1951, 11, 495.

120. White, J.M., Ross, G.A.L., McHenry, E.W., Cancer Research 1954, 14, 173.

121. Harrington, C.R., Van Slyke, D.D., J. Bio. Chem., 1924, 61, 575.

122. Van Slyke, D.D., Neill, J.M., J. Bio. Chem., 1924, 61, 523.

123. Van Slyke, D.D., J. B. C., 1911, 9, 185.

124. ibid., 1912, 12, 275.

125. Levine & Van Slyke, D.D., J. Biol. Chem., 1912, 12, 301.

126. Bethke, R.M., Kennard, D.C., Sassaman, H.L., J. Biol. Chem., 1927, 72, 695.

127. Pollard, C.B., Carr, R.H., Am. J. Physiol., 1924, 67, 589.

128. McFarlane, W.D., Fulmer, H.L., Jukes, T.H., Biochem. J., 1930, 24, 1611.

129. Krebs, N.A., Henseleit, K., Klin. Worchschr., 1932, 11, 757.

130. Braganca, B.M., Quastel, J.H., Schucher, R., Arch. Biochem & Biophys., 1954, 52, 18.

131. Levy, A.L., Chung, D., Anal. Chem., 1953, 25, 396.

132. Williams, J., Kirby, H., Science, 1948, 107, 481.