A STUDY ON
THE PRIMARY CHARACTERISATION OF
AVIAN EGG-SHELL MEMBRANES

A THESIS

PRESENTED BY

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D E C L A R A T I O N

I hereby declare that the following thesis is a record of the experiments carried out by me. It is my own composition and no part of it has previously been presented for a Higher Degree.

The experiments were carried out in the Department of Biochemistry, University of St. Andrews, under the supervision of Professor G.R. Tristram.

C E R T I F I C A T E

I hereby certify that Khalida Anwar has spent four terms in Research work under my supervision and that she has fulfilled the conditions of the Resolution of the University Court 1974 No 2, and that she is qualified to submit the accompanying thesis in application for the degree of Master of Science.

UNIVERSITY CAREER

I was an undergraduate at Royal Holloway College, University of London, from October 1976 until I graduated in August 1979 with B.Sc. Honours in Biochemistry.

I then enrolled as a Research Student in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St Andrews, in October 1979.

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I. INTRODUCTORY REVIEW

INTRODUCTORY REVIEW

SECTION 1 - GENERAL

Normally the Hen egg is studied as it is easily available and is of great importance to us as a source of nutrient. Eggs of other birds have been studied. These include among the many, the Duck, Turkey, Goose, Quail and Pheasant. Such eggs are of delicatessen value.

To the housewife, the egg is food and the eggshell is a protection of the egg from physical damage and barrier to dirt and microorganisms. To the hen, the egg is a potential chick and the shell a protective covering and a source of calcium for the growing embryo (Taylor 1970).

The egg is a self-contained life support system for the developing chick. All the nutrients, minerals, energy sources and water are already present in the freshly laid egg. It, however, requires warming by the parents and periodic turning to prevent the adhesion of the embryo to the shell membranes. The egg also needs to breathe.

Breathing is by diffusion through thousands of microscopic pores in the shell. The oxygen goes in, carbon dioxide out and water is lost at a controlled rate (Lomholt 1976). If this were not to occur, the water content of the egg would increase, since, during embyronic development the burning of fat from yolk yields energy and water. Therefore for the chick to hatch, 15% of the initial mass of the egg must be lost as water over 21 days of incubation.

Gas diffusion is dependent upon Fick's law (Rahn, Ar and Paganelli 1979). That is, it is dependent on the ratio of pore area to pore length. The pore area is found to be proportional to the mass of the egg. On average, 10,000 pores are found in the shell of a hen's egg. The aggregate geometry of the pores thus determines the diffusing capacity of the eggshell. This increases with increasing incubation time. It is known that the removal

of the membranes results in increased permeability. It has been observed during incubation that the membranes are much thinned down, probably due to head movements of the embryo (Candlish 1972). This thinning of the membranes may result in the increased permeability of the egg.

In addition to the eggshell being porous enough to permit respiration of the embryo and contain various inorganic salts in amounts sufficient to satisfy the greater part of the mineral requirements of the developing embryo, it must meet two further requirements.

(1) It must be sufficiently strong and rigid to withstand the weight of the adult bird, and

(2) compact enough to prevent entrance of microorganisms and escape of too much moisture.

The strength of the shell is derived from its shape and structure. It is gracefully curved to fit the taut egg membranes, the domed architecture of which contributes to its strength. It utilises the principle of the arched stone bridge, designed to bear extraordinary weight on its convex surface. The shell has additional strength due to the radial orientation of the crystals in its outer surface. If they were parallel to the surface, they may scale and the shell would be weakened (Romanoff and Romanoff 1949).

When the egg is laid, the shell is usually sterile, soon after bacteria and moulds are present in profusion on its surface. On cooling some bacteria are drawn in through the pores. Under normal conditions further entry into the egg of these bacteria will be prevented by the physical barrier of the membrane. Some lysosomal activity is thought to be associated with the membranes (Board 1968). Further defence is provided by the proteolytic action of the albumen.

Under unhygienic conditions, the entry of bacteria is greatly facilitated. Soiled eggs when especially exposed to moisture are instantly attacked. Thus washing dirty eggs merely increases their vulnerability

especially if the water happens to be colder. At refrigeration temperatures, most bacteria are almost completely inhibited, but moulds are capable of fairly vigorous growth, if they are provided with sufficient moisture.

In order to ensure good recovery and to be able to store eggs for keeping (for not more than one year) adequate precautions are necessary. Firstly, eggs should not be allowed to soil. They should be collected as soon as they are laid. The floors of pens and cages should be cleaned regularly. Secondly, the eggs must be kept dry. Water levels must be hen-neck high (Joyce and Chaplin 1978). Following these conditions the eggs must be washed in clean disinfected water which is of the temperature of the eggs (Ostlund 1971). Such washed dried eggs are then ready for storage after they have been candled.

Candling is a procedure involving observation of the eggs over a light source. Contaminated eggs show characteristic patterns and can then be discarded. Mottled eggs are not necessarily contaminated or old. Such eggs have increased areas of translucent shells as opposed to fewer of these areas in normal eggs (Romanoff and Romanoff 1949).

Open eggs can be dehydrated and stored in the form of a powder. Intact eggs are usually stored in cold refrigeration conditions. Presence of carbon dioxide in storage atmosphere is a good preservative. Shell sealing treatments have also been used and can be expensive and tedious (Romanoff and Romanoff 1949).

In industry, eggs are opened at egg breaking plants. The eggshell and adhering albumen are waste products. The albumen is centrifuged off and the eggshell is discarded. In the USA alone, 50,000 tons of such waste is produced annually. It is fast becoming a pollution hazard. Steps are being taken to conserve this waste and to utilise it, especially as animal feed. This is further discussed later in the introduction.

The usefulness of the egg contents in the foodstuff industry is immense. This usefulness is due largely to its colloidal nature.

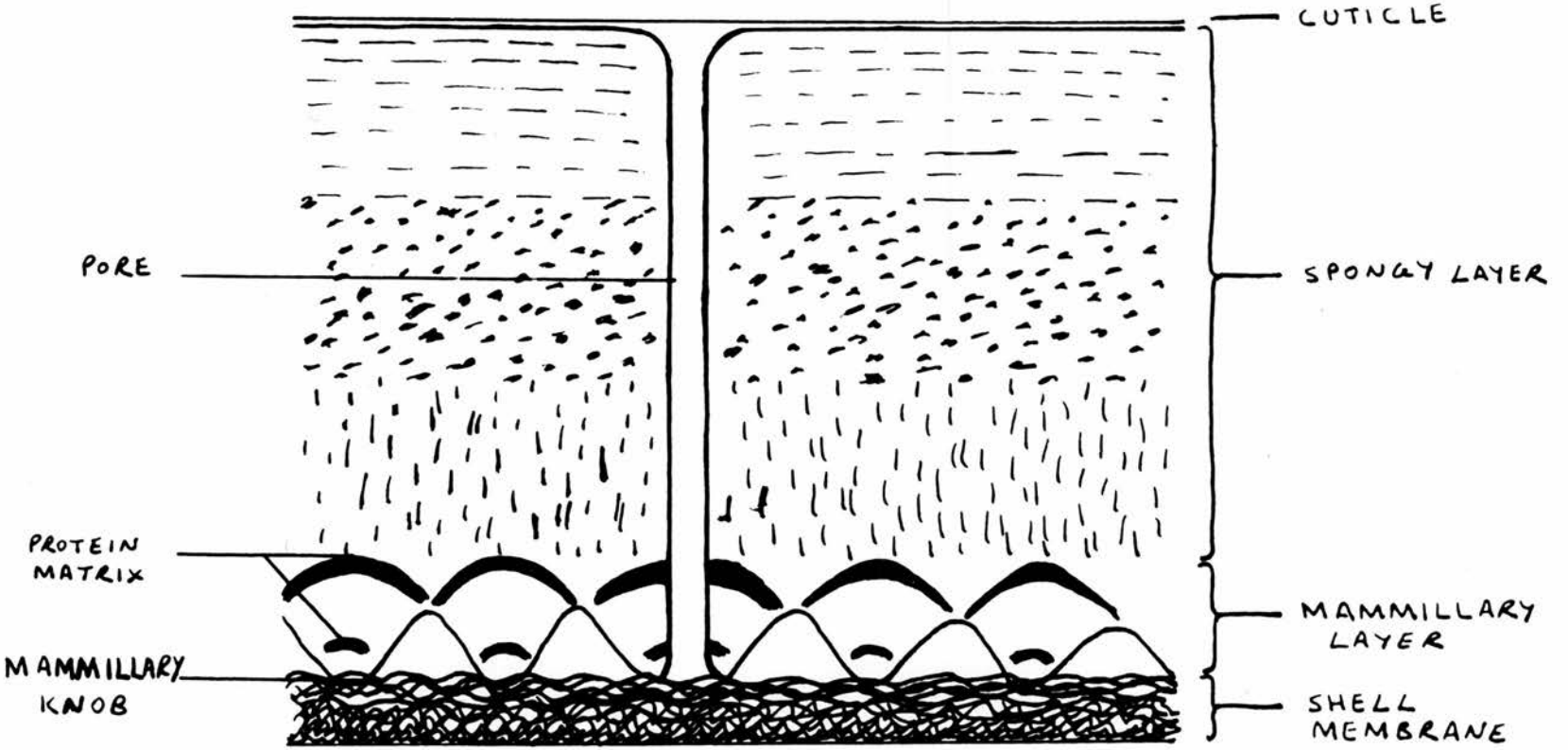

DIAGRAM 1

A VERTICAL SECTION OF THE EGGSHELL

(MODIFIED VERSION KAPLAN AND SIEGESMUND 1973)

Today, besides animal feed, fertilisers and imitation jewelry are made from its shell. The eggshell mosaics and the easter eggs have their traditional uses.

The eggshell as is known consists of an outer cuticle, the true shell and two shell membranes. These three main components have been investigated by histochemical, biochemical, ultrastructural and mineralogical techniques. The following discussion is therefore presented as a review of the most important studies of the composition of the eggshell proteins that have been made to date. It may be of interest to know that the most important reviews that have been written to date are by Stewart (1935), Romanoff and Romanoff (1949), Simkiss (1968) and Candlish (1972). The review by Candlish (1972) is concerned solely with the membrane.

The discussion to be carried out, is on all three components of the eggshell. The reason is the close relationship of the various layers of the shell and membranes as shown opposite.

It may be mentioned that the words "membrana testae" and "membrana putaminis" refer to the outer membrane and the inner membrane respectively and are now obsolete.

The word "shell" has been used synonymously by many authors to mean the true shell or shell with cuticle and membranes or both. Attempt has been made in here to use "shell" in its true context and eggshell for all the three components.

The discussion below has been divided into chapters. The membrane is in three parts: the structure, its composition and its formation. Then the mamillary layer is discussed, followed by the shell with the cuticle. Lastly, the importance of the membrane is summed.

SECTION 2 THE STRUCTURE OF THE MEMBRANE

Enclosing the albumen is the inner layer of the inner membrane or lamella. Next to this is the inner membrane. The inner membrane is separated from the outer at the air space. The air space is not present in freshly laid eggs, but forms as they cool. The air space always appears at the blunt end of the egg because the greatest number of pores occur in that area. Eggs of cold-blooded animals do not have an air space (Candlish 1972).

In freshly opened or boiled eggs, the membranes appear whitish. Running down from their blunt to their pointed end are dark stripes spaced out at regular intervals. According to Landois (1865) these stripes were impregnated with protein. It is now known that the whitish sheen is due to large masses of very fine crystals embedded in the outer membrane and the stripes are the areas in which the crystals are absent. These crystals have no relationship with the mammillary cores (Candlish 1972). In bulked preparations both the inner and the outer membranes are always pink, due to the presence of a very small amount of porphyrin pigment, oöporphyrin (Klose and Almquist 1937).

Both the inner and outer membranes may be separated from the shell by placing in dilute acid (Simkiss 1968) or boiling 2.5% NaOH (Tyler and Geake 1953). The inner membrane may be separated from the rest of the eggshell by blowing through either end of the egg of which the contents have been removed. The mammillary layer along with the membranes may be removed by treating the eggshell with 5% EDTA (ethylene diamine tetra acetic acid), pH 8.5. The mammillary layer as indicated in Diagram 1 lies between the outer membrane and the shell.

Unlike the inner membrane, the inner layer is not possible to separate and examine on the laboratory bench. All knowledge of it has been gained by

electron microscope studies. This layer is 0.1μm thick and has the same electron density and granular structure as the mantle substance which surrounds the fiber cores of the outer and inner membranes (Tung and Richards 1972). This layer was first recognised by Baer in 1837. He showed that the inner membrane had a smooth surface inside. Romanoff and Romanoff (1949) found the inner membrane to be smooth inside. They proposed, this was due to a thick coat of the "cementing material" which binds the inner and outer membranes together. They supposed this material to be albuminous in nature. The chemical analysis of this layer is discussed under membrane composition. Masshoff and Stolpmann (1961) also described the inner membrane as being smooth on the inside. It was not until the following decade that any progress was made to understand this structure.

By transmission electron microscope (TEM) work, Simons (1971) described the outer zone of this layer to be fused with the mantles of the adjoining fibers of the inner membrane. He found 16 membrane fibers were attached to the inner layer over a length of 20μm. Tung, Garland and Gill (1979) showed the inner layer to be continuous with the mantles of the fibers (Rahn et al 1979; Candlish 1972). Froix et al (1977) have described this layer to be homogeneously dense. The fibers of the inner membrane appear to terminate in it. The surface of this is coated with flocculent material that is less dense than the layer itself. Similar material is found in the interstices of the membranes. This may be the less condensed material of the mantle substance. A strong evidence thus exists for the inner layer to be an integral part of the inner membrane.

Simons (1971) also demonstrated 0.03μm width areas of greater electron density to be present in this layer. Many of such areas were found to be in the centre. On the outside of the dense centre he observed a loose structure bordered by a discontinuous array of small openings. A more ordered row

of similar openings formed inwards from the outer zone of this layer. Another interesting structure shown by Simons (1971) was the 0.03μm wide canal passing through the entire layer. Its diameter was so small that microorganisms could not penetrate it. However, this was seen only once during all preparations.

Since the fibrous reticulum of the membranes offers no barrier to entry by spoilage organisms, Candlish (1972) reported the function of the inner layer may be to inhibit the penetration of these microbes. Invading organisms had been found accumulated against the surface of the inner layer with mechanical restraint for a period up to 15-20 hours (Walden et al 1956). In contrast to these findings, Tung, Garland and Gill (1979) observed microorganisms to be present throughout the inner and outer membranes. How they breach the fiber mantles and the inner layer remains to be elucidated.

Von Nathusius (1868) was the first to distinguish between an inner and an outer membrane. Sajner (1955) was the first to recognise the mammillary layer. He termed this "Körnchenhäutchen" or granulated membrane. Purkinje (1825) described the membrane to be of a fibrous nature. Hays and Sumbardo (1927) demonstrated a lamellar structure. They showed that the different lamellae are not quite separated, the fibers of one entering the other. The same authors tried measuring membrane thickness, but found difficulty in mounting the membrane.

Romanoff and Romanoff (1949) stated that the membrane thickness was proportional to the size of the egg. As the egg size diminished so the membrane thickness decreased. They also stated that the eggs of species with thick calcified shells have thin membranes and vice versa. Tyler and Geake (1964) did not find this relationship in ratite birds. Furthermore, Candlish (1972) found no correlation between the thickness of the membranes and thickness of the shell for individual eggs or strains. However, Simons

(1971) was able to show that the second egg of a two-egg clutch had thinner membranes than the first egg; the second egg in a three-egg clutch had the thickest membranes and eggs from larger clutches varied with the seasons. Balch and Cooke (1970) were able to show that eggs laid in the springtime had thinner membranes than the eggs from the same bird which had lain in the summer season. The explanation for this is unknown. A greater understanding is yet needed. It is also known that eggs from older birds have a thinner membrane. Romanoff and Romanoff (1949) also mentioned that membranes are not of uniform thickness from one end of the egg to the other, but are thickest at the blunt end. Simons (1971) was able to show that membrane thickness was more constant round a particular latitude than along any longitude.

It is known that the outer membrane is thicker than the inner membrane but there is considerable variation in published values (as shown below). However, there is a significant correlation between weights of inner and of outer membrane. The mean ratio of the two membranes being 5.8:1 (Balch and Cooke 1970). This ratio implies a considerable difference in the tightness of packing of the fibers in the two membranes.

It may be of interest to mention here that the mean ratio for thickness of outer membrane to inner membrane is about 3:1. Hays and Sumbardo (1927) found a total membrane thickness of between 56 and 75μm of which the inner membrane varied from 13-17μm, and the outer from 43-58μm. Moran and Hale (1936) gave values for the membrane between 70-84μm. The inner membrane measured 40-48μm, the outer 30-36μm. This was the only published work found with the thicker inner membranes. Romanoff and Romanoff (1949) gave 65μm thickness for the membrane of Leghorn with the inner membrane being 15μm and the outer 50μm. Similarly for the Bantam the membrane measured 50μm, the inner being 10μm and the outer 40μm. Simons and Wiertz (1963) gave a value of about 70μm thick membrane, the inner being 22μm and the outer being 48μm thick. Simons (1971) gave a

total thickness of 70μm with the inner being 22μm and the outer 48μm, thereby confirming previous results. Tung and Richards (1972) gave a total thickness of 73-114μm.

Electron microscope studies indicate that the inner membrane is more dense than the outer membrane. This is contrary to expected results since the outer membrane is six times heavier and three times thicker and would be expected to be twice as densely packed (Balch and Cooke 1970). That this is not a coating artefact is obvious from the reticular character of the fibers of the membranes.

Attempts have been made by many authors to separate each of the membranes into a number of layers. The results are variable.

Blasius (1867) and Von Nathusius (1868) were the first to recognise that more than two layers could be separated from the membrane. As mentioned earlier, Hays and Sumbardo (1927) were able to demonstrate that the lamellae were interlayered. Any real attempt at separating the different lamellae was carried out by Moran and Hale (1936). They found the inner membrane to be composed of two layers; the outer of three layers, each layer having a mesh of fibers. The explanation given by these authors was that the outer layer of the outer membrane is composed of coarse fibers of keratin (2.5μm diameter). The other two layers contain finer fibers (0.8μm diameter) of mucin. The indistinct layers of the inner membrane consist of keratin and mucin fibers.

Simons (1971) examined both the inner and outer membranes and detected a number of layers in each. He observed in the inner membrane (from inside to outside), first a layer of transversely cut fibers (2-3 fibers thick), then a second layer of longitudinally cut fibers (1-3 fibers thick), and a third layer of again transversely cut fibers (4-10 fibers thick). In the first layer he noticed irregular open meshes of about 2x3μm. The second layer was very dense. The third layer had more open meshes than the first of size 5x7μm.

In the outer membrane he observed six layers of fibers (Simons 1971), again lying in the two perpendicular directions. In the longitudinal section from side of inner membrane, the layers were 5-9, 3-5, and 1-2 fibers thick. In the transverse section they were 5-8, 1-4, and 4-6 fibers thick from side of inner membrane. The meshes in this membrane were 8-10μm in size. The importance of the size of the meshes will be further discussed later in the introduction. Simkiss (1968) having observed the results of Simons and Wiertz (1963) (similar to Simons, 1971, above), criticised the work of Moran and Hale. This author wrote that the keratin and mucin fibers may be represented as various thicknesses of mantle and core.

Simons (1971) noticed that at the line of contact between the inner and outer membranes only eleven fibers of the inner membrane were fused by their mantles to those of the outer membrane fibers. Two fibers of the outer membrane were fused with those of the inner membrane. The contact between the two membranes appeared to be loose.

Later Tung (1970) and Fujii and Tamura (1970) considered that in both the inner and outer membranes the fibers are randomly orientated in directions parallel to the egg surface. Candlish (1972) was able to show a typical radial section with the membranes randomly orientated in the tangential plane. It was considered natural that some fibers should run parallel. Moreover, Candlish (1972) reported

> "The egg probably rotates in the oviduct .... and fibers deposited on a moving surface will tend to be 'spun' so that tracts with preferential orientation are bound to arise."

It is at such points that the membrane can be teased apart.

Furthermore, Candlish (1972) indicated that the distinctness between the inner and outer membranes may be illusory. The abrupt thickening of the fibers one third of the way from the inside may produce a natural

cleavage plane rather than a functional division. Adequate proof of this statement is not yet available. However, some eggs with poor shells having only one membrane (Candlish 1972) have been found. Such eggs are abnormal, perhaps thickening of the fibers has not taken place and the plane of cleavage does not therefore exist. It is interesting to know that Romanoff and Romanoff (1949) do not mention any such eggs in their list of abnormalities.

Coste (1847), Blasius (1867), Von Nathusius (1868), Masshoff and Stolpmann (1961), Simons (1971) and Tung and Richards (1972) among many more have shown that the outer membrane has thicker fibers than the inner one. Von Nathusius (1868) noticed that the broader fibers, especially in the outer layer, may be formed by more than one fiber. This author stated that the membrane fibers of larger eggs were not thicker than those of smaller eggs. Later Simons (1971) was to show composite bundles of fibers in both the membranes, more so in the outer membrane. In the outer membrane he could trace bundles of up to four fibers. Such thick fibers were found to run almost parallel forming diamond shaped meshes. However, Kaplan and Siegesmund (1973) observed varying sizes of anastomosing fibers in the inner membrane. The larger fibers were shown to be composed of parallel accretions of smaller fibers. In the outer membrane similar fibers were apparently homogeneous. It is obvious that much more study is needed for a clear understanding of the fiber layout.

The thickness of the fibers is also variable. However, Simons (1971) concluded that on the whole the values given by some of the authors are comparable with that of his own. The average fiber diameter in the inner membrane is about 0.9μm with maximum size being 1.5μm. The average value compares well with that of Moran and Hale (1936), 0.8μm, and also that of Wolken (1951), 1.0μm. The outer membrane has a maximum diameter of over 3μm

and an average diameter of about 1.3μm. Once again the values are comparable. Simons (1971) also found the fibers of the duck and turkey eggs to be about the same in diameter as those of the hen egg.

The structure of the fibers was first shown by Romankewitsch (1932) using a silver impregnation method. It was possible to distinguish a dense outer layer and a less dense core. It was not until 1961 that Masshoff and Stolpmann saw fibers with cores surrounded by a less dense mantle of thickness 0.5μm. Between the core and mantle was a cleft 0.3-0.1μm wide. By enlargement of the fiber core they could determime fibrils with a diameter of 3-4nm. They reported that the fibrils were embedded in a matrix. These results have yet to be confirmed.

Simons (1971) was also able to show a dense core with a less dense mantle. This author observed holes in the cores of especially thick fibers. They had a maximum diameter of 0.3μm. He reported that these holes were formed by a shrinkage of the mantle-like material present in the cores. Candlish (1972) described them to be an artifact probably due to inefficient penetration of the fixative. The clefts observed by Simons (1971) were less thick than those mentioned by Masshoff and Stolpmann (1961). It is possible that these are also an artifact (Candlish 1972; Tung and Richards 1972).

Balch and Cooke (1970) noticed that the sheath (mantle) to core ratio was different in the inner and outer membranes. They believed that this could account for the difference in the composition of the two membranes (see later in introduction - next chapter). These authors found that the compositional difference between the mantle and the core may well account for the difference in chemical composition between the two membranes.

Simons (1971) showed that the mantle was thicker in the fibers of the outer membrane. Tung and Richards (1972) considered the thickness of

the mantle to be variable in both the membranes in the two different strains they studied. However, they showed the core diameter varied in the outer membrane of the white Leghorn. This was not observed in the New Hampshire strain. They also showed that the inner membrane of the white Leghorn strain had a more open structure. Whether real differences do exist in the structure of different strains has yet to be proved.

Romanoff and Romanoff (1949) mentioned that a cementing material was present in the interstices of the fibers. Candlish (1972) has described an amorphous material that is visible only in some sections round the membrane fibers. The nature of the material is unknown. Froix et al (1977) also mention such a material to be present around the fibers of the membrane. However, they report this to be the less condensed form of the mantle substance. Fujii and Tamura (1970) had previously mentioned that the mantle was derived from a "cementing material".

The mantles of the outer membrane fibers are connected by fine structures or lamellae which are about 90 $\AA^{o}$ thick. These are protein in nature (Candlish 1970). These connections endow the membrane with a secondary reticulum thereby reinforcing the existing reticulum of the membrane. Due to the fine nature of these connections, they can be easily broken by contact with buffer or even water. The breakage may be caused by removal of a soluble component or by a physiochemical effect, for example osmotic swelling. These broken threads are seen as buds on the fibers under the electron microscope. Since then the presence of these buds have also been shown by other groups of workers (Simons 1971; Kaplan and Siegesmund 1973; Froix et al 1977). Simons (1971) mentioned that these threads are also present in the inner membrane.

Romanoff and Romanoff (1949) observed that the membranes are pliable when moist but brittle when dry. Though they are thin, they are tough in

the moistened state. This toughness compensates for the brittleness of the shell.

Candlish (1972) also noted that the sheets of the membranes are quite strong. When in strips, the doublet can be pulled hard without rupture until the sudden split. Thus Candlish (1972) wrote "... it is elastic up to a distinct failure point".

Froix et al (1977) carried out an excellent set of experiments using the relaxation technique to show the physical properties of the membrane. In their own words:

> "The structure is capable of adsorping water in excess of 2.5 times its weight. The water containing species give rise to dielectric and n.m.r. relaxations associated with tightly bound water.
>
> "An additional relaxation observed at low temperatures is the facility of the membrane to change its deformation characteristics from that of a brittle to a low modulus elastomeric material."

They noted that this was only true of the water-saturated non-heat treated material. Such a heat-treated material they observed failed in a "brittle manner".

The above study has shown that a further exploration of some areas of this subject is necessary. This may reveal a clear structure of the membranes.

In summary, both the membranes are composed of fibers randomly orientated in a tangential plane. The two may be loosely connected. The fibers of both consist of a central core surrounded by an electron dense mantle, the boundaries of which are diffuse. The dense fibers of the inner membrane fuse to form the granular structure of the inner layer.

SECTION 3 - THE CHEMICAL COMPOSITION OF THE MEMBRANE

As mentioned earlier, the fresh membranes in bulked preparations always appear pink. This is due to the presence of the porphyrin containing pigment Oöporphyrin. At first it was believed that Oöporphyrin was derived from the bile pigments biliverdin and bilirubin. This theory has since been disproved. The Oöporphyrin pigment is now said to be formed from haemoglobin of red blood corpuscles by a series of transformations (Romanoff and Romanoff 1949). A faint purple pigment, non-porphyrin in nature, was originally thought to be present (Brooke 1937) in the membranes. However, nothing more of it has been mentioned in the literature.

Lipids other than the porphyrin pigments are also present in the membranes. It is believed (Candlish 1972) that these lipids are probably not firmly bound as lipoproteins or lipopolysaccharides since they can be extracted at low temperatures with neutral methanol and chloroform. Wedral et al (1974) using a similar procedure were able to extract such lipids from the separated inner and outer membranes. The inner membrane was found to have a higher lipid content (3.5-4.2%) than the outer membrane (1.9-2.7%).

Previously Hasiak, Vadehra and Baker (1970a,b) had carried out an extensive study of the lipid components of the eggshell structures. They had shown both the neutral and polar lipids to be present in the inner and outer membranes. The total lipid content varied from strain to strain and was found to decrease with age. The total neutral lipid and total phospholipid also varied from strain to strain. In the White Leghorn, an average 1.5% of total lipid was present in the outer membrane and an average 3.1% in the inner membrane. However, Suyama et al (1977) have shown the eggshell membranes to have an average total lipid content of 1.35%.

The ratio of neutral lipid to polar lipid is apparently 6:1 (Suyama et al 1977; Hasiak et al 1970b). Thus 86% of the total lipid fraction

is composed of neutral lipids and 14% of polar lipids. The neutral lipid fraction mainly consists of cholesterol, cholestryl ester and diglycerides. The minor components are free fatty acids, triglycerides and monoglycerides. This pattern of the constituents of the neutral lipid fraction is not unique to the eggshell membranes but forms a general pattern throughout, in all animal tissues.

However, the polar lipid composition of the membranes is rather unusual. Phosphatidylcholine which is a major component in phospholipids of almost all animal tissues is a minor component in the eggshell membranes. In this structure sphingomyelin is the major component (Suyama et al 1977). Other phospholipids which have been identified as minor components of the membrane include phosphatidylethanolamine, phosphatidylglycerol, phosphatidic acid, and lysophosphatidyl choline.

About 14 different fatty acids (Hasiak et al 1970a) were found to be present in the membrane. Suyama et al (1977) have shown that there may possibly be as many as 17 different fatty acids in this material. Moreover, Hasiak et al (1970a) had described the fatty acids in the neutral and polar lipid fractions to be similar. A 19:1 fatty acid was shown to be the major component of the neutral and phospholipids of both the inner and outer membranes. This fatty acid was not present in the interior of the egg. They also showed that ageing birds had progressively more of a 21:1 fatty acid in the inner membranes and less in the outer membranes. However, three fatty acids, palmitic (16:0), stearic (18:0) and oleic (18:1) have been more recently shown to be present in highest concentrations in both the fractions (Suyama et al 1977). Two more fatty acids arachidonic (20:4) and docasanoic (22:0) are also present in high concentrations, but only in the polar fraction.

This obviously indicates that the true identity of the fatty acids in both the lipid fractions has yet to be identified.

That the membranes are almost entirely protein in form has long been known. At first it was believed (Landois 1882) that they were a felt-like mass of fibrinous or albuminous fibers. This idea was based on the possible precipitation of albumen in water. However, Von Nathusius (1882) considered the membranes to be composed of elastin. The cuticle too was considered to be elastin in nature. The composition of the cuticle will be discussed later in the introduction. Lindwall (1881), and Liebermann (1888) nevertheless had shown that the protein in the membranes was keratin-like. This theme persisted into the present day.

Calvery (1933) isolated such a protein by suspending the eggshells in acid and pepsin. The dried material was subjected to amino acid analysis. His conclusion was based on the tentative definition of a keratin as stated by Block and Vickery (1931):

> "A keratin is a protein which is resistant to digestion by pepsin and trypsin, which is insoluble in dilute acids and alkalis, in water and in organic solvents, and which, on acid hydrolysis yields such quantities of histidine, lysine and arginine that the molecular ratios of these amino acids are respectively approximately as 1:4:12.".

The authors then mentioned that these proteins also yield large proportions of cystine though this is not necessarily a characteristic feature. However, present-day definition states a keratin to be a tough fibrous protein containing much sulphur and occurring in the epidermis of vertebrates (Abercrombie et al 1972).

Later, Wolken (1951) on the basis of x-ray diffraction analysis and infra-red absorption spectra claimed that the membrane is in the form of unorientated α-keratin helices. Terepka (1963) criticised the quality of Wolken's x-ray diffraction patterns and reported that he was unable to find spacings typical of a keratin constituent. Moreover, Terepka (1963) stated:

> "Calvery's chemical amino acid analysis done in 1933 can hardly be accepted today. Amino acid analyses even by present day methods are not relied upon for such identifications.".

Terepka (1963) was definitely not convinced that the membrane was a keratin.

Yet this belief continued. Using histochemical techniques, Moran and Hale (1936) and Simkiss (1958) showed that the membranes mainly consist of keratin. As reported earlier, Moran and Hale (1936) had described both the outer and inner membranes to be composed of different layers of keratin and mucin fibers. Simkiss (1958) established a positive reaction for sugars bound in the membrane fibers.

The differentiation of the membrane fiber into core and mantle as seen under the electron microscope immediately led to the supposition that they are chemically distinct. On the basis of the filamentous appearance of the core material, Masshoff and Stolpmann (1961) concluded that the core consists of "ovokeratin". Since the mantles were eroded by the action of hyaluronidase and papain, it was proposed that they contain mucopolysaccharides.

The first amino acid and carbohydrate analysis by modern methods was carried out by Baker and Balch (1962). They used the combined shell membranes from a Light Sussex x Rhode Island Red strain. These authors, also, concluded that the membranes were mainly keratin. They found their preparation had a high content of cystine, glutamic acid, histidine and proline. Traces of sulphate were also to be found. The ash and nitrogen content were determined. Later, Simkiss (1968) also reported high values for cystine (9.59%), histidine (3.71%) and proline (8.05.).

A small amount of carbohydrate was present in the membranes (Baker and Balch 1962). This contained hexosamines and galactose with traces of the sugar mannose. Uronic acid and fucose were not detected. The authors reported that the carbohydrate is unlikely to be a contamination from egg white since the latter contains more mannose than galactose and the ratio hexosamine to neutral sugar varies considerably between the two components. Since the membrane fibers and the mammillary cores are so tightly complexed, the authors proposed that the carbohydrate may in fact originate from the cores.

TABLE I

The carbohydrate content of the shell membranes (as percentage dry matter) of the various strains of Hen

CONSTITUENT	THORNBER '404' CANDLISH 1972			LS X R1R BAKER AND BALCH 1962			WL X R1R BALCH AND COOKE 1970		
	1	2	3	1	2	3	1	2	3
Hexosamine:	0.8	0.6	-	-	-	1.45	-	-	-
Galactosamine	-	-	-	-	-	-	0.12	0.14	-
Glucosamine	-	-	-	-	-	-	0.51	0.92	-
Neutral sugar	0.9	0.9	-	-	-	1.97	2.55	2.54*	-
Sialic acid	4.0	3.5	-	-	-	-	0.18	0.15	-
Uronic acid	0.03	0.03	-	-	-	0	-	-	-

1 refers to inner membrane

2 refers to outer membrane + mammillary cores

3 refers to inner + outer membrane

* refers to hexoses

According to Balch and Cooke (1970) the eggshell membranes are mainly protein and less than 4% is carbohydrate. This contains mostly the neutral sugars and hexosamines (see Table I). In the neutral sugars, the concentrations of galactose and fucose far exceed that of glucose and mannose. Sialic acid is present in small amounts. The authors observed that the carbohydrate is more concentrated in the inner membrane. Moreover, the sialic acid and glucosamine content is greater than in the outer membrane.

During autoclaving, more carbohydrate was found to dissolve from the membranes than the total dry matter (Balch and Cooke 1970). This led to the suggestion that the carbohydrate was not uniformly distributed. Electron micrographs confirmed this result. The mantle was much more dissolved than the core. It was therefore proposed that the mantle was glycoprotein in nature. The core was considered to be keratin. However, Balch and Cooke (197)b) indicated that some neutral sugar may also be present in the core. To further enhance the difference between the core and mantle, hydroxyproline was reported to be present only in the mantle of the fibers.

The outer membrane was much more affected than the inner membrane during the autoclaving. Moreover, it had turned yellow quite rapidly. The yellow colour was due to the decomposition of the sialic acid. The authors therefore claimed that the inner and outer membranes were different, not just physically but also chemically. They believed that the carbohydrate was bound differently in both the membranes. However, no evidence is yet available to verify this statement.

Since hydroxyproline is to be found in both collagen and elastin, Simons (1971) carried out specific histochemical tests to prove the presence of one or the other in the membranes. This author obtained a positive staining reaction for elastin but not for collagen. Moreover, Simons (1971) claimed that the hydroxyproline content in elastin and his membrane preparation

TABLE II

Analysis of Lipid-free Shell Membranes and the fibrous Proteins

Values as percentage dry mass

	INNER[1] MEMBRANE	OUTER[1] MEMBRANE + MAMILLARY CORES	MICRO-[2] FIBRILS ENZYMATIC DIGEST	KERATIN[3] WOOL MERINO SHEEP	COLLAGEN[3] SKIN (GELATIN) BOVINE	ELASTIN[3] LIGAMENTUM NUCHAE 0.1N NaOH at 98°C
Aspartic acid	6.6	6.4	9.25	5.86	5.79	0.60
Threonine	5.3	4.3	4.73	5.52	1.88	0.85
Serine	5.1	4.2	5.28	7.97	3.49	0.74
Glutamic acid	10.1	9.7	9.83	12.74	10.00	2.11
Proline	6.8	6.9	7.35	5.70	13.79	12.82
Glycine	4.9	4.4	14.2	4.17	20.9	20.40
Alanine	2.6	2.3	8.26	3.41	8.78	18.20
Cystine	7.8	8.4	5.63	10.77	0	0
Cysteine	-	-	-	n.d.	0	0
Valine	3.9	4.5	6.97	4.80	2.19	15.40
Methionine	3.3	3.4	1.30	n.d.	0.78	TRACE
Isoleucine	2.0	2.1	4.38	3.21	1.48	3.02
Leucine	3.9	3.1	6.55	7.71	2.87	7.59
Tyrosine	2.2	1.3	2.76	4.97	0.26	1.44
Phenylalanine	1.9	1.5	3.28	3.54	1.99	4.90
Lysine	2.9	3.1	3.67	2.92	3.94	0.53
Histidine	2.8	3.2	1.15	1.62	0.69	0
Arginine	5.6	5.8	4.23	4.58	7.89	0.72
Hydroxyproline	1.2	0.8	0.17	0	12.17	1.29
Hydroxylysine	0.3	0.2	0.07	-	0.86	-
Tryptophan	2.3	2.7	1.19	n.d.	0	0.09
Amide nitrogen	0.8	1.7	n.d.	n.d.	(0.11)	n.d.
% Amino acids	82.3	80.0		89.5	(100.9)	90.7

1 DATA from Candlish (1972)

2 DATA from Ross and Bornstein (1969)

3 DATA from Tristram and Smith (1963)

was the same. The specific orcein stain for elastin was located in the mantles of the fibers. Simons (1971) therefore proposed that the mantle contains elastin and the core consists of keratin.

The presence of the hydroxyproline in the membranes led Candlish and Scougall (1969) to examine the hydrolysates for hydroxylysine (Balch and Cooke 1968). Hydroxylysine is considered to be exclusive to collagen (3-10 moles/10^5g of protein) in animal tissues. However, claims have been made for its presence in keratin (Eastoe 1967). Kersten and Zürn (1959) found it in hydrolysates of wool (1.2 moles/10^5g protein). On the analyser they obtained a double peak composed of hydroxylysine and allo-hydroxylysine. Besides these claims, many different keratins have been studied (Fraser et al 1965; Beyer and Schenk 1969), in none of which has hydroxylysine been detected. It has also been reported to be present in intercostal cartilage and in a non-collagenous fraction from invertebral disc (Steven, Jackson and Broady 1968,1969).

The hydroxylysine was detected in hydrolysates of both the inner and outer membranes by ion-exchange and paper chromatography (Candlish and Scougall 1969). The dinitrophenylated hydrolysates were applied to paper chromatograms and the N-ε-DNP-hydroxylysine determined. Complete amino acid analysis of the hydrolysates showed hydroxylysine to be the least abundant of the known amino acids in the membranes (1-2 moles/10^5g protein). Furthermore, the results indicated that hydroxylysine was derived from lysine by a post translational hydroxylation step.

It so appears that the membrane contains both collagen and elastin. Yet according to Ross and Bornstein (1969) elastin contains hydroxyproline but not hydroxylysine or cystine. Cystine is plentiful in the membranes but it too is absent in collagen* (see Table II). However, reports indicate that non-fibrous cystine-containing proteins are present in various connective tissues; for example, the microfibrillar protein associated with elastin

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* More recent studies show that collagen does contain cystine, for example as in the skin tissue, the aorta and other blood vessels.

fibers (Ross and Bornstein 1969) and the acidic structural protein of the skin and cartilage (Furthmeyer and Timple 1970).

Candlish (1972) believed that the membrane proteins are related to those in the vitelline membrane, the shell matrix and the cuticle in all of which cystine is present. Further evidence was provided from N-terminal amino acid studies (Candlish 1972). Aspartic acid was common to all four sources. Later, Tung, Garland and Gill (1979) questioned this proposition.

Since the cystine-containing proteins of the matrix, cuticle, and vitelline membrane contain neither hydroxylysine nor hydroxyproline, it appears that all four (including the membrane) possess a common protein, which is insoluble and acid in nature, perhaps very similar to that described for skin. Candlish (1972) wrote:

> "This can no longer be called ovokeratin; if 'insoluble, acidic cystine-containing protein of the egg coverings' is too lengthy a term 'ovocapsin' might be a suitable name.".

It was indicated by Candlish (1972) that ovocapsin has a great tendency for cross-linkages with other proteins. In the membranes it may be with collagen. This author reported that the cross-linkage is alkali-labile, which seemed to implicate a role for carbohydrates. Many glycoproteins are known to contain such a linkage in which the heterosaccharide moiety is joined to the peptide chains. Whether ovocapsin is a glycoprotein like the rest of the egg proteins has not yet been definitely proved. Candlish (1972) proposed that ovocapsin may constitute the greater part of the cores and not ovokeratin. Some collagen may also be present. This author indicated that the mantles are probably not mucopolysaccharide in nature because the carbohydrate content of the membranes is too low (<6%).

It now seems reasonable that keratin should be excluded as a constituent of the shell membranes and instead collagen can be considered a

strong possibility. This is in view of the presence of hydroxylysine and the ability of collagenase to digest these membranes (Candlish 1972). Furthermore, on comparing the properties of the membrane with the other structural proteins (see Table III), Candlish (1972) concluded that collagen is a definite component. However, the membranes are not very soluble in classical collagen solvents and nor have the typical banded fibrils been detected, although this does not necessarily occur in all collagens.

When Britton and Hale (1977) studied the amino acid analysis of membranes from young and old hens, they carried out performic acid oxidation prior to hydrolysis. They found that the membranes contained relatively large amounts of the sulphur containing amino acids, cystine (9-10%) and methionine (3-4%). They did not detect any hydroxyproline. The authors concluded that the membranes were keratin in nature. Previously Cantoni and Beretta (1976) on the basis of their amino acid analysis had concurred.

However, in 1978 Leach and coworkers definitely showed that the eggshell membrane contains both hydroxyproline and hydroxylysine. Moreover, Leach and Rucker (1978) stated that the major component of the membrane is an extensively insoluble fibrous protein. They found the membrane remained insoluble even after performic acid oxidation. This suggested the existence of cross-links other than disulphide cross-bridges which make the protein resistant to solubilization.

Previously, Leach and coworkers (1978) working with copper deficient laying hens had found that the eggshell membranes of such hens were abnormal. They were both physically and chemically deranged. Amino acid analysis showed that the lysine content of the membranes had considerably increased. This was also known to occur in copper-deficient connective tissue proteins (Franzblau 1971). Therefore, the most likely explanation for the role of copper in membrane formation appeared to be analogous to the role that this element played in connective tissue cross-linkages.

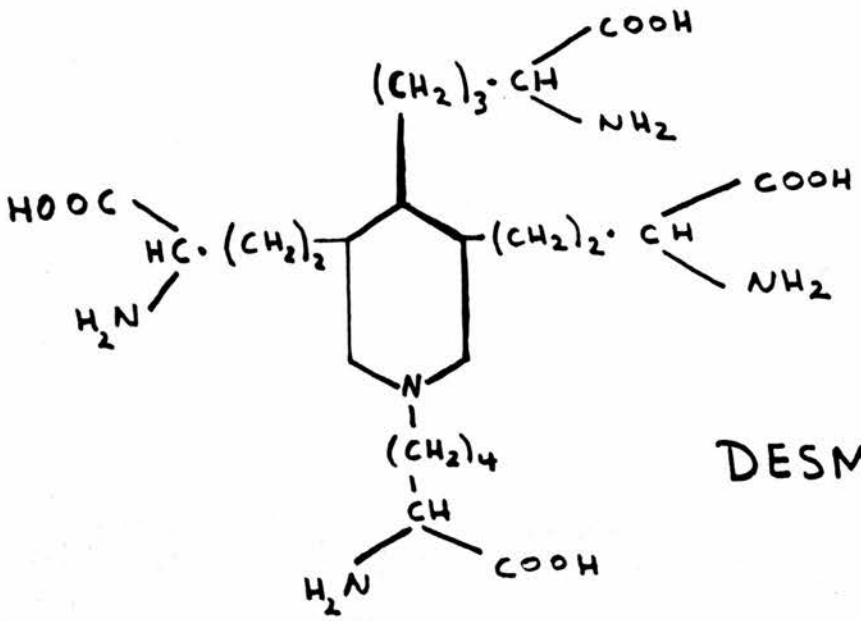
TABLE III

Comparison of "Key" amino acids and other properties of various structural proteins with those of the egg coverings (adapted from Candlish 1972)

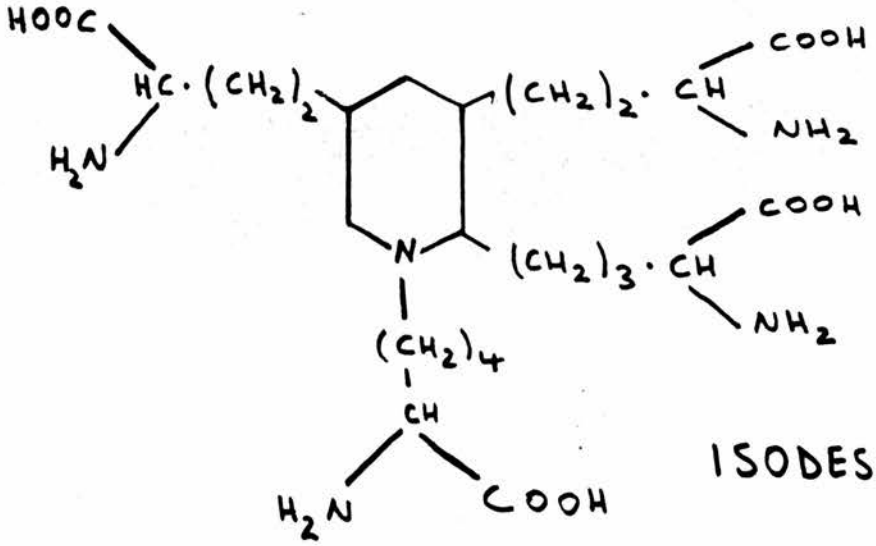
	CYSTINE	HYDROXYPROLINE	HYDROXYLYSINE	INTRACELLULAR	EMBRYONIC ORIGIN	PROMINENT SOLVENTS
Shell Membrane Proteins	+	+	+	-	Mesodermal	NaOH
Shell Matrix Proteins	+	-	-	-	Mesodermal	NaOH
Egg cuticle	+	-	-	-	Mesodermal	NaOH
Vitelline membrane	+	-	-	-	Mesodermal	?
Elastin	-	+	-	-	Mesodermal	None
Keratin	+	-	-	+	Ectodermal	Reducing Oxidising Agents
"Connective Tissue Proteins"	+	-	-	-	Mesodermal	NaOH
Vertebrate Collagens	-	+	+	-	Mesodermal	Dilute acid Hot TCA Hot water
"Primitive collagens"	+	+	+	-	Mesodermal	"α-Amylase"

FIG. 2.

It can be seen from the structure of these polyfunctional amino acids that they can cross-link up to four polypeptide chains.



DESMOSINE



ISODESMOSINE

DIAGRAM 2

The effects of copper-deficiency and the lathrogen, β-amino-propionitrile or BAPN appear to be alike with respect to connective tissue proteins (Franzblau 1971). So when BAPN was fed to the hens, this resulted in the production of eggs, similar in appearance to those observed with copper-deficiency. Moreover, the copper content of the BAPN-treated membranes was normal. This suggested that copper was not directly involved in membrane structure.

BAPN, it is known, inhibits connective tissue cross-link formation by acting as an irreversible substrate inhibitor to lysyl oxidase. Lysyl oxidase selectively oxidises the ε-amino groups of specific lysine residues to α-amino adipic acid- -semialdehyde residues. Copper is necessary for the activity of this enzyme (Franzblau 1971). This enzyme has very recently been isolated from the hen oviduct, thus strongly implicating the role that it may play in membrane formation (Leach et al 1980).

The aldehydes formed by the reaction are precursors of a variety of cross-links found in connective tissues (Tanzer 1973). This difference in cross-links is dependent upon the tissue of origin. However, the exact nature of these cross-links in the membranes is unknown at the present time, though it is reported to involve desmosine and isodesmosine (see Diagram 2). The two amino acids have been isolated from the membrane (Leach and Rucker 1978). These are unique to elastin. It is believed that the two form the major cross-links in this compound (John and Thomas 1972). However, an elastin-like compound has so far not been isolated. This has led to the conclusion that the major protein of the membranes is a unique protein. Like keratin, it contains a high number of disulphide cross-links. It also contains the amino acids specific to elastin. However, the amino acid composition of the protein differs considerably from these well known fibrous proteins.

All this time attempts had been made to isolate and characterise the

membrane proteins. It was Balch and Cooke who first extracted a protein-polysaccharide complex in 1968. It contained all the amino acids, but more hydroxyproline and glycine and less cystine, arginine, lysine, and histidine. It also contained the neutral sugars, the sialic acid and the hexosamines.

In 1974, Wedral, Vadehra and Baker claimed that they had isolated three different kinds of protein from both the membranes. They labelled them water soluble, salt soluble and salt insoluble. The water soluble protein was the extract from the water homogenised membrane which they had taken to dryness. The salt soluble protein was the salt extract from the water homogenised residue. The final residue contained the salt insoluble protein. Overall 6% of the membrane consisted of salt and water soluble proteins. No further study of these proteins was made.

However, the authors did observe that 1.6-1.7% of the membranes were carbohydrate in nature (cf Balch and Cooke 1970) and that xylulose was probably present. They also reported the presence of sialic acid.

The validity of the keratinous nature of the membranes was questioned by these workers. On the strength of their experiments and the report of the hydroxylysine, they proposed that the membrane had a collagen-like protein structure. Furthermore, the authors reported the presence of large quantities of calcium (25-31µg/g dry matter) in the membrane, especially the inner membrane. They indicated that calcium may therefore play a structural role. One other report by Plimmer and Lowndes (1924) claimed that chicken membranes contain about 0.2mg calcium.

Leach and coworkers (1978) isolated a protein from the membranes in order to characterise it for keratin. They showed that 10.7% of the membranes were soluble in 0.2M potassium thioglycollate in 6M urea followed by alkylation with iodoacetic acid. They ran a disc gel electrophoresis without success. No further experiments were made on this protein. In their experiments on keratin dispersion, Jones and Mecham (1944) had tried

isolating a protein from the membranes under similar conditions. They reported that 15% of the membrane was soluble.

A real attempt to try to characterise the membrane proteins was made by Paul-Gardais and corworkers (1973,1974). They isolated two sulphated glycopeptides A and B from pronasic and tryptic digests, precipiated by cationic detergents and separated by preparative electrophoresis. Small quantities of glucuronoglycosaminoglycans that were present were removed. Both the glycopeptides were homogenous.

In sulphated glycopeptide A, the molar ratio, hexose:hexosamine was 0.4:1 and the molar ratio, sulphate:hexosamine was 0.1-0.25:1. The hexosamine was mainly composed of N-Acetylglucosamine. The hexoses were galactose, glucose and mannose. Fucose and sialic acid were also present. The composition thus indicated the presence of chains with many glycosyl groups and a few amino acids.

The sulphated glycopeptide B had a hexose:hexosamine molar ratio of 1:1 and a sulphate:hexosamine molar ratio of 0.8-1.0:1. Equimolar quantities of N-Acetylglucosamine and N-Acetylgalactosamine were present. Galactose was common, with some glucose fucose and sialic acid being present. Main amino acid residues were serine and threonine. This glycopeptide probably consists of short carbohydrate chains linked to polypeptide through O-glycosidic bonds involving N-Acetylgalactosamine and serine and threonine.

Moreover, the authors found that the glycopeptides had a turnover rate ($t_{\frac{1}{2}}$) of 1.2-1.5 days. The chondriotin sulphate had a turnover rate of 4 days and the sugar nucleotide, UDP-N-Acetylgalactosamine-4-sulphate they studied had a turnover rate of 1.2 days.

These results indicated that the chondroitin sulphate was not a part of the glycopeptides. Previously Balch and Cooke (1970) had also shown that it was absent from the membranes. Paul-Gardais and coworkers also indicated that the sugar nucleotide was not a precursor. Its slow turnover rate was incompatible with the possible role that was envisaged. The authors proposed that it was possible, however, that it may play a role in embryonic develop-

ment. Furthermore, the results gave some idea of the structural link up of the carbohydrates with the proteins. Both glycopeptides appear to be differently combined. It cannot be speculated at this stage to suggest that this may form the basis of the chemical difference between the outer and inner membranes, as proposed by Balch and Cooke (1970).

Although definite assertions have been made as to the precise composition of the membranes, they have not been accepted. Over the last decade, many workers have tried to elucidate the chemical composition of this unique structure which is neither wholly collagen nor keratin and nor elastin but very much similar to all three of the fibrous proteins. The carbohydrate component of the membrane has been termed a mucopolysaccharide, a glycoprotein, yet the uncertainty remains. However, if we examine some of the carbohydrate-containing proteins, especially those associated with elastin and other connective tissues, that might lead us nearer to an answer which we have been seeking for so long.

Nevertheless, any further attempt to try to characterise these proteins would not be correct unless as Candlish (1972) explained, a complete recovery of the organic dry mass in terms of known constituents is achieved. He wrote:

> "until this is done, and the macromolecules isolated and elucidated, the precise chemical nature of the shell membranes will remain speculative."

SECTION 4 - THE FORMATION OF THE MEMBRANES

It is generally agreed that membrane formation takes place in the isthmus region of the oviduct. Early workers were able to recognise that as soon as the albumen enclosed egg noses into the isthmus, the forward end is immediately covered with the membrane (Coste 1847; Asmundsen 1931; Burmester 1940). By the time the egg has fully entered the isthmus, it is enclosed in a very thin tight-fitting inner membrane. During the time period (1-1.25 hours) the egg remains in the isthumus, the formation of the outer membrane is also fully complete.

At first the membranes fit the egg snugly but later stretch to a flaccid state before the egg enters the uterus. However, during the plumping process the membranes become taut once more.

According to Giersberg (1921) the stimulus of the egg's presence causes the tubular glands of the isthmus to secrete a granular like material into their ducts. The granules swell by taking up water and coalesce into viscous strands. The sticky fibers on issuing from the apertures of the numerous tubular glands, become closely felted into a lace-like membrane (Richardson 1935) and are applied to the surface of the albuminous sac.

However, according to Simons (1971) both fibrillar and granular materials are secreted by the glands. Supposedly the fibrillar material forms the inner layer. Then the cores are laid down. These originate from the accretions of the fibrillar material. The loosely arranged granular material penetrates the core to form the mantles.

By using the scanning electron microscope (SEM) in their studies, Fujii and Tamura (1970) found support for Giersberg's (1921) and Richardson's (1935) theory of granular secretion. Moreover, they proposed that the mantle is subsequently derived from a "cementing material".

As the fibers are being secreted from the glands these are apparently

not differentiated into cores and mantles until after they are firmly enmeshed in the membrane itself (Draper et al 1972). Whether this differentiation is a result of some polymerisation/depolymerisation change in the secreted fiber or whether the two are formed separately in different cells is unknown at the present time.

However, some histochemical work does suggest that in fact the mantle may be derived from the isthmal surface epithelial cells. On the other hand, the amorphous material visible in the fiber interstices may well be produced by this source. Yet another possibility exists (Candlish 1972). The oviduct is open to the exterior, and these cells may merely form the mucus type ciliated lining common in such tissues. If this were so, it is obvious that the core and mantle would have to be derived from the tubular glands by some phase change.

Candlish (1972) proposed that the evidence for the most economic synthesis would be the secretion of the fiber cores from the tubular glands and the derivation of the polysaccharide-rich mantle from the surface epithelial cells. This would then explain the formation of the inner layer.

According to Candlish (1972), the deposition of the membranes begins with the oncoming egg being quickly surrounded by a smooth sheet of the epithelial secretions. This is interrupted by the secretions of the core which bind it (epithelial secretion) as mantles. As the egg enters further into the isthmus, the secretions of the tubuluar glands are increased. However, the epithelial secretions are relatively constant. This may explain the formation of the coarser fibers and the lower mantle/core ratios in the outer membrane. Whether such a proposed model is true remains to be seen.

Most recent work by Leach and coworkers (1980) suggests that the membrane may be formed through a process not unlike that which forms other connective tissue proteins. Their results indicate that lysyl oxidase is involved in the synthesis of the membrane protein and in the post-translational formation of cross-links. They have evidence that cross-linking is initiated

at a specific section of the isthmus before and during the passage of the egg to the shell gland.

A study by Paul-Gardais and coworkers (1973,1974) indicates that part of the membrane may well be synthesised in the magnum. They isolated two sulphated glycopeptides A and B from the membranes and similar sulphated glycopeptides A and B were found in the isthmus and the magnum regions of the oviduct respectively. It is possible that the membranes may well be contaminated with the egg white derived from the magnum.

Very little has been done on membrane formation, but if the membranes were to be formed in the magnum, then the whole process of the membrane formation would need to be re-examined.

SECTION 5 - THE MAMMILLARY LAYER

The Mammillary layer forms the connecting point between the shell and the membrane.

As described earlier, this layer can be separated from the shell by use of 5% EDTA, pH 8.5.

The layer is 17μm thick and is considered to be a specialised part of the outer membrane (Robinson and King 1967). Purkinje (1825) first noticed the "knob like processes adjacent to the membranes". The mammillary knobs which are present in profusion on the surface of the membrane are roughly conical, and oval to circular in cross-section (Romanoff and Romanoff 1949). They are tightly compressed side by side in a single stratum. Their broad domed tops are somewhat flattened or indented at the sides where they fit and cement against each other.

At the tapered bases, gaps interconnect to form an uninterrupted network of air channels throughout the lower portion of this layer. Frequently, very small canals issue outwardly between the tops of the mammillae to form the intermammillary ventilation system. This will eventually give rise to the pores in the shell.

This layer rests on the outer surface of the membrane and is partially embedded in it. Indeed, the membrane fibers are associated with the calcified mammillary knobs and penetrate in to associate with the core region of the knobs (Bunk and Balloun 1977a).

The mammillary knobs consist of a central core of organic matrix with an outer granular matrix surrounded by a homogenous solid, that is crystalline in nature. These are the radial core crystals. Arranged concentrically around them is a layer of unorganised crystals of the mammillary knob proper. These become calcified as the shell is deposited. It may be interesting to note that in the eggs of each species, the shape and size of the knobs and

the arrangement of the matrix within it are characteristic.

Simkiss (1968) reported that the membranes lie 20μm deep in the shell. This is to be expected, since the membranes are firmly attached to the shell through the bases of the mammillary knobs. Furthermore, Terepka (1963) reported that the concentrically arranged fibrous rings on the tops of the mammillae are responsible for the firm attachment of the membrane to the shell. This has yet to be verified.

Earlier, Kelly, Clevisch and Schmidt (1924) had reported that the knobs were large spherulite crystals of calcite. Calcium phosphate was also reported to be present. Stewart (1935) reported that the mammillary cores were organic in nature since they dissolved in alkali and were disintegrated by heat, but remained insoluble in acid.

Later, Robinson and King (1967) using histochemical techniques showed that the cores consisted of a central zone of neutral mucin surrounded by weakly acidic substances, probably sialomucins. On this basis, the authors concluded that the cores were a specialised form of the membrane fibers containing intra-chain disulphide bonds. Simkiss (1968) also using similar methods concluded that the cores were protein-polysaccharide in nature and that they had reducing groups, probably sulphydryls and/or phenols.

In 1970 Balch and Cooke, using modern chemical methods, were able to detect that the cores were rich in hexosamine, sialic acid and hexose. Uronic acid was absent. They suggested that the cores were neutral muco-polysaccharide in nature, but the sialic-acid containing material on the outer surface of the cores was probably a part of the matrix, since it could be washed away. Moreover, they suggested that chondroitin sulphate played no part in shell initiation since uronic acid was absent in the cores, but it may well be involved in crystal growth in the shell gland.

The organic core of the mammillae was believed to be composed of two portions (Tamura 1971), an inner central core, that is firm and compact,

and an outer superficial portion that is fibrous but also compact. Tamura (1971) showed by histochemical studies that the central core contained neutral mucopolysaccharide and a certain amount of concentrated lipoprotein. The outer superficial portion contained acid mucopolysaccharide.

These studies were based on the oviducal tissue. As mentioned earlier, Balch and Cooke (1970) had shown that the "sialomucin" material referred to by Robinson and King (1967) was not part of the core; Tamura (1971) showed otherwise. He found that the central core was derived from the isthmouterine cells which also stained for lipoprotein. The superficial portion was derived from the uterine mucous cells, and the shell matrix was also derived from the same cells. This obviously explains why Balch and Cooke (1970) believed that the outer core was part of the matrix. Since the knobs are formed from two different regions of the oviduct, it is apparent that these are only fully formed in the uterus when the shell is deposited.

The formation of this layer was originally thought to begin in the uterus until it was realised that the membrane appeared granular in the isthmus. In his review, Simkiss (1968) reported that the formation took place in the lower isthmus. It is now certain that it starts in the mid-isthmus region of the oviduct (Leach et al 1977).

Small grains of size 1-10μm appear on the surface of the membrane in the mid-isthmus region of the oviduct. They are seen under the microscope only after staining. They stain positively for calcium. It is possible that the grains contain calcium citrate since the isthmus mucosa is rich in both ions. Unless this is verified the exact nature of the grains will remain speculative. The grains occur at a frequency of about $270/mm^2$, increasing to about $2700/mm^2$ in the posterior isthmus. These grains stain negatively for carbonate and are acid soluble, thus indicating the absence of organic matter which is not present at this stage of the formation. Moreover, these grains seem to be loosely attached since they agglomerate during staining.

As the egg passes into the tubular shell gland, the region situated between the isthmus and the uterus, the grains diminish in number. The particles on the surface in this region range from 28-90μm in diameter. These are the mammillae which are enmeshed in the fibers of the outer membrane. They give a positive reaction for carbonate. It is possible that the grains act as nuclei for the deposition of the secretions of the tubular shell gland. This region is akin to the isthmo-uterine region described by Tamura (1971). There is a great likelihood that it is in this region and stage of formation that the central organic core of the knobs is deposited. Taylor (1970) stated that the knobs are calcified as soon as the cores are laid down, so that they can act as nuclei for crystal growth. Candlish (1970) observed that the cores obviously orientate crystal growth but they do not initiate it, which is rather surprising.

When the egg has passed into the shell gland, the grains are almost absent. The mammillae enlarge in this region to size range from 60-150μm in diameter. They stain uniformly with nine different histochemical stains, showing a relatively even chemical composition. They grow in size during deposition of shell mineral and eventually touch each other, forming small irregular gaps which become part of the pores of the finished shell.

Creger and coworkers (1976) proposed that the mammillary knobs are genetically controlled. They also suggested that the initial number of knobs determine the future strength of the shell and therefore the difference in shell quality. Later Bunk and Balloun (1977) were able to show the validity of these statements. They found that in the weak shell, the mammillary layer is disorganised. The mammillae formed either singly or in partly fused groups. This gives rise to a false nucleation plane which produces waves and bulges on the surface of the shell. These authors observed that the extensive core formation as in the normal mammillae was missing in the weak shells. From this it can therefore be concluded that normal mammillary

formation is necessary for the cone shaped knobs to cement together to form a rigid but fragile foundation for the growth of the shell. However, at this stage it is not known what controls the normal formation of this layer. Much more work is necessary in this important and economic field, in order to produce strengthened shells for transport and storage.

Apart from strengthening the shell, another physiological function of the mammillary knobs is to separate the membranes from the shell in the incubated egg just before hatching takes place. It has been observed (Simons 1971; Candlish 1970) that such separated membranes have a "crumbling" appearance. It so appears that decalcification is taking place, but the exact mechanism is obscure. The dissolution of the organic matter may be mediated by enzymes or the mineral may be mobilised by organic acid. There is more of a possibility that the latter is true. Simkiss (1967) had indicated that citric acid may be involved. Simons (1971) was able to show that more citric acid was present in the shells of incubated than in non-incubated eggs. However, this has yet to be verified before any conclusions can be made.

It is clear that the mammillary layer is better understood but there are still gaps in this field which require a further study.

SECTION 6 - THE SHELL OR "SPONGY" LAYER

The shell has been described as a tertiary membrane of the egg (Wilbur and Simkiss 1968). Originally, it was believed that the shell was composed of the mammillary and the spongy layers. Stewart (1935) asserted that the two were separate and discrete layers. They were formed separately and moreover happened to be physically different. The name the "spongy" layer originated with Landois (1865) who depicted it as being mucin like and structureless, except by having many inward gaps. Rather unfortunately, indiscriminate use of strong acids gave illusionary results leading to a poor choice of name.

In fact, this layer is fibrous and very compact, with numerous microscopic canals traversing its entire depth at irregular intervals. Kelly (1901) showed that the spongy layer is chiefly made up of small calcite crystals, very thickly interlayered. However, x-ray studies reveal (Herzog and Gonell 1925) that the shell becomes progresssively denser and more crystalline towards the surface. Moreover, protein stain techniques show three fairly distinct strata in the spongy layer. Within each stratum, the distribution of matrix fibers is fairly uniform (see Diagram 1). They are plentiful in a wide stratum adjoining the mammillary layer, sparse in a narrow zone at the surface of the shell, and moderately abundant in the middle zone of intermediate thickness.

Pore canals either single or branched, form connecting passages between the exterior of the shell and the network of air spaces in the mammillary layer. These are widest at the mouths where they open into grooves on the surface of the shell. The pore system is filled with matrix of protein fibers which stain as for membranes and are referred to as pore plugs. Tung, Garland and Gill (1979) also refer to a secondary pore plug. The pores are distributed unevenly over the surface of the shell.

The cuticle, a thin transparent coating of protein about 10μm thick, covers the entire surface of the shell including the mouths of the pores. This layer is nevertheless permeable to gases. Although it appears to be homogenous it contains two distinct layers. The inner layer contains pigmented granules, otherwise it is clear and highly vesiculated with many open spaces (Cantoni and Beretta 1976). The outer layer stains for fats.

In some birds, especially sea birds, the outer coating is in the form of a vaterite cover. Vaterite is a polymorph of calcium carbonate and chalky in appearance. This layer serves the same function as the cuticle. Due to the physiological nature of the bird, the cover is more suitable.

The organic shell matrix consists of 70% protein and 11% polysaccharide. 35% of the polysaccharides are present as chondroitin sulphates A and B (Baker and Balch 1962), the others are galactose, mannose, fucose galactosamine, iduronic acid and sialic acid.

Originally Almquist (1934) had wrongly proposed that the matrix was collagen in nature. Baker and Balch did not find any hydroxyproline in the shell. Instead the authors proposed that the matrix was a non-collagenous protein-polysaccharide complex more closely resembling the non-collagenous proteins found in supporting tissues and referred to as the cementing substances. Frank et al (1965) confirmed these results.

In 1970, Cooke and Balch proposed a type of glycopeptide linkage for the matrix, similar to one found in bovine cartilage. Xylose was shown to be present in the matrix. In bovine cartilage, this pentose and two molecules of galactose form a trisaccharide that links the chondroitin sulphate to the protein. It is possible that xylose fills a similar role in egg shell matrix. Furthermore, the authors noticed that the differential liberation of the sugars from the hydrolysed matrix corresponded to the commonest structure for a glycoprotein. The structure was that of a backbone of

hexose and hexosamine units with fucose and sialic acid as terminal units.

Attempts were then made at separating the proteins from the matrix. Using gel-filtration and ion-exchange chromatography, Krampitz and Engels (1974) detected 20 different polymers. Robinson and Heaney (1973, 1976) using electrophoresis and ion-exchange chromatography were able to separate three different glycoproteins from the decalcified shell. 'A' and 'B1' proteins were very similar but 'B2' was different, in being rich in uronic acid. This B2 was later isolated and purified and shown to be in fact hyaluronic acid. It thus appeared that B2 was bound to A and B1 in some fashion. No more is known about the nature of these proteins.

The cuticle according to Tamura (1971) consists of a neutral polysaccharide. It also contains the porphyrins which lend colour to the surface of the egg.

In all birds shell formation occupies about 24.5 hours. Shell calcification occupies 15-16 hours. The cuticle is added shortly before the egg is laid. However, the matrix is secreted throughout shell calcification and is complete halfway in the uterus. The matrix is laid down as a fibrous network between which crystals of calcium salts are deposited.

The primary source of calcium is the blood. The secondary source is the medullary bone and this is under hormone control. Carbonic anhydrase (and maybe Calcium binding protein) are thought to be involved in the calcification procedure.

Shell calcification was studied by surgically removing eggs from the hen at regular time intervals after oviposition (Creger et al 1976).

In the uterus at 3.75 hours after oviposition a calcium-rich material was deposited which then aggregated into pentagon-shaped crystals. This aggregation took place at the 'bonding' sites. Sites where crystalline growth is said to occur.

At 4.50 hours, crystal formation increased but definite crystalline

growth was replaced by a more amorphous type deposition and calcium was deposited in small irregular clumps covering the surface of the crystalline structure.

At 5.50 hours the crystals were almost completely covered so as to resemble a "cinammon roll" - like structure. As the shell grew vertically a "slurry-like" material was spread over the whole surface in an attempt to fill the crevices, separating individual columnar structure.

At 6.00 hours no crystalline structure could be observed and the shell continued to grow vertically.

It appeared that the pores were a result of random incomplete closure of spaces between the crystal structure.

The cuticle is derived from the uterine ciliated cells (Tamura 1971). It is now believed that a change in the phosphate balance of the oviduct acts a signal for vaterite or cuticle deposition (Tullet et al 1976). It is possible that phosphate ions may cause a disruption in the calcium carbonate lattice of the shell. Greater concentration of phosphate has been found near the surface of the shell. Tullet and coworkers proposed that a low concentration of phosphate, 0.33-0.55%, causes a change from calcite formation to vaterite formation. However, a high concentration of about 2.5% terminates all crystallisation. This may then act as a signal for the cuticle to be secreted from the cells. This proposition has really yet to be confirmed, even though it does provide information as to why "glassy" shells (Almquist and Burmester 1934) are produced.

SECTION 7 - THE IMPORTANT ROLE OF THE MEMBRANE

During plumping, when the membranes are taut, an ideal surface is produced for the formation of the shell. This appears to be an essential preliminary process for shell calcification. It has been observed (Britton and Washburn 1977) that membrane weight is greater in eggs with better shell quality. The membrane therefore must play a significant role in best quality eggs.

The membranes are generally not thought to contribute significantly to the strength of the intact shell as assessed by cracking or crushing tests (Tyler and Geak 1964). The isolated membrane does however show very striking mechanical properties (Candlish 1972; Froix et al 1977). As described earlier, the water-saturated non-heat treated membrane behaves as a low modulus elastomeric material, that is, it is elastic up to a distinct failure point when it suddenly ruptures. It is tempting, but probably facile, to assume that this provides an ideal immediate covering for the growing embryo.

Although the membranes may not in general contribute to the strength of the shell, their thickness, nevertheless, plays a significant factor. The two are highly correlated (Vandepopuliere et al 1974). This may provide the necessary strength at a point when the egg is cracked or crushed and the membranes very often remain intact. This aspect alone would seem to justify much more research on membrane fragility.

Another important role served by the membranes is to provide an effective defence barrier to spoilage organisms. Previously it was believed (Salvatori 1936) that the outer membrane was freely permeable, but the inner membrane was selective, preventing the entry of colloids and such like. Later the inner layer was considered to provide the effective barrier since the interstices of the membranes were found to be large enough to allow the passage of almost all microorganisms (Haines and Moran 1940).

Candlish (1972) was able to find support for this role of the inner layer by showing accumulated bacteria against its surface. Moreover, he observed that the membrane reticulum was a very efficient culture medium for some type of organisms. The invading organisms were digesting the membrane mantles.

A first SEM study of the invading bacteria provided a better understanding of the membrane as a defence structure (Tung, Garland and Gill, 1979). The eggs were immersed in a suspension of P.fluorescens. After four days, it was observed that the cuticle and the pore plugs were digested but the membranes and the inner layer remained intact. (The cuticle and the pore plugs also provide an effective barrier to penetration by microorganism under dry condition.)

Bacteria were found accumulated against the surface of the outer membrane. Thereafter rapid invasion of the membranes occurred. They were then found throughout the fiber interstices with no particular accumulation at the continuous boundary of the inner layer. The fibers and the inner layer had still remained intact, suggesting that lysis was not involved.

The authors found support for their work, from studies made by Wedral (1971). He showed that the composition of the normal and infected membranes was the same, that the proteolytic and non-proteolytic bacteria (P.aeruginosa) penetrated the membranes at the same rate, and lastly, commercial or bacterial enzymes failed to produce a signficant change in the permeability of the inner membrane.

These results showed that lysis was definitely not taking place and moreover any holes that may be present were not a significant factor in the bacterial invasion. This differential lysis of the egg structures can only be explained by the diverse chemical nature of all four parts.

It thus appears that the bacteria are able to digest their way through the cuticle and pores to accumulate against the surface of the membrane. They may then be able to penetrate the membrane by sheer mass force. Whether this will prove to be true remains a question.

A possible function of the acellular membranes is that of a quasi-metabolic one (Candlish 1972); in which they conduct calcium to the chorio-allantoic membrane. Their detachment from the mamillary cores during incubation is suggestive of enzyme activity. Any possible evolutionary advantage that the membrane may serve is as yet unknown.

In industry, the eggshell waste from egg breaking plants poses a dire problem. As mentioned earlier, 50,000 tons are produced annually in the USA alone (Vandepopuliere 1977). If stored the waste develops an off-odour. Dump sites are diminishing or will not accept this material. Bad weather prevents its spreading on farmlands. Disposal costs are increasing (Vandepopuliere 1973). It is therefore a pollution hazard.

However, there is now strong evidence that instead, it can be turned into a valuable byproduct of the industry. The waste can be dehydrated in an especially designed equipment connected with the breaking plants. The product can then be substituted for laying rations.

Results prove that the flocks when corrected for age differences, show a normal pattern of performance and can even support the highest egg production (Vandepopuliere 1975). This feedstuff replaces all calcium from ground limestone and the protein from a combination of DL-methionine, meat and bone meal with wheat middlings. Therefore on economic considerations, depending on the type and size of equipment and plant, the costs can be far less than in using commercial rations.

Such a product when very finely ground has been added to the dried egg (Dawson 1947). It does not seem to affect the palatability of the cooking. However, it does not appear to have gained any significance.

In the foodstuff industry, boiled eggs are used in pies and salads. The degree of adhesion of the membranes to the boiled albumen or "peelability" as described by Candlish (1972) is an important factor that needs to be assessed.

To end on another rather traditional role; an interesting but hitherto unknown medical use of the membranes was cited by Romanoff and Romanoff (1949). It was that of an old folk custom of healing open wounds and ulcers by covering them with the membranes from eggs which had been boiled for 5 minutes. The resulting beneficial effect was probably due to the attached albumen.

It can be concluded from this brief review that the membrane plays a significant role in the structure of the egg and for this role to be strengthened in terms of industrial production, the many unsolved problems would need to be elucidated.

The aim of this thesis has been to reinvestigate the chemical composition of the hen eggshell membrane, to relate this with the more recent ideas which have been proposed and to compare these results with other avian eggshell membranes.

The further aims of this work have been to extract the hen eggshell membrane protein(s) by chemical means rather than by use of enzymes or harsh physical conditions and if possible to characterise the isolated protein(s).

The physical nature of the hen eggshell membrane is also to be studied with the electron microscope; the purpose being, to try to relate the chemical composition of the membrane to its physical structure and so evaluate the possible relationship with the fibrous proteins.

II. EXPERIMENTAL METHODS

EXPERIMENTAL METHODS

The marketed eggs of the hen were studied, but comparison was drawn with the following freshly laid (April) eggs received from the Poultry Research Centre in Edinburgh:

Bantam (mixed breeding)
Quail
Turkey (Ross super-midi)
Duck
J-Line (Brown Leghorn)
T-Line (medium-heavy cross layer)
S-Line (White Leghorn)

Goose eggs were received from a nearby farm. These had been freshly laid in March.

Apart from the eggs of the Quail, the eggs from the other birds numbered 6 each. The Quail eggs numbered 12.

Most of the eggs were kept at room temperature for just one week, over which time eggs of one bird or two were cracked open, their eggshells washed under rushing tap water and then immersed in distilled water overnight at room temperature.

The following morning, the membranes were separated from the shell. First the inner membrane was very gently rolled off. The outer membrane was then pulled away from the shell. During the separation, the mammillary layer was probably half torn away with the membrane. Nevertheless, many times, the membrane separated quite neatly. The whole of the outer membrane slipped off the shell.

The well-known 'classical' methods of separation by acid or alkali

were not used. The membrane is a delicate structure and by exposing it to such harsh conditions, would be damaging it. The water, even, has been described to be rough on the membranes (Candlish 1972). However, nothing better is available.

After separation, the membranes were spread out on filter paper to remove excess water. They were then placed in a 2:1 mixture of chloroform and methanol, for 20 minutes. This was repeated two more times, in order to remove the lipid component of the membranes. The waste solvent was disposed into a solvent bin and the membranes allowed to dry. Soon afterwards, the dried but whitened membranes were placed in liquid nitrogen and ground to a very fine powder with a pestle and mortar. The membranes were stored in sample bottles at room temperature. They were ready for use.

However, one little snag became apparent. After completion of hydrolysis of some of the membranes, the cracked tops of the test tubes exploded on application of the hot glass rod. It was decided to place the membrane containing bottles on top of the oven to remove the excess solvent. Thereafter no such hazards occurred.

The experimental methods have been divided into two section of which the chemical composition will be discussed first. This will be followed by the chemical structure and an electron microscope study.

I. CHEMICAL COMPOSITION

1. Amino Acid Analysis

An analysis for amino acids is one of the first steps in the elucidation of the chemical structure of a protein molecule. Moore and Stein (1963) using the automatic analyser perfected this technique to such an extent that the accuracy of determination is limited only by

(1) the precision of determination of the amino acid composition, and

(2) the difficulties associated with protein hydrolysis.

Tristram and Smith (1963) reported that the instrument is capable of standard and repeated accuracy. They found the lowest recovery was 89% for methionine sulfone and all other amino acids ranged between 99-100%.

During hydrolysis, liberation of some amino acids is slow whereas losses of others is significantly measurable.

The slow liberation is due to the difficulty in breaking the peptide linkages formed by stable amino acids (Eastoe 1972). Most of the aliphatic, basic and acidic amino acids are stable. Those with long chain aliphatic side residues, for example valine and isoleucine, are especially stable under such strong acid conditions. Valylvaline and isoleucylvaline bonds are only broken after long hours ($\geq$ 70 hours) of hydrolysis.

The unstable amino acids include cystine and methionine, which are partially oxidised by the acid (more so in the presence of air) to cysteic acid and methionine sulfoxide. Tyrosine is converted to chlorotyrosine in the presence of free chlorine in the acid. The β-hydroxyamino acids, serine and threonine, gradually decompose over the 24 hour period. Serine decomposes by 10.5% and threonine 5.3% (Eastoe 1972). Moreover, these values are unaffected by the presence of carbohydrates (Pigman and Downs 1969) unlike the rest of the amino acids.

It has been known for a long time that glycoproteins on hydrolysis with mineral acids at 100°C turn brown, then darken and finally produce considerable amounts of soluble and insoluble black material. This is humin, and the development of the colour denotes the extent of the side reactions. The colour may vary from pale violet or a light straw, through various shades of yellow, to dark brown or even black. Under the conditions described, sialic acid readily decomposes with the formation of this material (Gottschalk 1972). Tryptophan is especially conducive to humin formation particularly where there is access to oxygen (Eastoe 1972).

The side reactions just mentioned are due to the interaction between the saturated and unsaturated dicarbonyl and α, β-unsaturated carbonyl compounds formed from the constituent sugar and the amino acids released from their peptide linkages (Gottschalk 1972). Such interactions result in disintegration of the amino acids, thus diminishing their yield.

Although every effort should be made to improve the accuracy of amino acid determination, it may be noted that despite the humin formation, the destruction of amino acids is small indeed (Eastoe 1972).

(i) Total Amino Acid Analysis

Procedure

A 2mg sample of the air-dried material was placed into a thick heavy-walled 16x125mm pyrex test tube. A 2ml volume of 6NHCl (a 1:1 dilution of reagent concentrated hydrochloric acid) was added to the membrane. The finely ground membrane floated onto the surface of the acid.

Then the test tube was gently placed into a polypropylene beaker half-filled with liquid nitrogen. After 5-10 minutes, the test tube was removed and held in the air for 20 seconds so that the acid mixture appeared crystal white. The test tube was connected to an oil vacuum pump by a piece of tygon tubing.

The air evacuation was continued until the mixture had fully thawed. Whilst the pump was on, the lower half of the tube was placed into the liquid nitrogen and gently shaken to remove any air bubbles still clinging to the side walls.

The frozen test tube was removed from the pump and immediately sealed. After the mixture had fully thawed, the test tube was wiped dry and placed into a Pye-Unicam heating block set exactly at $110^{o}\pm1^{o}C$.

All the membrane divisions (that is, inner, outer and inner plus outer membrane) were hydrolysed for 24 hours. The S-line inner plus outer membrane (IO) and the Duck outer membrane (O) were also hydrolysed for 48 and 72 hours.

After hydrolysis was complete, the test tubes were allowed to cool, before being filed and cracked open. The hydrolysates were evaporated at $42^{o}C$ under vacuum with a rotary film evaporator. The flasks were covered and stored until required.

Before analysis, the flasks were removed from the refrigerator and left out to warm to room temperature. A 10ml volume of pH 2.2 citrate buffer containing the internal standard nor-leucine was added to the dried hydrolysates. A 0.8ml aliquot was injected into the Jeol Full-Automatic amino acid analyser JLC-5AH.

The procedure just described is a modification of the Moore and Stein method (1963). It is rather unfortunate that some amino acids are not satisfactorily analysed in this system. Those specifically in mind are the tryptophan and the hydroxyproline. The hydroxyproline does not give a satisfactory ninhydrin test. The recovery of tryptophan is low, about 40-60%.

(ii) Tryptophan Analysis

Mild hydrolysis conditions are required for the analysis of this amino acid so many experimental methods were designed.

The use of solid ion-exchange resin in the presence of very dilute acid has been suggested as one possibility for achieving protein hydrolysis under mild conditions. However, Paulson and Deatherage (1953) found that after treating bovine serum albumin and edestin with 5x their weight of sulphonic acid resin (Dowex 50) and 50 or 100 parts of 0.05N HCl at 100°C, considerable amounts of peptides remained, even after heating had been continued for 100 hours. So it was suggested that this technique may be effective for substances that are readily hydrolysed, and has been used in connection with the determination of hexosamines (Anastassiadis and Common 1955).

Liu and Chang (1971) showed that tryptophan and other amino acids are recovered quantitatively using proteins in the presence of 3M toluene-p-sulphonic acid with 0.2% 3-(2-aminoethyl)indole at 110°C.

Bennet et al (1975) reported that with hydrolysis using Sepharose-bound peptidases, even asparagine and glutamine are recovered.

Udenfriend and coworkers (1975) described a form of fluorescence detection using fluorescamine (4-phenyl spiro [furan-2(3H),1'-phanalan]-3,3'-dione) as a detection reagent. Quantities as small as 50pmoles can be analysed.

Despite the many excellent techniques, the method adopted in this laboratory for determining tryptophan was that of Matsubara and Sasaki (1969). Nevertheless, the procedure by Penke and coworkers (1974) was first tried. These authors claimed that their technique using mercaptoethane sulphonic acid was superior to that of Liu and Chang (1971) and also to that of Matsubara and Sasaki (1969). They showed that the recovery of tryptophan

was high even in the presence of carbohydrates. However, despite the claims, the eggshell membranes remained undissolved in the acid even after 48 hours of hydrolysis. Larger volumes of the acid also had no effect.

Procedure

Matsubara and Sasaki (1969) reported that 4% mercaptoacetic acid in 6NHCl was sufficient for optimum recovery (91%) of tryptophan. However, in this laboratory, 6% mercaptoacetic acid (BDH laboratory reagent) was found to give the same optimum value.

Therefore, hydrolysis for tryptophan was carried out using 6% mercaptoacetic acid in 6NHCl at 110°C for 24 hours. Instead of freezing to evacuate the tubes, they were flushed with nitrogen before being sealed, otherwise the conditions for hydrolysis and analysis were all the same as described previously for total amino acid analysis. However, evaporation of the hydrolysate was carried out at 60°C. It may be of interest to note that total amino acid analysis could not be determined in the presence of mercaptoacetic acid, since this interferes by giving a large absorption peak in the area of hydroxyproline.

(iii) Hydroxyproline

Using the Cessi and Serafini-Cessi method (1964), many problems were solved. The distillation allows the complete separation of the chromogen from the non-volatile products of tyrosine and tryptophan, which otherwise interfere to give a strong colour reaction (~2%) with p-dimethyl amino benzaldehyde as in the method of Neumann and Logan (1950).

Other workers used complicated and difficult methods to separate the imino acid from the interfering substances. Partridge and Elsden (1961) suggested chromatographic techniques. Prockop and Udenfriend (1960) worked out an oxidation technique using chloramine T with extraction of the

resulting pyrrole in toluene. Blumenkrantz and Asboe-Hansen (1974) used a modification of this technique.

Besides the ease with which the separation of the chromogen occurs, this method by Cessi and Serafini-Cessi (1964) is also unaffected by the humin content of the hydrolysates. Therefore, using this method, minute quantities of the hydroxyproline can be detected.

Materials

1) Hydroxyproline (Sigma Ltd)

Stock solution. 1mg/ml was prepared. It was stored at $4^{o}C$.

2) Cupric sulphate (BDH AR)

0.05M.

3) Sodium hydroxide (BDH AR)

2.5N.

4) 30% Hydrogen peroxide (BDH AR)

Diluted before use to 6%. (v/v). Stored at $4^{o}C$.

5) Potassium chloride (BDH)

Saturated aqueous solution.

6) Hydrochloric acid (May and Baker AR)

3N.

7) Stannous chloride crystals (AR)

10-15mg single weight.

8) Ehrlich's reagent

Prepared fresh before use.

1g p-dimethylaminobenzaldehyde (AR) in 55ml glacial acetic acid (AR) with 10ml 10N H_2SO_4 (BDH AR).

Sample preparation

Since comparison of these readings was to be made with those from amino acid analysis, it was decided to hydrolyse 4mg of the membrane samples at 110°C for 24 hours. The method of hydrolysis was the same as described previously. To each 4mg of the evaporated hydrolysate residue, 2.5ml of distilled water was added.

Standard

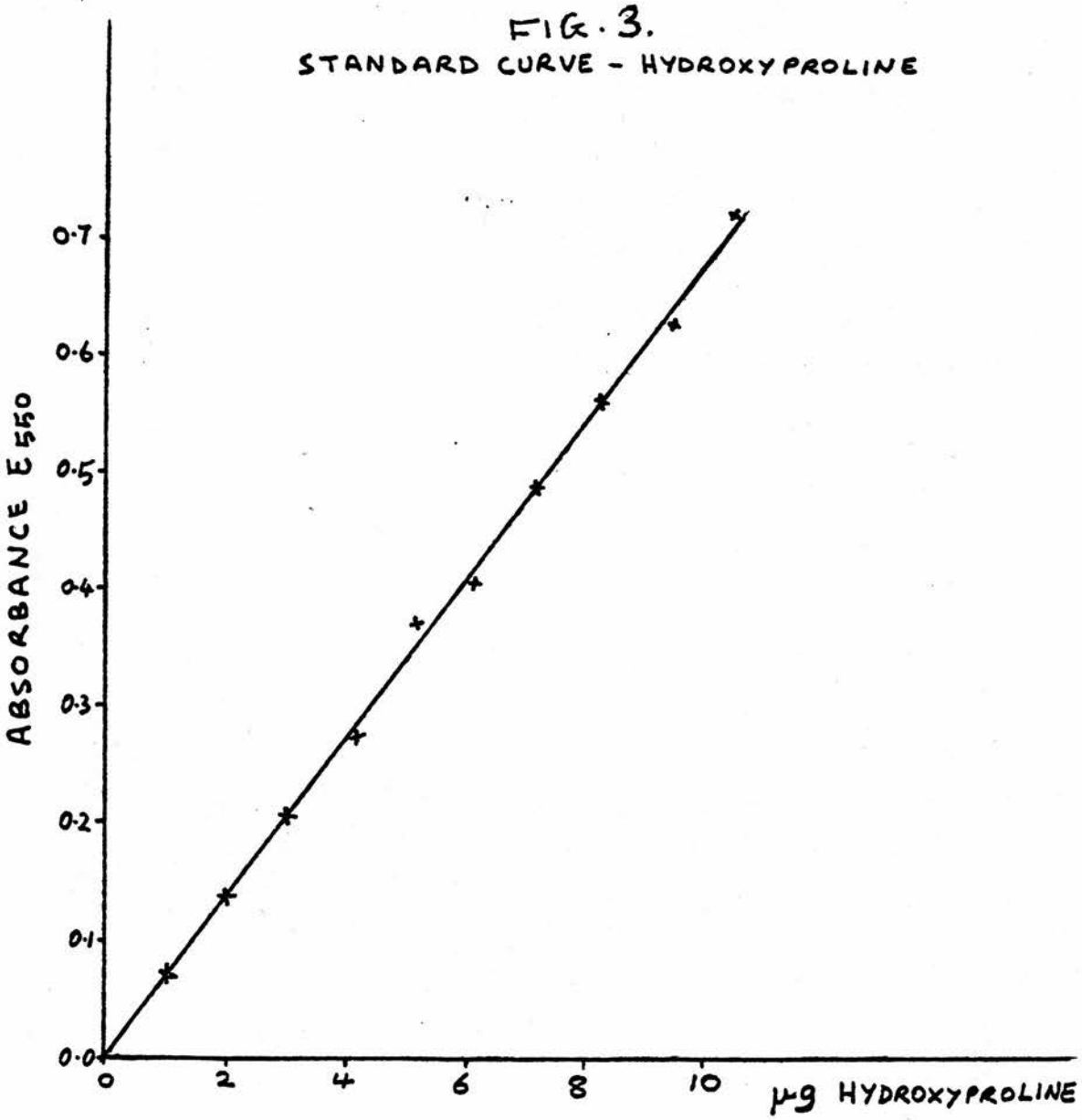
A standard was prepared (1-10µg) (see Fig. 3). The extinction is a linear function of the concentration of hydroxyproline in the range 2-10µg and is reproducible. Duplicate readings were taken.

Procedure

To 1ml of sample standard or blank, 1ml of 0.05M copper sulphate and 1ml of 2.5N sodium hydroxide were added in test tubes. The tubes were placed in a water bath at 40°C, and 10-15 minutes later 1ml of 6% hydrogen peroxide was added. Frequent shaking of the tubes removed the excess oxygen. After 10 minutes the tubes were placed in an ice bath until distilled. Immediately before distillation, 2ml saturated potassium chloride, 1ml 3N hydrochloric acid, and one crystal of stannous chloride were added. The contents of the tubes were transferred to the apparatus described by Cessi and Serafini-Cessi for the estimation of hexosamines. 3.5ml of the distillate was collected into a 10ml volumetric flask containing 6.5ml of the Ehrlich's reagent. The distillation rate was 1ml/minute.

The colour which developed immediately, was read straight away at 550nm on the Pye Unicam SP6/550 spectrophotometer. The colour is stable for only 15 minutes. It is reported that the rate of change with time is higher at other wavelengths. The readings were repeated with the next 1ml of the sample.

FIG. 3.
STANDARD CURVE - HYDROXYPROLINE



2. Polysaccharide Content

The quantitative estimation of sugars in a glycoprotein is an extremely complex problem. At first, all the glycosidic bonds must be split, and these bonds vary in their ease of cleavage depending on the individual sugar concerned and its mode of linkage to other sugars.

The two principal techniques used for cleavage of glycoproteins are acid-catalysed hydrolysis and methanolysis. No single method exists for the cleavage of all the sugars in all the different forms of combination. For the complete release, it is most inevitable that some destruction of individual sugars will take place, the extent varying from sugar to sugar.

The N-Acetylhexosamines which are found in the majority of glycoproteins pose a special problem. Unless they are cleaved with a highly concentrated acid, the fission of the acetamido group can result in the formation of the deacetylated hexosaminides, which are resistant to the action of acids (Conchie 1976). Due to the high concentrations of the acid, considerable destruction of the neutral sugars takes place.

Under acid conditions of 2N or 6N HCl at 100^{o}C, the neutral sugars have little tendency for glycosylamine formation (Eastoe 1972). Instead, hexoses are degraded to hydroxy methyl furfural (HMF) and 6-deoxyhexoses (methyl pentoses) to 5-methyl-2-furaldehyde. HMF may then undergo ring fission with the production of levulinic and formic acids under such strong acid conditions.

It is therefore apparent that the destruction of carbohydrates is very great since they are degraded irrespective of the carbohydrate amino acid interactions. So in order to carry out a complete analysis, more than one set of conditions would be necessary, if specific methods are to be used. However, recent automated techniques such as ion-exchange chromatography and gas-liquid chromatography enable analyses of most, if not all,

sugars in a glycoprotein to be performed on a single sample with increased sensitivity and accuracy. Acid-catalysed hydrolysis is necessary for determination by ion-exchange chromatography, whilst methanolysis is used when determining by gas-liquid chromatography (GLC).

Methanolysis has a greater advantage over acid catalysed hydrolysis, since loss of neutral sugars and sialic acid are avoided. It reduces the possibility of side-reactions of sugars with amino acids by protecting the liberated reducing group as the methyl glycoside. These methyl glycosides, as well as the methyl esters formed from sialic acid are highly stable under such strong acid conditions. They are also suitable derivatives for gas chromatography (Bhatti et al 1970,1972).

Many attempts were made to use the GLC but rather unfortunately the columns happened to be rather old, so inaccurate results were obtained. This method could not be used, instead only the specific tests were carried out.

(i) Neutral Sugars

The neutral sugars include the hexoses and L-fucose in this text. They are determined by the phenol-sulphuric acid colorimetric method (Dubois et al 1956; Hodge and Hofreiter 1962). In actual fact, except for the hexosamines, the reaction is sensitive and general for all classes of sugars, including sugar derivatives, oligo- and polysaccharides (Hodge and Hofreiter 1962).

It is probable that the reaction occurs in several stages (Racusen 1979):
Firstly, hydrolysis of the glycosidic bonds must take place. Then the sugars are probably dehydrated to furfural derivatives and finally condensation of the furfural with the phenol may take place to yield the quinoidal pigments.

TABLE IV

RELEASE OF NEUTRAL SUGARS WITH TIME

Time (Hours)	% Neutral Sugar
4	4.32
6	0.89
9	1.12
12	0.82
24	0.89
36	2.64
48	2.68

Apart from its applicability to almost all sugars, the other virtues of this technique are its simplicity, its speed plus accuracy and above all, its insensitivity to non-carbohydrate lipids, amino acids and proteins in general. Moreover, the reagents are stable, inexpensive and readily available.

Materials

1) D-glucose (BDH Laboratory Reagent)
   Stock solution 1mg/ml. Stored at $4^{o}C$.

2) Phenol Reagent 5% (W/V)
   Stock solution prepared by dissolving 5g of phenol (BDH AR) in 100ml of distilled water.

3) 96% Sulphuric acid (BDH AR)

Preparation of sample

For neutral sugar hydrolysis, the method of Kido and coworkers (1977) was adopted.

To 2mg of the membrane, 1M H_2SO_4 was added. The tubes were flushed with nitrogen for a minute before being sealed. Hydrolysis was carried out at $100^{o}C$ for 4 hours in the Pye Unicam heating block. The time factor was established by carrying out hydrolysis of the Goose membrane at 4, 6, 9, 12, 24, 36 and 48 hours, as shown in Table IV.

The hydrolysate was evaporated at $40^{o}C$ under vacuum. 2ml distilled water was added to the dried residue.

Standard

A glucose standard (0-100µg) was prepared immediately before use (see Figure 4). Duplicate readings were taken.

Spectrophotometer

The Pye Unicam SP6/550 spectrophotometer was used. Cells used were 1cm in light path.

Procedure

To the 1ml sample in each of the test tubes, 1ml of 5% phenol was added. The contents were thoroughly mixed. From a fast-flowing pipette (a portion of the tip was removed) 5ml of the concentrated sulphuric acid was added to the mixture. The continuous stream of acid was allowed to hit the liquid surface of the solution, so as to produce good mixing and an even heat distribution. The tubes were agitated after the addition of the acid and 10 minutes later re-shaken before placing in a water bath at 25-30^{o} for 20 minutes.

The absorbance was read soon afterwards at 490nm against a reagent blank. The yellow-orange colour is stable for several hours.

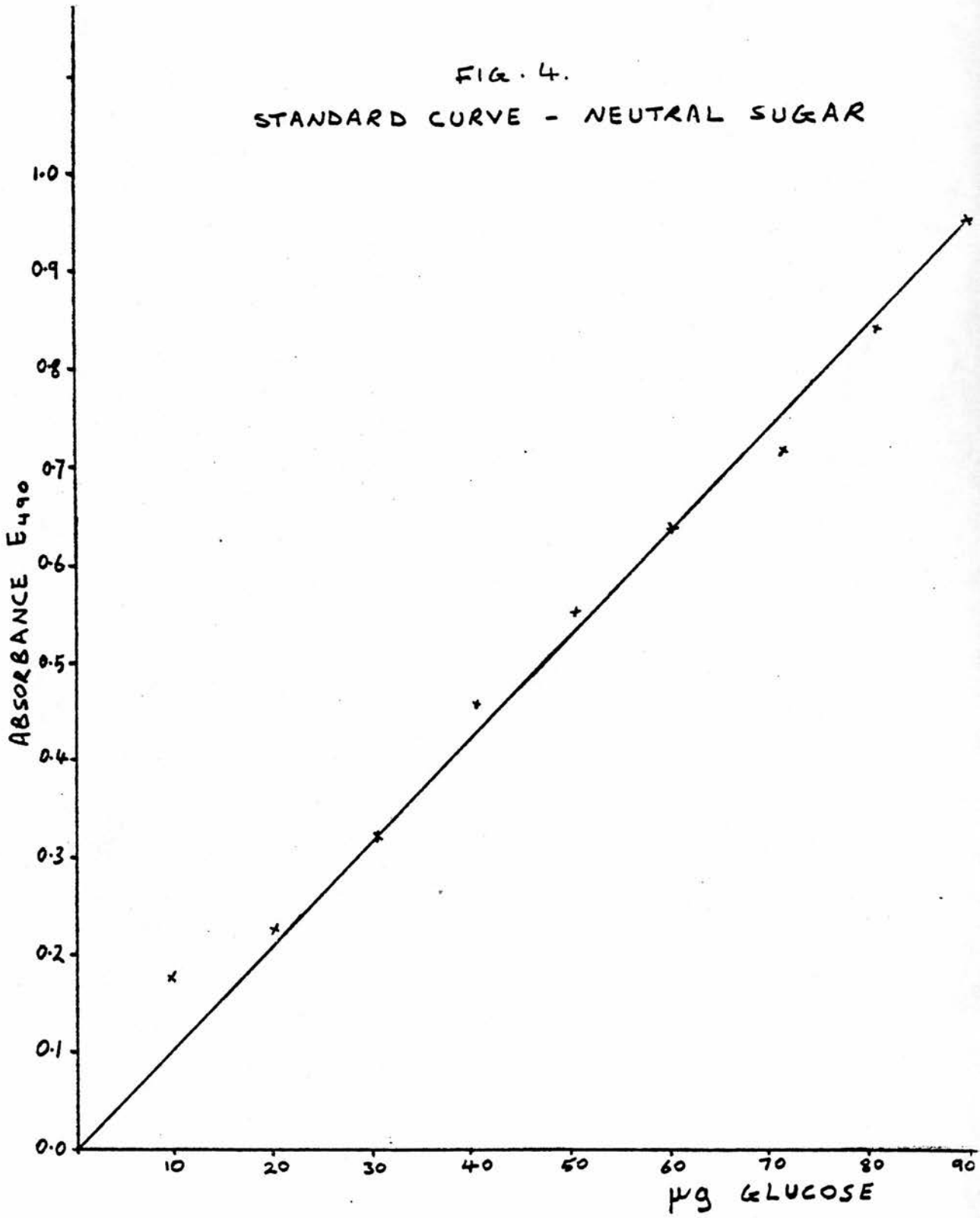
(ii) Hexosamines

The hexosamines were determined by the method of Cessi and Piliego (1960). However, preliminary experiments were carried out using the Elson-Morgan method (Wheat 1966; Belcher et al 1953; Partridge 1948). The modifications designed by all these authors are originally based on the reaction of amino sugars with acetylacetone in an alkaline medium and subsequent development of a red colour with p-Dimethylaminobenzaldehyde.

The Elson-Morgan method has one drawback; the presence of other sugars and amino acids in the reaction strongly interfere with the estimation

FIG. 4.

STANDARD CURVE - NEUTRAL SUGAR



of the hexosamine. Lysine and glucose prove to be serious contaminants. Therefore Schloss (1951) proposed a heating method whereby such interference could be overcome.

By use of the distillation procedure (Cessi 1952; Cessi and Piliego 1960) the volatile hexosamine chromogen is easily separated from the other non-volatile chromogens thereby easily overcoming any serious contamination. The interference by hydroxyproline is only about 0.3% of that of glucosamine.

Later, on the basis of the mechanism of the reaction the authors (Cessi and Serafini-Cessi 1963) were able to show a method for the determination of D-galactosamine in the presence of D-glucosamine. The mechanism of the reaction is still obscure but is believed to involve an intermediate which, under alkaline conditions, converts to the stable chromogen, 2-methylpyrrole. However, no attempt was made to distinguish between D-glucosamine and D-galactosamine.

Materials

1) D-glucosamine hydrochloride (Sigma Ltd)

Stock solution 1mg/ml. Stored at $4^{o}C$.

2) Acetyl Acetone Reagent

This reagent was prepared fresh daily, and kept in the refrigerator at all times.

To 1ml Acetylacetone (Sigma Ltd, AR), 100ml of pH 9.8 buffer containing 0.1M NaCl was added.

The pH 9.8 buffer was obtained by dissolving 23.02g of sodium carbonate (BDH AR), 2.76g of sodium bicarbonate (BDH AR) and 5.84g of sodium chloride (BDH AR) per litre of distilled water.

The pH of the reagent (pH 9.8) was checked after the addition of the buffer and adjusted if necessary.

TABLE V

RELEASE OF HEXOSAMINES WITH TIME

Time (Hours)	% Hexosamine
6	1.45
8	2.32
10	1.55
24	1.58

3) p-Dimethylaminobenzaldehyde reagent

80mg of p-Dimethylaminobenzaldehyde (BDH AR) was dissolved in 100ml of absolute ethanol containing 3.5ml of concentrated Hydrochloric acid. The solution was almost colourless and could be stored at $4^{o}C$ for several days. However, fresh samples were prepared at all times.

Distillation apparatus

An all glass apparatus was used. It consisted of a 100ml spherical flask with a short neck, fitted tightly to a 20cm condenser. The apparatus was heated directly over a gas burner and brought to boiling in about 1 minute. The rate of distillation was 1ml/minute.

Preparation of sample

The hydrolysis of the membranes for hexosamine determination was followed from Kido and coworkers (1977).

To 2mg of the membrane, 4ml of 4M hydrochloric acid was added. The sample was frozen and evacuated as described previously. The tubes were sealed and hydrolysis carried out at 90^{o} for 8 hours. The time period was determined over a number of hours (see Table V).

The hydrolysate was evaporated at $40^{o}C$ under vacuo. 2ml of distilled water was added to the dried hydrolysate.

Standard

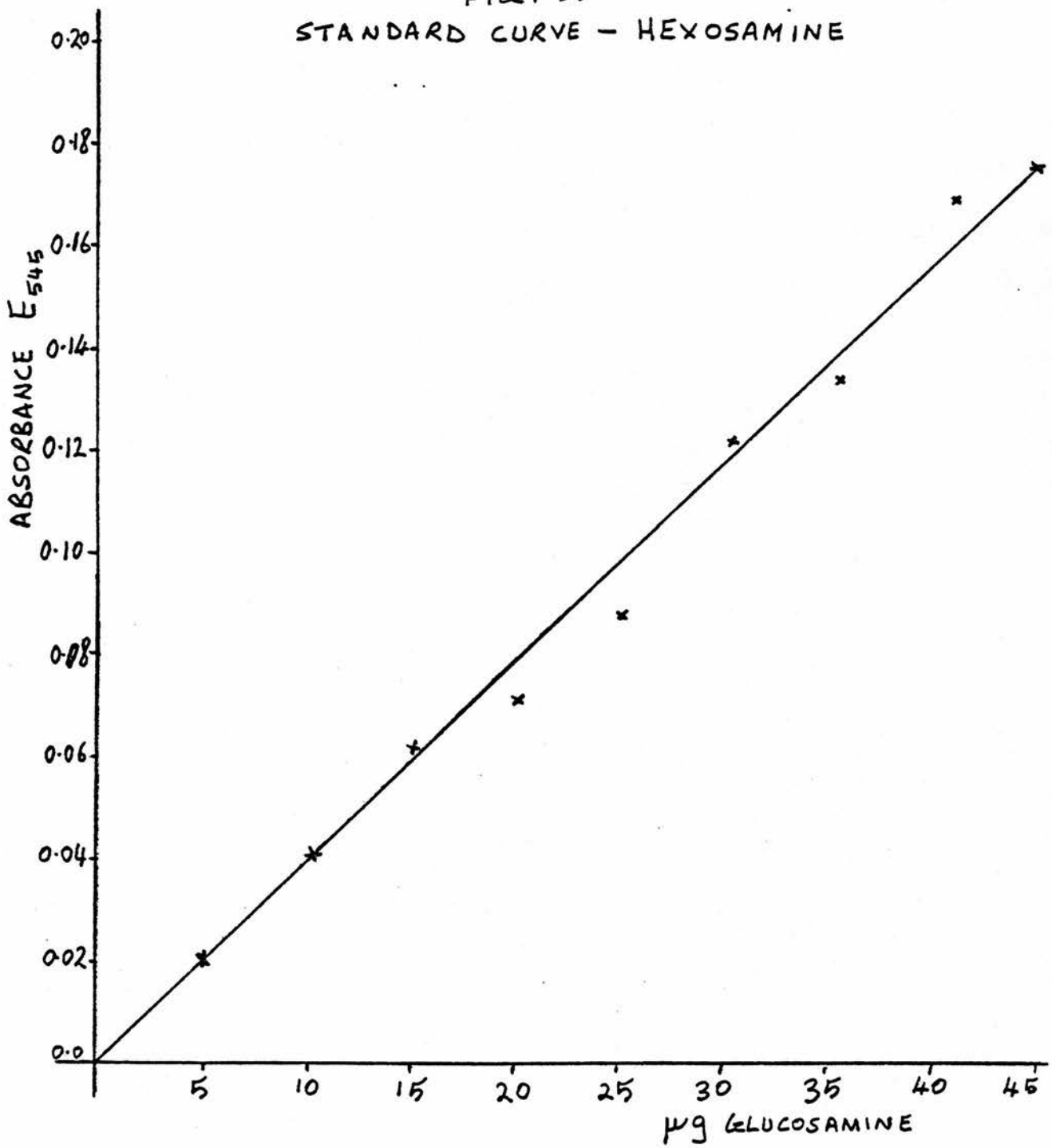
A range of concentrations 5-50µg of D-glucosamine were prepared. Duplicate readings were taken (see Fig 5).

Spectrohotometer

The absorbance E_{545} was measured against a reagent blank. The spectro-

FIG. 5.

STANDARD CURVE – HEXOSAMINE



photometer used was a Pye Unicam SP6/550. Cells of 1cm light path were used.

Procedure

2ml solutions of the sample, blank, or standard, were mixed with 5.5ml of acetylacetone reagent, pH 9.8 in teflon-capped pyrex hydrolysis tubes and heated in a boiling-water bath for 20 minutes. After placing in cold water for a while, the tubes were transferred to an ice bath before the contents were distilled. It may be of interest to note that the chromogen is only stable for 30 minutes in this state (Schloss 1951).

Prior to distillation, the reaction mixture and 3 washings of 2ml of water (from a dispensing pipette) were transferred from each tube to the distillation apparatus. 2ml portions were distilled into 10ml volumetric flasks containing 8ml of the p-dimethylaminobenzaldehyde reagent. The colour is fully developed after 30 minutes at room temperature. Its intensity is unchanged after 24 hours. However, absorbance readings were taken 1 hour later at 545nm.

(iii) Sialic Acid

In the estimation of this sugar, the conditions of hydrolysis such as those described above may lead to its destruction.

Sialic acids, since they are located in peripheral positions of a sugar unit, may be determined directly on the unhydrolysed glycoprotein. The most sensitive method (Jourdain et al 1971) which is also least influenced by the presence of amino acids, other carbohydrates and lipids, is a recent modification of the original resorcinol procedure (Svennerholm 1957). This method was adopted.

However, preliminary experiments were carried out using the very sensitive thiobarbituric acid method (Aminoff 1961). This method requires the glycoprotein to be hydrolysed in 0.1N H_2SO_4 at 80°C for 1 hour prior to analysis. The membranes remained almost insoluble, but this was not the sole reason for discontinuing with this technique.

The iodine vapour which was supposed to be liberated at room temperature from the solution in 1-2minutes, was still present even after heating in a boiling water bath for 20 minutes. The only explanation that can be made, is that the periodate was somehow lodged in the membranes and could not be immediately available to the action of the arsenite. Furthermore, the colour of the solutions (including the blank) became rather intense on standing. At the end of one hour, they all appeared to be of one shade and hue.

Materials

1) Sialic acid (Sigma Ltd)

Stock solution 80.0µg/ml. Stored at 4°C.

2) Periodic acid (BDH Laboratory Reagent)

0.04M

3) Resorcinol Reagent

To 0.6g resorcinol (BDH laboratory reagent), 25µmoles of $CuSO_4$ in 60ml of 28% HCl and 40ml water were added.

4) 95% tert-butyl alcohol (BDH Laboratory Reagent)

Sample

2mg of the membranes were placed in 0.5ml of distilled water in test tubes.

Standard

A range of concentrations 1-16µg of sialic acid were taken. An average of duplicate readings was calculated (see Fig 6).

Spectrophotometer

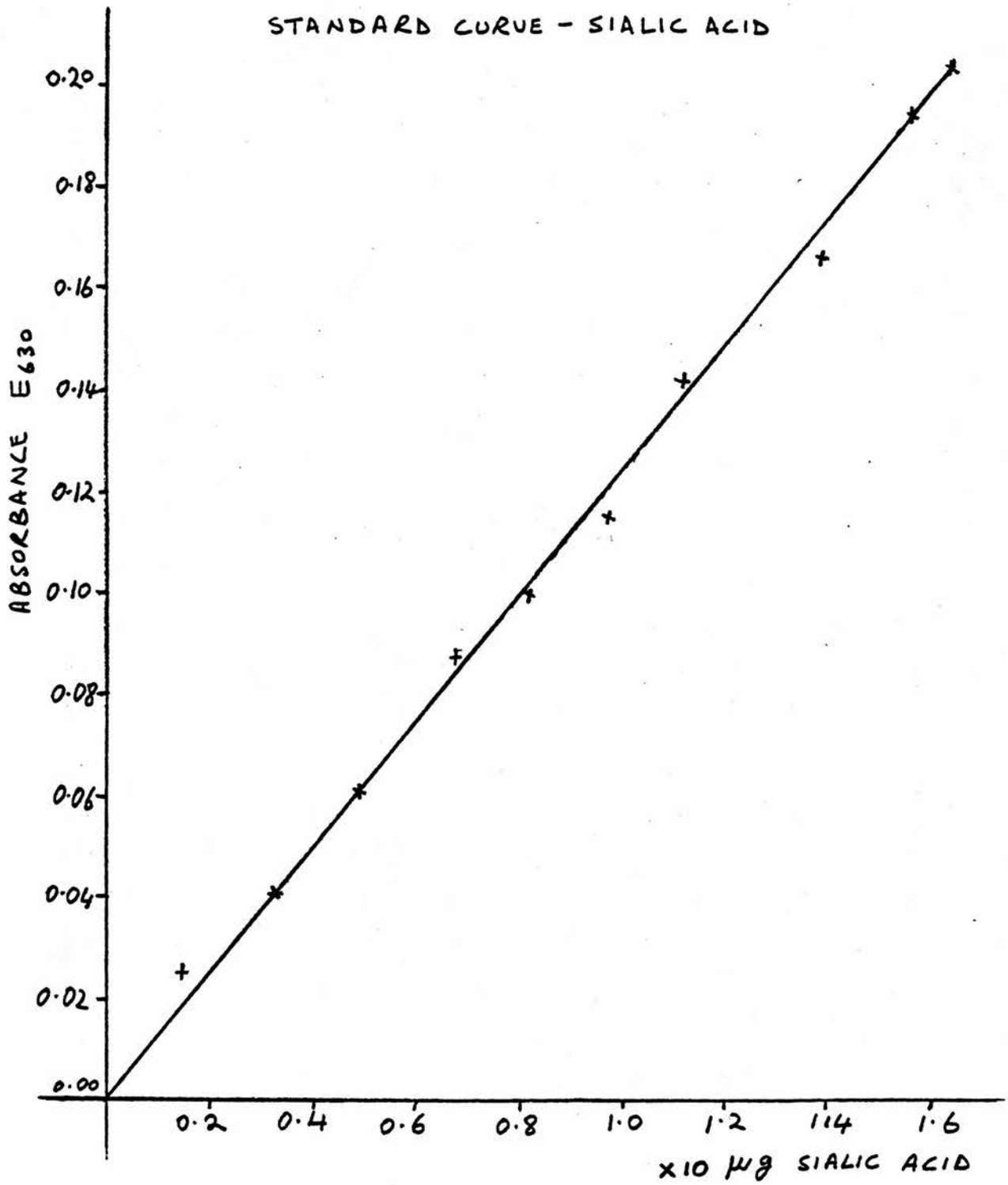
Absorbance at 630nm was measured with the Pye Unicam SP6/550 spectrophotometer. Cells of 1cm light path were used.

Procedure

To 0.5ml of the sample, standard or blank, 0.1ml portions of 0.04M periodic acid were added. The contents were thoroughly mixed and the tubes were allowed to stand in an ice bath for 20 minutes. Soon after, 1.25ml of the resorcinol reagent was added, the contents once more thoroughly mixed and the tubes were again placed in the ice bath, but only for 5 minutes. Then the tubes were removed and placed in a boiling water bath for 15 minutes. After cooling in ice water, 1.25ml of tert-butyl alcohol was thoroughly mixed with the contents. The tubes were placed in a 37^{o} water bath for 3 minutes and then cooled to room temperature. 10 minutes later absorbance readings were taken. The colour is fairly stable over 24 hours.

FIG. 6.

STANDARD CURVE - SIALIC ACID



3. Desmosine and Isodesmosine Content

Elastin is believed to be unique in its cross-linking amino acids, desmosine and isodesmosine. Due to the cross-linking, this compound has a high degree of insolubility in all solvents that have been studied. Some of this insolubility is due to the other two cross-linking rather rare amino acids lysinonorleucine and merodesmosine. Due to their structure, these amino acids can cross-link up to 4 polypeptide chains (see Fig 2). Alongside this study, x-ray diffraction analysis suggests that the elastin is a 3-dimensional network of randomly coiled polypeptide chains joined by covalent cross-links (Franzblau 1971).

Another more pronounced characteristic of elastin is its ability to exhibit a strong blue-white fluorescence in the Ultra-Violet. This is now believed to be due to the two amino acids desmosine and isodesmosine described above (Partridge et al 1963; John and Thomas 1972). Previously La Bella and Lindsey (1963) had reported 3 activation/fluorescence maxima at 340/405, 350/440, and 370/460nm, believed to be due to a single unidentified compound in the elastin. However, Blomfield and Ferrar (1967) found only two such peaks. John and Thomas (1971) also found only two peaks, one at 250/374 and another at 330/384. Could this decrease in number of unidentified peaks be due to purification of the elastin (Blomfield and Ferrar 1967)? The question has yet to be answered.

Many attempts were then made to isolate and separate the cross-linking amino acids. Moczar et al (1972) used high voltage electrophoresis. Others used gel-filtration and ion-exchange chromatography (Green et al 1973). However, these methods either failed to separate desmosine from isodesmosine or they were time consuming and cumbersome. It was Starcher (1977) who described a rapid and simple method whereby the amino acids could be resolved and quantitated from whole tissue hydrolysates. On this basis measurement

of elastin as low as 1% as in some tissues could be determined. This method was adopted for the determination of desmosine and isodesmosine in the membrane hydrolysates. Preliminary experiments with just the untreated hydrolysate gave trace peaks for the two amino acids.

(i) Determination of desmosine and isodesmosine by amino acid analysis

Materials

1) Whatman 3MM chromatography paper
2) N-butanol (BDH analytical laboratory reagent)
3) Glacial Acetic acid (BDH ")

Preparation of sample

The hen eggshell membrane (inner and outer or IO), 2mg was hydrolysed in 4ml 6N HCl for 24 hours at 110°C as described previously for total amino acid analysis.

To the dried hydrolysate 0.5ml of distilled water was added.

Amino Acid Analyser

For this purpose the Locarte Full Automatic Analyser was used.

Procedure

The hydrolysate was streaked along a 7-inch wide strip of Whatman 3MM paper, and dried in a stream of warm air. This was continued until all the hydrolysate had been applied. The paper was serrated at the bottom to allow constant free flow of the solvent and then placed in a large chromatography tank to develop for 48 hours. The developing solvent used was a 4:1:1 mixture of N-butanol, acetic acid and water respectively.

After 2 days, the chromatogram was removed from the tank and dried inside a fume cupboard. Since the desmosines are insoluble in butanol and

remain in place at the origin, a strip of paper was cut that included and extended about 3mm beyond each side of the original streak. The resultant strip was approximately 2.5cm wide.

One end of the strip was cut into a point, and a wick was attached to the other end with a staple. The strip and wick were hung from the staple joint, over a glass rod placed in a small developing tank. The wick dipped into a beaker of distilled water, whilst the pointed end lay just above a 5ml beaker. The whole chamber was enclosed. The strip was eluted until 1ml had been collected. The exact volume was measured with an Ependorph pipette. For analysis 0.5ml was applied to the analyser.

(ii) U.V. and fluorescence spectroscopy

The same prepared samples were used in both the cases.

1) 2mg of the hen eggshell membrane (IO) was hydrolysed in 4ml of 6N HCl at 110^{o}C for 1 hour, the purpose being to solubilise the membrane. Three such hydrolysates were prepared. They were evaporated before being subjected to different solvents.

Hydrolysate 1 was dissolved in 1M HCl

" 2 " " " pH 6.8 phosphate buffer

" 3 " " " 1M NaOH

2) Elastin was hydrolysed in a similar manner.

Hydrolysate 4 was dissolved in 1M HCl

" 5 " " " 1M NaOH

Fluorescence spectroscopy was not carried out.

3) The oxidised and reduced pyridine nucleotides NAD^+ and NADH respectively were also studied for comparative purposes. 3mg of NAD^+ (6) was dissolved in water as was NADH (7).

U.V. Spectra

All three of the samples, including elastin and the pyridine nucleotides were fully scanned from 200nm to 500nm with the Unicam SP 800 spectrophotometer. 1cm light path silica cells were used.

Fluorescence Spectra

NAD^+, NADH, and all three of the membrane hydrolysates were fully scanned, from 200 to 800nm. Activation wavelength settings were taken at 10nm intervals between 200 and 400nm. The Aminco-Bowman Spectrofluorimeter was used. Special fluorimetric cells were used in this instrument.

II. CHEMICAL AND PHYSICAL STRUCTURE OF THE MEMBRANE

4. The Membrane Protein

In the past, the extracted membrane proteins have been analysed for their chemical composition but no attempt has been made to examine their chemical structure. Harsh physical methods such as autoclaving have been used by one group of workers whereas an enzymic method of extraction has been used by another. Still others have used chemical methods but without real success.

Since harsh physical conditions may have deranged the chemical nature of the extracted product and enzymic extraction alone does not provide an answer to the possible chemical structure of the protein, use was made of the traditional chemical denaturing agents.

Recent work by Kido and coworkers (1975,1977) on the Vitelline membrane, was an inducement to examine the validity of Candlish's (1972) statement, that the eggshell membrane protein is related to other organic components of the egg covering. The Vitelline membrane was reported to be soluble in 5% SDS and 6M guanidine hydrochloride after 2 hours of vigorous stirring. The maximum solubility was at a concentration of 10mg/ml in either solvent. In 8M urea, however, the membrane dissolved rather slowly giving a viscous solution. This low level of solubility was reported to be due to the rather high hydrophobic amino acid content of the protein. The authors believed the vitelline membrane protein to be keratinous in nature.

(i) Egghsell membrane extraction

Materials

1) Hen eggshell membrane (IO)

2) Guanidine hydrochloride (Sigma Ltd)
   6M

3) Sodium dodecyl sulphate (SDS) (Sigma Ltd)
   5% and 10% (W/V)

4) Urea (BDH Ltd)

8M

5) Thiourea (BDH Ltd)

1.2M

6) Cellophane Visking Tubing 3cm width

7) Trichloroacetic acid (TCA) (BDH Ltd)

Procedure

At first small quantities of about 5mg membrane were placed in 10ml of the denaturing reagents and stirred vigorously at room temperature. No protein could be detected in any one of the reagents. The membrane remained insoluble. Precipitation with TCA was tried and readings measured at 280nm with the Pye Unicam SP6/550 spectrophotometer, without success.

Then 0.5g of the membrane portions were placed in 50ml of the denaturants at 40°C in a water-bath and stirred overnight. Protein was only detected in the guanidine hydrochloride suspension.

Since the results were encouraging, 2g of the membrane was placed in 100ml of guanidine hydrochloride and vigorously stirred at 40°C for 48 hours. After two days the suspension was filtered and placed into a visking tubing and dialysed against running tap water.

The membrane residue was dried on top of the oven and reweighed. It appeared that 0.1g of the membrane had dissolved.

After five days of continuous dialysis, the volume of solution was reduced by drying the tubing in air before centrifuging. The recovered precipitate weighed 60mg. The supernatant was tested for any remaining protein; none was present.

The same reweighed membrane was once more subjected to 6M guanidine hydrochloride, under similar conditions as before, and the solution dialysed. No precipitate was obtained, since the protein was found to be water-soluble. The solution of protein was dried in air and the residue which was recovered weighed 40mg.

The extracted proteins were analysed for their total amino acid content as described previously. The guanidine hydrochloride insoluble residue from the first extract was also similarly analysed.

(ii) Gel-filtration

Attempts were made to redissolve the extracted guanidine hydrochloride soluble protein under the same conditions as for extraction but to no avail. Instead a new extracted solution had to be used in this experiment. It is possible that the extracted protein may have renatured in the tap water. Reports of the matrix protein renaturing in the presence of calcium ions have been mentioned (Robinson and Heaney 1973).

Procedure

The gel, Sepharose CL-6B was used. It is especially stable in the denaturing agents such as guanidine hydrochloride. The gel which is available as a suspension was deaerated and packed into a column of size 1.8 x 90cm. The Merthiolate which is present in the gel was removed by passing large quantities of deaerated water through the column. The gel was equilibrated with the guanidine hydrochloride.

The 20ml sample was loaded onto the column. The sample volume is within the recommended 1-5% ratio to bed volume. 3ml fractions were collected at a flow rate of 0.3ml/minute. This is well below the recommended 2.5ml/minute (Pharmacia 1979). The void volume was measured using the Blue Dextran 2000. Alternate fractions were measured with the Pye Unicam SP6/550 spectrophotometer.

5. Electron Microscope Studies

The physical structure of the membrane has been carefully examined by many authors, however the major concern in this study was to examine the chemically treated membrane, to compare it with the natural one and to compare some properties of the latter with that of the fibrous proteins.

Materials

1) Sodium cacodylate (Taab) and HCl (May and Baker AR) buffers:

(i) 0.08M cacodylate buffer pH 7.3

(ii) 0.2M cacodylate buffer pH 7.3

2) Paraformaldehyde (BDH) and 25% glutaraldehyde (BDH) fixative:

4% paraformaldehyde and 5% glutaraldehyde in 0.08M and 0.2M cacodylate buffer pH 7.3.

3) Osmic acid (BDH)

1% Osmic acid in 0.08M cacodylate buffer pH 7.3 (W/V)

4) Araldite epoxy resin (Taab)

(i) Resin 506

(ii) Hardener - DDSA (dodecenyl succinic anhydride)

(iii) Accelerator - DMP30 (tri-dimethylaminoethyl phenol)

(iv) Plasticer - (di-butyl phthalate)

5) Absolute alcohol (BDH)

6) Epoxy propane (BDH)

7) Uranyl Acetate (BDH)

2% solution in 50% alcohol (W/V)

8) Sodium hydroxide (BDH AR)

0.1N

9) Lead Citrate (BDH)

0.4% solution in 0.1N NaOH (W/V)

Procedure

The membranes were fixed in formaldehyde/glutaraldehyde mixture for 1 hour before being centrifuged and washed three times with the 0.08M cacodylate buffer pH 7.3.

Postfixation was for 1 hour in the osmic acid. Again the fixed membranes were centrifuged and then washed three times with the same buffer.

After dehydration with increasing concentration of the alcohol, the fixed blocks were washed with the alcohol/epoxy propane intermedium, before being rewashed three times with the epoxy propane medium and finally centrifuged.

The pellets were cut into cubes and placed overnight in an epoxy propane/araldite epoxy resin mixture. They were then embedded in the araldite epoxy resin for 24 hours prior to hardening at 60^{o}C for 36-48 hours. Thin sections were cut on the Reichert UM-3 ultramicrometer and fixed onto 300 mesh grids.

Next the grids were stained with uranyl acetate. The excess stain was washed off with water and the grids dried. They were restained in lead citrate. Excess lead citrate was removed with sodium hydroxide. The sodium hydroxide was washed with water. The grids were dried and stored. They were ready for use. Photographs were taken on the AEI EM6B electron microscope as shown in the results section.

III. RESULTS AND DISCUSSION

III. RESULTS

Firstly, the results of the chemical composition of the membranes will be presented, followed later by that of structural analysis. To avoid repetition of too many words, the following symbols have been used:

I refers to inner membrane

O refers to outer membrane, and

IO refers to inner plus outer membrane.

Amino Acid Analysis

Since both the losses and the slow liberation of amino acids occur during hydrolysis, analysis at 48 and 72 hours was performed. The corrected values and percentage recovery of the Duck outer membrane are presented in Table VI. Analysis of the S-Line IO also yielded similar values. These values were used to correct all other values of the different membranes.

All values have been expressed as gram of anhydro-amino acid per 100g of dry membrane.

The individual amino acid values of the hen membrane (Table VII) were used to compare the relative amino acid values of the other birds' membranes (Tables VIII to XIV).

The concentration of the amino acids in the inner membrane of the hen is higher than in the outer membrane. This was also observed to be the case in the different strains of the hen (Tables VIII to XI). In the other species, however, the relative amino acid concentration is greater in the outer membrane. This may in fact be due to sheer difference in weighing out 2mg of the membranes. However, the values in the two membranes are high enough and too consistent to be due to difference in mass alone.

TABLE VI

AMINO ACID COMPOSITION OF DUCK OUTER MEMBRANE

AMINO ACID	TIME OF HYDROLYSIS 24 hour	48 hour	72 hour	CORRECTED VALUES	RECOVERY AFTER 24 HOUR HYDROLYSIS
4 OH-Proline	0.59	Trace	-	-	-
Cysteic Acid	3.42	4.15	4.13	4.15*	82.4%
Aspartic Acid	8.02	7.19	6.63	8.38	95.7%
Threonine	5.34	4.71	4.17	5.80	92.1%
Serine	5.64	5.03	4.56	6.20	91.0%
Glutamic Acid	10.06	9.00	8.25	10.55	95.4%
Proline	8.44	7.81	6.56	9.10	92.8%
Glycine	5.90	4.85	4.81	6.45	91.5%
Alanine	2.95	2.69	2.41	3.25	90.8%
Valine	6.82	6.89	6.63	6.89*	99.0%
Cystine	1.20	0.47	-	1.45	82.8%
Methionine	1.21	0.66	0.43	1.60	75.6%
Isoleucine	2.96	2.71	2.46	3.25	91.1%
Leucine	4.39	3.66	3.38	4.90	89.6%
Tyrosine	2.58	1.97	1.73	3.02	85.4%
Phenyl-alanine	2.36	2.14	1.95	2.56	92.2%
Tryptophan	2.57	-	-	2.57*	-
Hydroxy-lysine	0.16	0.19	0.18	0.19*	84.2%
Lysine	3.57	3.10	2.98	3.88	92.0%
Histidine	3.16	2.94	2.84	3.32	95.2%
Ammonia	1.84	2.16	2.43	2.43*	75.7%
Arginine	7.37	6.56	5.96	7.80	94.5%
Totals	89.86	78.83	71.43	97.74	

NB Composition expressed as g of anhydro-amino acid per 100g of dry membrane

* represents maximum value. All other values extrapolated to zero hours.

TABLE VII

AMINO ACID COMPOSITION OF THE LIPID-FREE HEN MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	0.43	0.58	0.82
Cysteic Acid	3.24	2.88	2.84
Aspartic Acid	7.96	6.00	7.58
Threonine	5.61	4.37	5.60
Serine	5.46	4.50	5.34
Glutamic Acid	11.83	8.59	11.27
Proline	8.43	6.26	8.02
Glycine	5.79	4.80	5.72
Alanine	2.65	2.48	2.68
Valine	6.24	4.42	5.77
Cystine	3.51	2.37	2.31
Methionine	2.30	1.75	2.53
Isoleucine	3.41	2.89	3.21
Leucine	4.69	4.49	4.62
Tyrosine	1.83	2.19	1.94
Phenylalanine	1.79	1.75	1.78
Tryptophan	1.80	1.06	-
Hydroxylysine	0.20	0.21	0.23
Lysine	3.59	2.97	3.45
Histidine	4.20	2.67	3.40
Ammonia	2.38	1.81	2.96
Arginine	8.27	6.07	7.41

TO SHOW SOME DIFFERENCES BETWEEN THE VARIOUS BIRD SPECIES

Table VII(a)

Amino Acid	Hen I	S-Line I	J-Line I	QI
Cysteic Acid	3.24	11.17	10.45	4.04
Aspartic Acid	7.96	8.20	7.76	5.00
Serine	5.46	6.59	6.24	3.43
Glutamic Acid	11.83	11.93	9.36	6.70
Glycine	5.79	6.89	6.44	3.88
Valine	6.24	6.12	5.82	3.37
Methionine	2.30	1.67	1.51	1.02
Leucine	4.69	6.17	5.47	3.09
Tyrosine	1.83	2.93	2.69	1.21
Phenylalanine	1.79	2.14	1.95	1.59
Lysine	3.59	4.40	4.22	2.89
Histidine	4.20	3.85	4.09	2.70
Arginine	8.27	7.48	8.11	4.25

TO SHOW SOME DIFFERENCES BETWEEN THE VARIOUS BIRD SPECIES

Table VII(b)

Amino Acid	Hen IO	T-Line IO	J-Line IO	Goose IO
Cysteic Acid	2.84	6.70	5.24	7.03
Aspartic Acid	7.58	5.91	5.38	6.86
Serine	5.34	3.74	3.48	5.42
Glutamic Acid	11.27	8.69	15.34	9.11
Glycine	5.72	4.58	4.15	5.26
Valine	5.77	4.75	4.20	5.03
Methionine	2.53	1.26	1.28	1.49
Leucine	4.62	3.77	3.38	3.82
Tyrosine	1.94	1.65	1.48	2.32
Phenylalanine	1.78	1.51	1.31	2.26
Lysine	3.45	2.88	2.66	3.58
Histidine	3.40	3.02	2.93	2.71
Arginine	7.41	6.14	5.66	5.61

TABLE VIII

AMINO ACID COMPOSITION OF THE LIPID-FREE S-LINE MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	0.68	Trace	0.77
Cysteic Acid	11.17	6.54	8.65
Aspartic Acid	8.20	5.88	7.88
Threonine	6.11	4.09	5.44
Serine	6.59	4.21	5.70
Glutamic Acid	11.93	8.62	11.25
Proline	9.01	6.28	8.51
Glycine	6.89	4.47	6.30
Alanine	3.40	2.02	2.95
Valine	6.12	4.46	5.91
Cystine	-	0.82	0.99
Methionine	1.67	1.57	1.47
Isoleucine	3.89	2.36	3.21
Leucine	6.17	3.44	5.02
Tyrosine	2.93	1.48	2.26
Phenylalanine	2.14	1.41	1.94
Tryptophan	0.90	0.91	0.79
Hydroxylysine	0.22	0.21	0.24
Lysine	4.40	2.80	3.79
Histidine	3.85	2.97	3.88
Ammonia	3.17	1.93	2.60
Arginine	7.48	5.63	7.59

TABLE IX

AMINO ACID COMPOSITION OF THE LIPID-FREE T-LINE MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	0.58	Trace	Trace
Cysteic Acid	9.14	3.63	6.70
Aspartic Acid	7.37	6.56	5.91
Threonine	5.54	4.69	4.27
Serine	5.34	4.45	3.74
Glutamic Acid	10.78	9.87	8.69
Proline	8.47	7.37	6.50
Glycine	6.13	4.88	4.58
Alanine	2.77	2.15	2.02
Valine	6.18	5.30	4.75
Cystine	0.78	2.78	1.17
Methionine	1.75	1.94	1.26
Isoleucine	3.54	2.83	2.60
Leucine	5.48	3.94	3.77
Tyrosine	2.53	1.78	1.65
Phenylalanine	1.91	1.67	1.51
Tryptophan	0.91	1.65	1.09
Hydroxylysine	0.26	0.24	0.20
Lysine	3.96	3.16	2.88
Histidine	3.58	3.05	3.02
Ammonia	2.55	2.34	1.88
Arginine	7.49	6.75	6.14

TABLE X

AMINO ACID COMPOSITION OF THE LIPID-FREE J-LINE MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	0.53	Trace	0.53
Cysteic Acid	10.45	4.39	5.24
Aspartic Acid	7.76	4.39	5.38
Threonine	5.85	3.19	3.87
Serine	6.24	3.18	3.48
Glutamic Acid	9.36	6.70	15.34
Proline	8.75	5.03	6.05
Glycine	6.44	3.30	4.15
Alanine	3.08	1.55	1.96
Valine	5.82	3.51	4.20
Cystine	0.30	1.21	1.23
Methionine	1.51	0.69	1.28
Isoleucine	3.64	1.98	2.37
Leucine	5.47	2.62	3.38
Tyrosine	2.69	1.24	1.48
Phenylalanine	1.95	1.17	1.31
Tryptophan	1.25	1.33	1.53
Hydroxylysine	0.26	Trace	0.19
Lysine	4.22	2.39	2.66
Histidine	4.09	2.82	2.93
Ammonia	2.34	1.74	1.76
Arginine	8.11	5.59	5.66

TABLE XI

AMINO ACID COMPOSITION OF THE LIPID-FREE BANTAM MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	0.34	0.43	0.48
Cysteic Acid	5.15	7.46	4.93
Aspartic Acid	8.02	6.19	10.89
Threonine	5.73	4.71	7.66
Serine	5.55	4.75	7.50
Glutamic Acid	12.09	8.81	16.24
Proline	9.49	6.90	12.49
Glycine	5.77	4.65	8.03
Alanine	2.63	2.46	3.68
Valine	6.41	4.51	8.48
Cystine	2.71	8.25	4.51
Methionine	1.09	1.59	3.28
Isoleucine	3.38	2.90	4.57
Leucine	4.59	4.35	6.50
Tyrosine	1.91	2.37	2.72
Phenylalanine	1.76	1.70	2.40
Tryptophan	1.25	0.74	1.25
Hydroxylysine	0.19	0.23	0.30
Lysine	3.85	2.86	4.66
Histidine	3.77	2.45	4.95
Ammonia	2.59	2.02	3.32
Arginine	8.13	6.29	10.76

TABLE XII

AMINO ACID COMPOSITION OF THE LIPID-FREE TURKEY MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	Trace	Trace	Trace
Cysteic Acid	5.47	2.88	3.75
Aspartic Acid	3.98	9.34	6.58
Threonine	2.77	6.44	4.54
Serine	3.23	6.74	4.77
Glutamic Acid	6.12	14.55	10.15
Proline	4.70	10.03	7.10
Glycine	3.18	6.33	4.59
Alanine	1.92	3.61	2.64
Valine	2.85	6.57	4.75
Cystine	-	5.15	2.74
Methionine	0.81	2.92	1.61
Isoleucine	1.87	4.62	3.27
Leucine	2.77	5.54	4.07
Tyrosine	1.77	2.64	2.12
Phenylalanine	1.13	1.86	1.55
Tryptophan	0.96	1.16	0.76
Hydroxylysine	0.15	0.24	0.15
Lysine	2.42	4.47	3.28
Histidine	2.13	5.00	3.61
Ammonia	1.68	3.54	2.83
Arginine	4.01	9.20	6.47

TABLE XIII

AMINO ACID COMPOSITION OF THE LIPID-FREE DUCK MEMBRANE

AMINO ACID	INNER MEMBRANE	OUTER MEMBRANE	INNER & OUTER MEMBRANE
4 OH-Proline	0.58	Trace	Trace
Cysteic Acid	6.89	4.15	2.12
Aspartic Acid	7.05	8.38	5.15
Threonine	4.80	5.80	3.55
Serine	5.25	6.20	3.82
Glutamic Acid	8.74	10.55	6.49
Proline	7.93	9.10	5.52
Glycine	6.32	6.45	4.00
Alanine	3.11	3.25	2.02
Valine	5.41	6.89	4.01
Cystine	1.87	1.45	2.31
Methionine	1.80	1.60	1.06
Isoleucine	2.45	3.25	2.36
Leucine	4.90	4.90	3.02
Tyrosine	3.14	3.02	1.85
Phenylalanine	2.71	2.56	1.65
Tryptophan	0.89	1.57	1.09
Hydroxylysine	0.23	0.19	0.18
Lysine	3.63	3.88	2.46
Histidine	2.36	3.32	2.20
Ammonia	1.54	2.43	1.73
Arginine	5.70	7.80	5.00

TABLE XIV

AMINO ACID COMPOSITION OF THE LIPID-FREE GOOSE AND QUAIL MEMBRANES

AMINO ACID	GOOSE INNER & OUTER MEMBRANE	QUAIL OUTER & INNER MEMBRANE	QUAIL INNER MEMBRANE
4 OH-Proline	0.68	0.43	0.48
Cysteic Acid	7.03	4.90	4.04
Aspartic Acid	6.86	8.82	5.00
Threonine	4.73	5.24	3.00
Serine	5.42	6.06	3.43
Glutamic Acid	9.11	13.04	6.70
Proline	7.68	9.06	5.01
Glycine	5.26	6.15	3.88
Alanine	2.65	2.99	1.87
Valine	5.03	6.35	3.37
Cystine	1.32	3.20	0.77
Methionine	1.49	2.67	1.02
Isoleucine	2.71	4.48	2.50
Leucine	3.82	4.59	3.09
Tyrosine	2.32	2.52	1.21
Phenylalanine	2.26	2.18	1.59
Tryptophan	1.89	1.16	0.52
Hydroxylysine	0.21	0.23	0.19
Lysine	3.58	4.61	2.89
Histidine	2.71	5.34	2.70
Ammonia	3.41	2.92	2.09
Arginine	5.61	7.62	4.25

On the whole, it can be said that the relative amino acids of all the relative membranes are comparable in value. Nevertheless, some subtle differences do exist and these are discussed with respect to each of the different membranes. Some of these differences are observed in Tables VII(a),(b).

First, the hen and S-Line membranes will be compared (Tables VII and VIII). The IO membrane values of the two birds so closely resemble that of each other, that it may be said the strain of the 'hen' is possibly S-Line. The eggs of both birds were of the same size and same colour. Nevertheless, differences are found between the inner membranes. The serine value is lower in the hen. This low value is not necessarily due to excessive decomposition in the presence of oxygen. The oxygen is certainly not available, since the cysteic acid content is low. Cysteic acid is readily formed during hydrolysis, under such gaseous conditions.

The other amino acids which are found to be significantly lower in the hen inner membrane include glycine, leucine and tyrosine. However, not all the amino acids are lower in hen I. A few amino acids, for example, arginine, histidine, methionine and valine, are just slightly higher. In S-Line O, leucine and tryptophan are somewhat higher than in hen O.

Comparison between the hen and T-Line (Tables VII and IX) indicate that once more the respective membranes are similar in amino acid content. Slight differences do exist, for example lysine, phenylalanine, tyrosine and leucine are variable. Although comparatively similar, the hen IO and T-Line IO differ to a certain extent. This difference is possibly due to a slightly higher mass ($\geqslant$ 2mg) taken for hen IO and a slightly lower mass ($\leqslant$2mg) taken for T-Line IO. The difference is very likely to be accentuated by the excessive decomposition in the presence of oxygen. The cysteic acid content of the T-Line IO is rather high.

The hen I compares very well with J-Line I (Tables VII and X) although the glutamic acid value is higher in the hen membrane. The J-Line O values

are slightly lower than in hen O. Again, this is probably due to the difference in weights. The glutamic acid content of J-Line IO is very high, even though the rest of the amino acid content is low.

The amino acid values in Bantam IO are higher than in hen IO (Tables VII and XI). The other two membranes of the Bantam and hen have comparable values.

Similarly, comparisons drawn between the different strains of the hen show that only some values are significantly different. In general, it can be seen that glutamic acid, methionine, leucine and histidine are most variable amongst all the membranes.

Comparisons were then drawn between the hen membranes and those of the other species. Results suggest that on the whole the Duck values are very much the same as the hen's, although the glutamic acid and methionine content of the hen is higher.

In the Turkey the outer membrane has a higher relative amino acid content than hen O. This is probably due to the difference in weights.

The Goose values differ in the amino acids, glutamic acid, arginine, valine, leucine, and aspartic acid, all of which are present in higher concentrations in the hen.

The Quail I has lower values than hen I. The amino acids lysine, histidine, proline, isoleucine, glutamic acid, and aspartic acid are higher in Quail IO than in hen IO.

In general, overall analysis of these results suggests that all the membranes are alike in their amino acid content. Any intrinsic differences that exist are not particularly important to certify that the membranes of one species or strain are different from the other.

TABLE XV

COLORIMETRIC DETERMINATION OF THE HYDROXYPROLINE CONTENT OF THE MEMBRANES

MEMBRANE		% HYDROXYPROLINE
Hen	IO	0.70
	O	0.58
	I	0.81
S-Line	IO	0.79
	O	0.69
	I	0.88
T-Line	IO	0.69
	O	0.68
	I	0.73
J-Line	IO	0.56
	O	0.51
	I	0.61
Bantam	IO	0.50
	O	0.44
	I	0.57
Turkey	IO	0.42
	O	0.43
	I	0.49
Duck	IO	0.64
	O	0.57
	I	0.68
Goose	IO	0.69
Quail	IO	0.63
	I	0.64

Colorimetric Determination of Hydroxyproline

These results definitely indicate that the ninhydrin test for this particular amino acid is not satisfactorily analysed.

Table XV shows that apart from the Turkey readings, all others are fairly high. On the analyser all three of the Turkey readings appeared as trace peaks.

The hen, S-Line and T-Line readings are higher than those of the J-Line and Bantam. The latter two strains produce small sized eggs. However, this criterion is no indication that as a consequence the hydroxyproline content should be less. Small sized eggs have the same thickness of membrane and the same proportion of weight as the large eggs (Balch and Cooke 1970). Moreover, the rest of the amino acid readings for the Bantam and J-Line are comparable with the other strains.

Table XV also shows that of all the membranes in each of the species, the hydroxyproline is more concentrated in the inner membrane.

Colorimetric Determination of the Sugar Content

The set of readings as shown in Table XVI were compiled from repeated observations.

The results of the neutral sugar analysis show that all the membranes have a fairly even carbohydrate content and that more is concentrated in the inner membrane. The average neutral sugar content for the inner membrane is 4.41% and that for the outer membrane is 3.76%.

The hexosamine content also follows a similar pattern. The concentration in the inner membrane ranges from 1.92% in the Quail to 2.32% in the Goose. In the outer membrane the concentration ranges between 1.48% and 1.63%.

TABLE XVI

SUGAR CONTENT

MEMBRANE		% NEUTRAL SUGAR	% HEXASOMINE	% SIALIC ACID
Hen	IO	4.08	1.90	0.74
	O	3.73	1.50	0.71
	I	4.55	2.25	0.77
S-Line	IO	3.85	1.96	0.63
	O	3.62	1.58	0.54
	I	4.43	2.28	0.66
T-Line	IO	3.64	1.88	0.75
	O	3.38	1.63	0.69
	I	4.43	2.11	0.78
J-Line	IO	4.20	1.83	0.66
	O	3.97	1.63	0.65
	I	4.48	2.19	0.79
Bantam	IO	3.85	1.86	0.66
	O	3.45	1.58	0.62
	I	4.43	2.08	0.74
Turkey	IO	3.90	2.04	0.67
	O	4.13	1.48	0.61
	I	4.67	2.19	0.73
Duck	IO	3.71	1.90	0.66
	O	3.38	1.58	0.61
	I	4.60	2.03	0.71
Goose	IO	4.32	2.32	0.64
Quail	IO	4.15	1.83	0.61
	I	4.39	1.92	0.69

NB IO refers to Inner Plus Outer membrane
O refers to Outer membrane
I refers to Inner membrane

The Sialic acid which comprises a small part of the total sugar content is also more concentrated in the inner membrane. The concentration range is between 0.66% and 0.77%. The concentration in the outer membrane ranges between 0.54% and 0.69%.

Thus overall the total sugar content in the membranes is approximately 8%. Candlish (1972) reported a total sugar content of just less than 6% (see Table I). The sialic acid content in the table is described to be rather high, it is my belief that it is an error and probably reads 0.9, 0.9. Instead the neutral sugar must read 4.0, 3.5 for the inner and outer membranes respectively. If that is so, then the readings described in Table XVI agree with Candlish's (1972) result for the neutral sugar and sialic acid.

The hexosamine content is much higher than what Candlish (1972) has proposed. However, the readings do agree with those of Baker and Balch (1962).

Desmosine and Isodesmosine Content

TABLE VII

AMINO ACID	HEN IO % CONTENT
Desmosine	0.028
Isodesmosine	0.022

NB Values expressed as g of anhydro-amino acid per 100g of dry membrane

FIG. 7

ABSORPTION SPECTRA
OF THE MEMBRANE AND ELASTIN SAMPLES
BEFORE REDUCTION

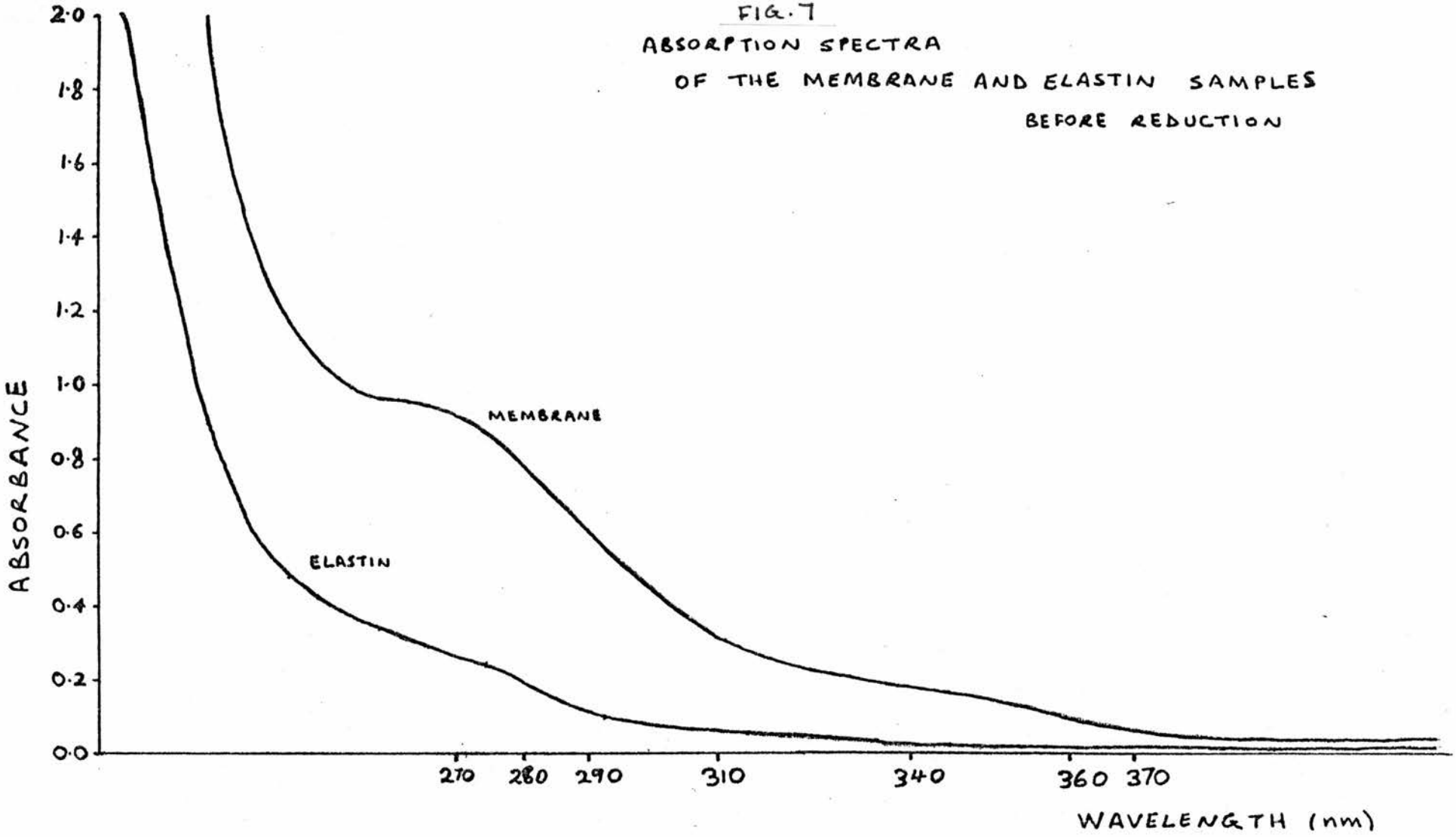


FIG. 8

ABSORPTION SPECTRA
OF THE MEMBRANE AND ELASTIN SAMPLES
AFTER REDUCTION

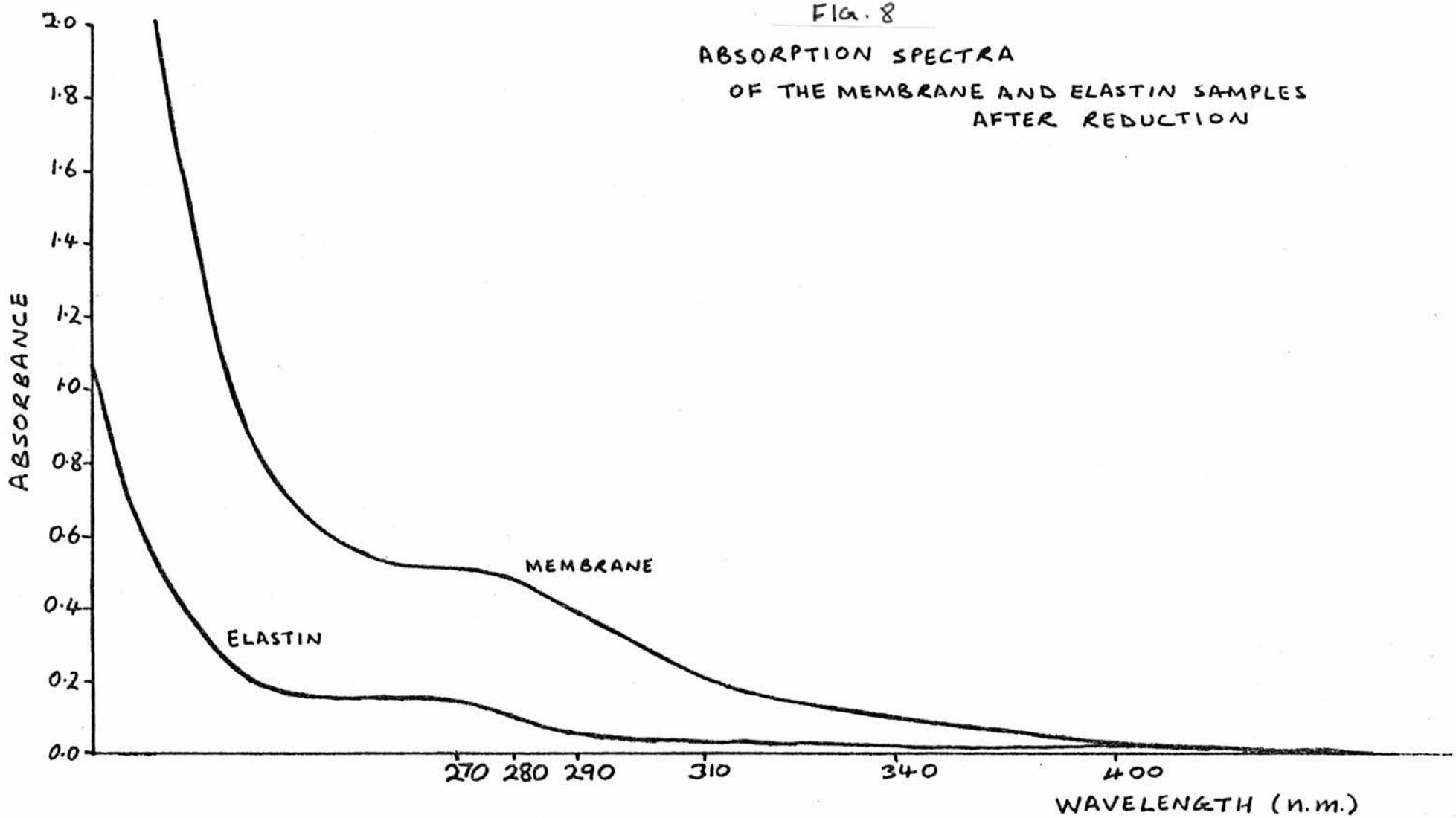
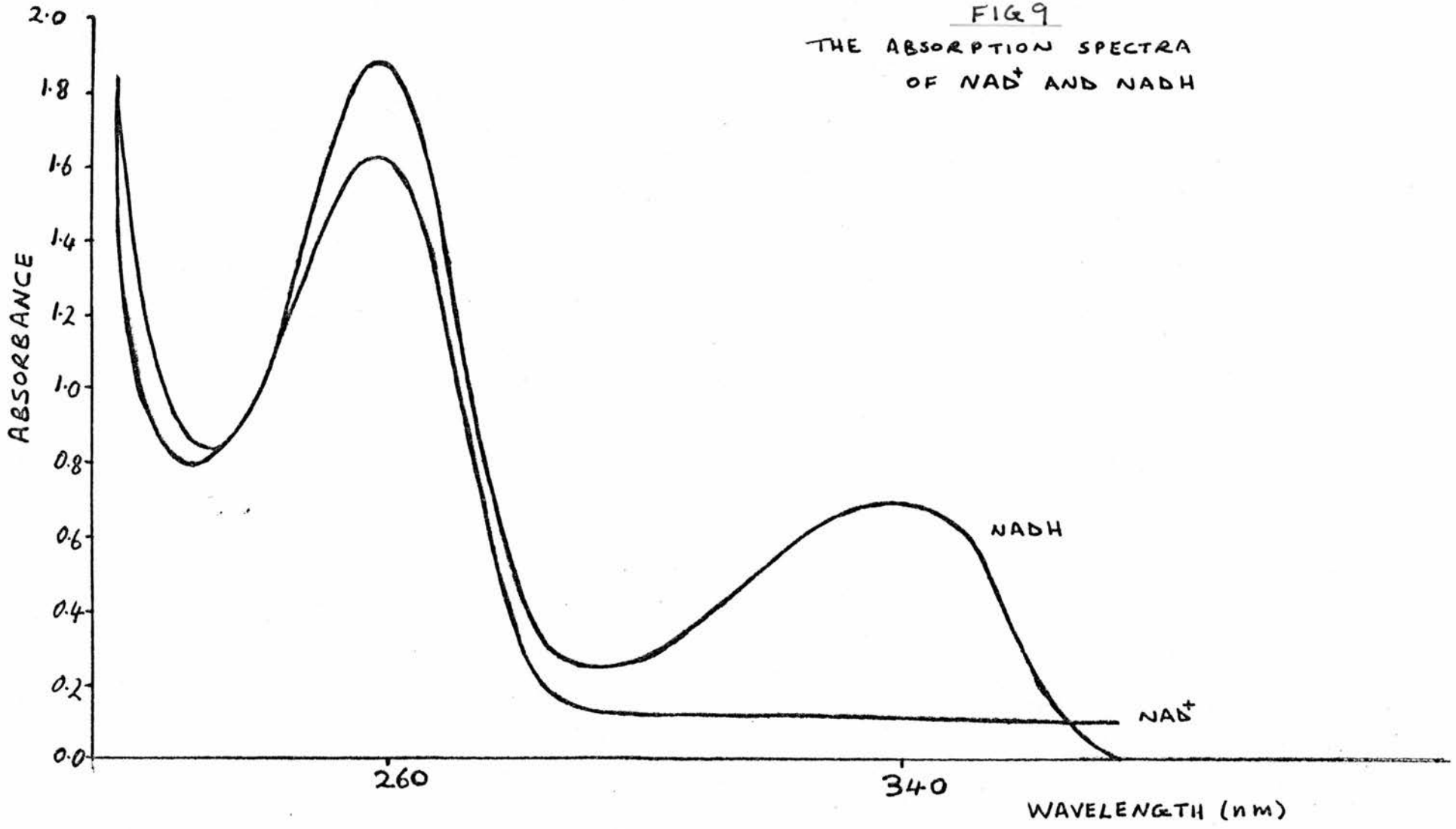


FIG 9

THE ABSORPTION SPECTRA OF NAD^+ AND NADH



Amino acid analysis shows that the desmosine content in the membrane is higher than the isodesmosine content. The values are, however, very low. Ross and Bornstein (1969) report values of 0.55% for desmosine and 0.39% for isodesmosine in samples of elastin. It so appears that the proportion of desmosine to isodesmosine in the membrane is not quite the same as in the elastin. Leach and coworkers (1978,1980) who first reported the presence of these two amino acids, however, do not quote a value for them.

Further comparisons drawn between the membrane and the elastin showed that in the UV both the structures behaved similarly. As can be seen in Figs 7 and 8, only one absorbance peak at about 270nm is apparent, both before and after reduction, the only difference being that after reduction the intensity of the peak decreases quite significantly.

Since desmosine and isodesmosine are shown to possess a pyridinium ring (Franzblau 1971) it was believed that in the reduced state, they would behave like the pyridine nucleotides and give a large absorbance peak at about 340nm as shown for NADH in Fig 9. However, as observed in Fig 8, this was not the case either in the elastin sample or the membrane.

It is possible that the attached proteins modify the molecules in such a way that the absorbance effect is cancelled.

Fluorescence spectroscopy studies show that NADH is not excited at any wavelength, but absorbs at 460nm (see Fig 10). Similarly, NAD^+ and the membrane hydrolysate 1 and 3 do not fluoresce below excitation wavelength 300nm, but absorb at one particular wavelength. NAD^+ absorbs at 400nm. Hydrolysates 1 and 3 absorb at 450nm. However, hydrolysate 2 is exceptional in that the absorbance peak is seen to shift from 390nm at excitation wavelength 230nm to 370nm at excitation wavelength 290nm.

Hydrolysate 1 as can be seen in Fig 11, has three excitation fluor-

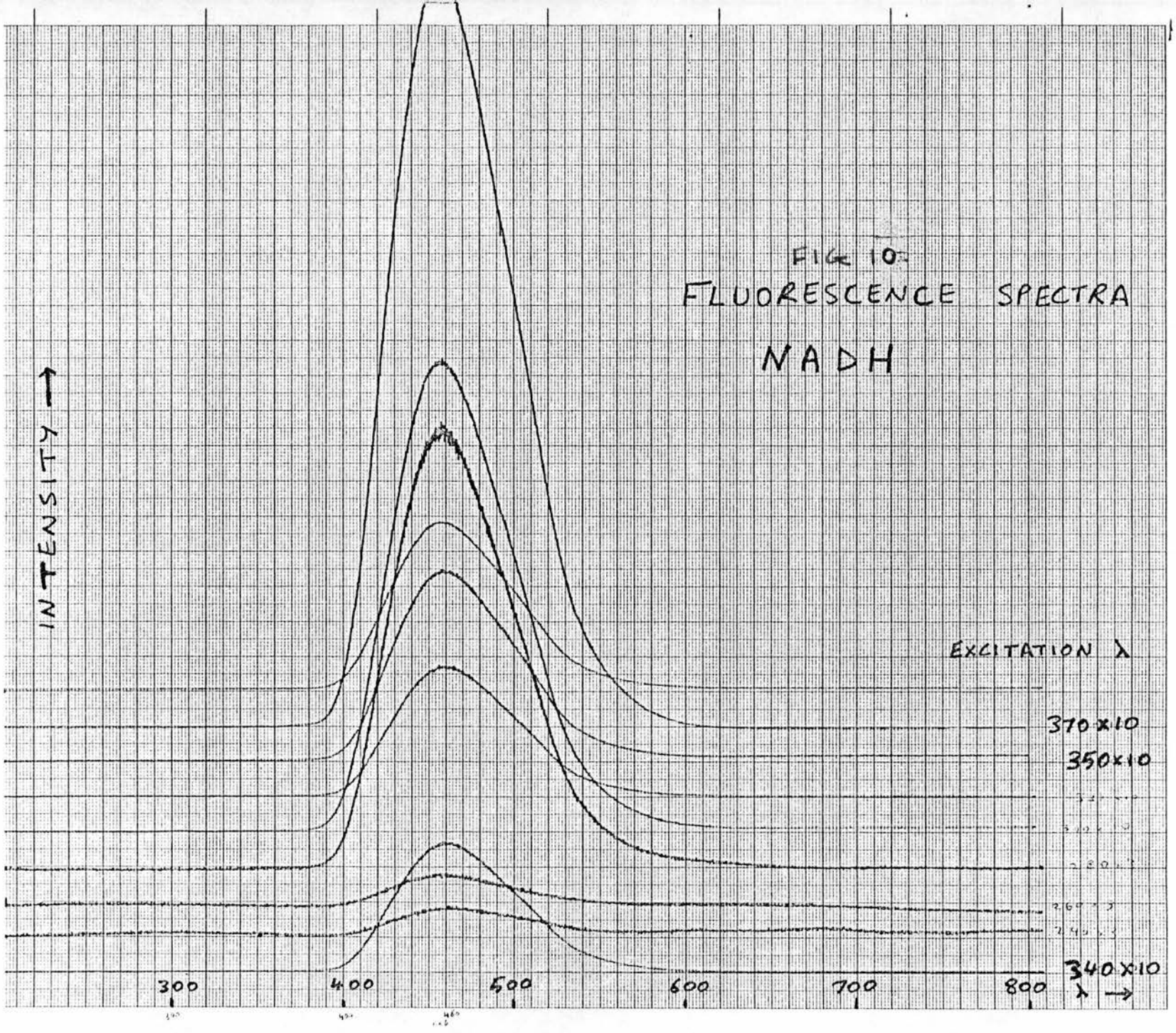


FIG 10:
FLUORESCENCE SPECTRA
NADH

FIG. 11

FLUORESCENCE SPECTRA

HYDROLYZATE I

EXCITATION / FLUORESCENCE MAXIMA

340 / 405

350 / 440

370 / 460

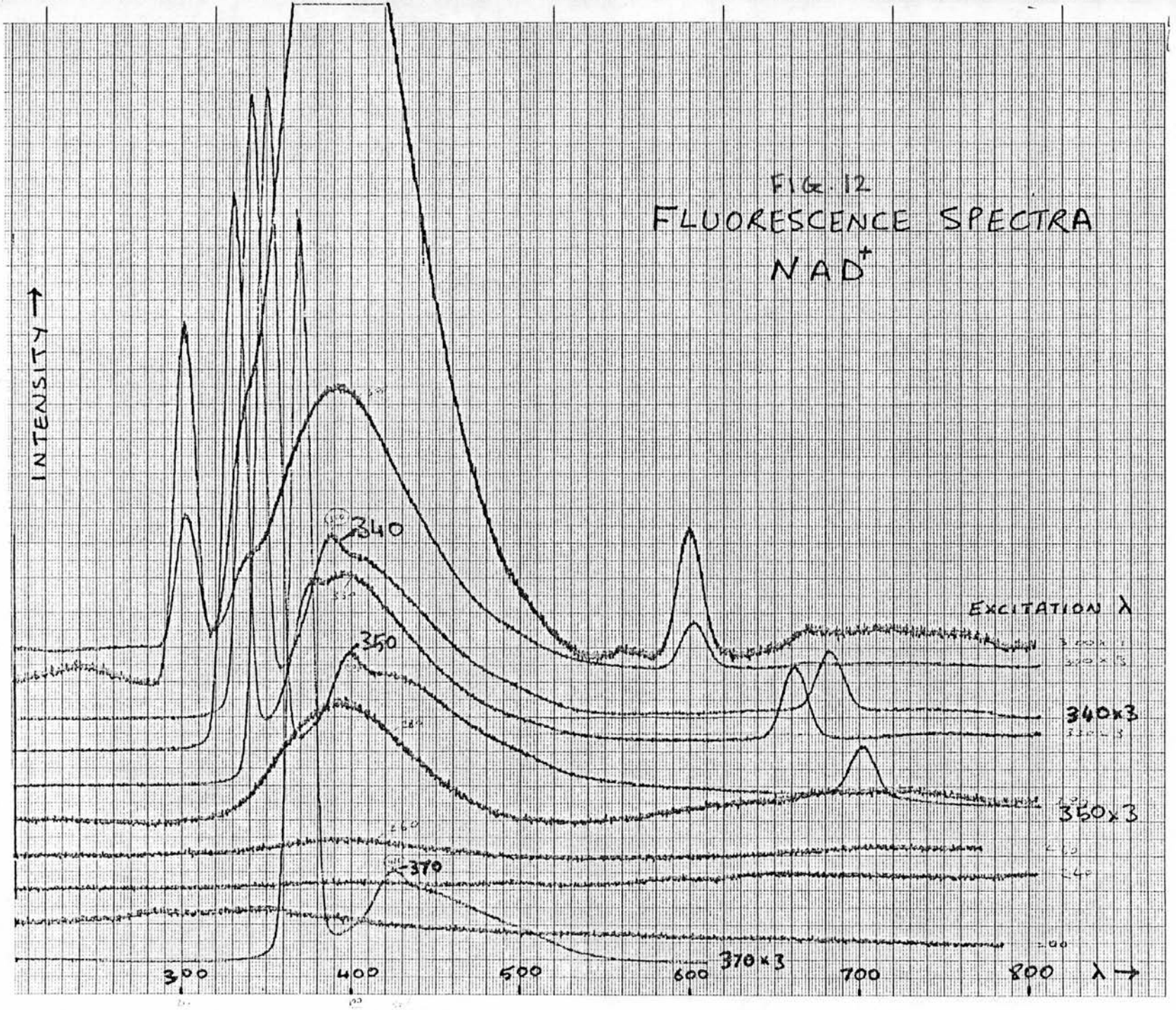


FIG. 12
FLUORESCENCE SPECTRA
NAD^+

escence maxima. Similarly, hydrolysates 2 and 3 also have such maxima. The 350/440 and the 370/460 maximum occur in all three hydrolysates. However, the 340/405 maximum in hydrolysate 1 is exceptional, as is the 340/435 maximum in hydrolysate 2 and the 340/440 maximum in hydrolysate 3. Thus, the 340nm excitation maximum is pH dependent.

In the NAD^+ (Fig 12), at the excitation wavelengths 340, 350, and 370nm, the peaks fluoresce at 390, 400 and 425nm respectively. The fluorescence peak range has far diminished.

In addition to the peaks just described, secondary small peaks can also be observed between the range 600 and 800nm. As the excitation wavelength is raised so the peaks shift to higher wavelength. Very small such peaks can also be seen in Fig 11. In hydrolysate 2, such peaks are almost equal in size to the NAD^+ peaks. It is believed that these peaks are peculiar to the pyridinium ring structure. Hence further evidence that desmosine and isodesmosine really do play a strong structural role in the membrane. However, fluorescence spectroscopy of the elastin sample would be necessary before any correlations can be made.

Chemical Structure

Amino acid analyses of the extracted proteins and the residue obtained from the first extraction are shown in Table XVIII.

As can be seen both the first extract and the residue show a similarity in their amino acid content. However, in the extract, cystine, methionine and hydroxyproline are absent. Cystine has obviously converted to cysteic acid. The methionine and hydroxyproline content are low anyway and may even be lower in this instance.

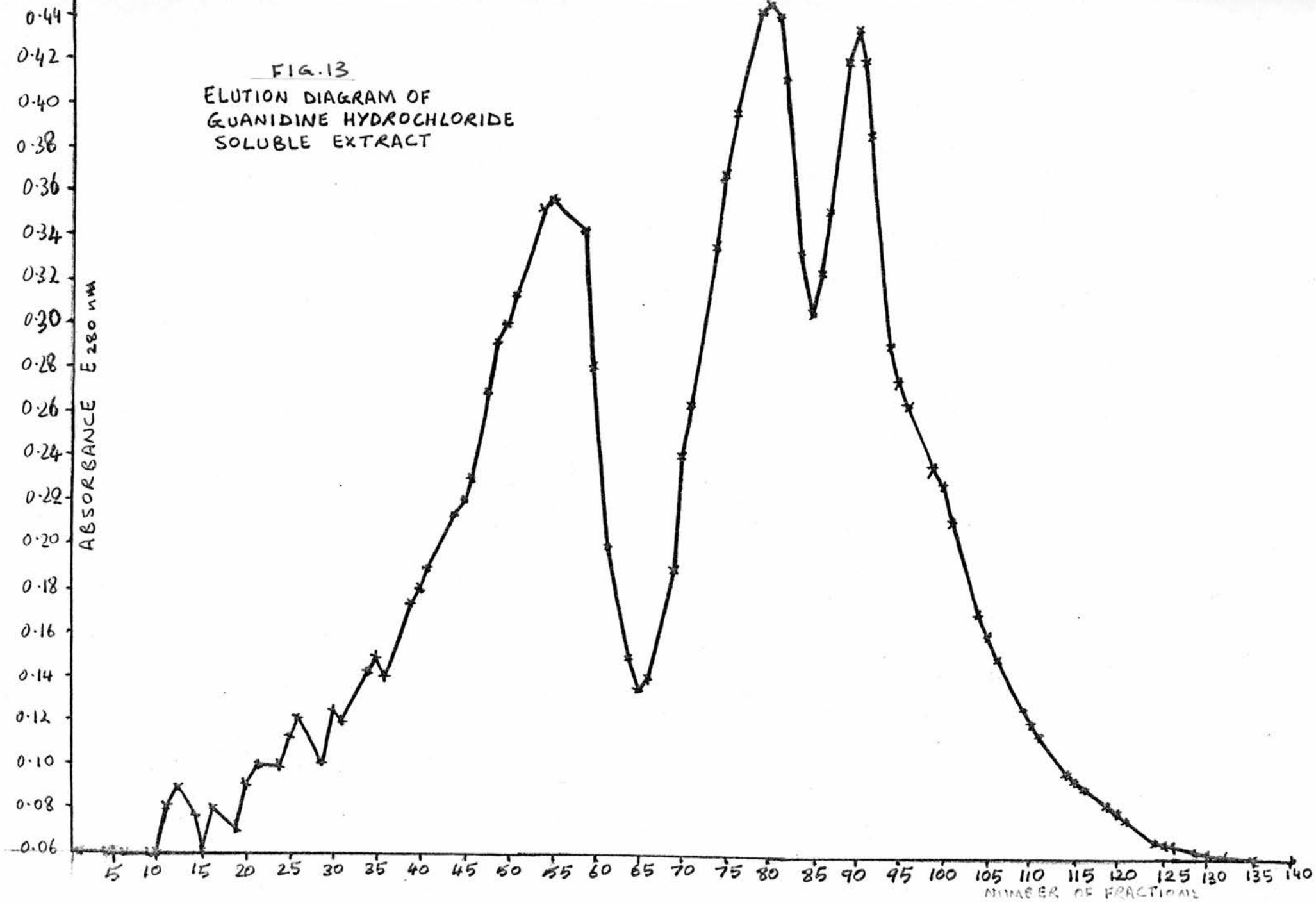
TABLE XVIII

AMINO ACID COMPOSITION OF THE EXTRACTED PROTEINS AND THE RESIDUAL MEMBRANE

AMINO ACID	GUANIDINE HYDROCHLORIDE SOLUBLE PROTEIN	WATER SOLUBLE PROTEIN	GUANIDINE HYDROCHLORIDE INSOLUBLE MEMBRANE
4 OH-Proline	-	-	1.11
Cysteic Acid	8.35	2.10	2.83
Aspartic Acid	10.51	2.70	6.59
Threonine	6.18	1.49	4.85
Serine	7.69	2.02	4.68
Glutamic Acid	12.45	-	9.83
Proline	7.27	-	7.75
Glycine	6.83	2.16	4.90
Alanine	4.95	1.59	2.24
Valine	6.62	Trace	4.90
Cystine	-	-	1.80
Methionine	-	-	2.10
Isoleucine	4.63	Trace	2.77
Leucine	7.40	Trace	3.87
Tyrosine	3.70	-	1.75
Phenylalanine	3.31	-	1.64
Tryptophan	0.15	Trace	0.12
Hydroxylysine	0.20	-	0.21
Lysine	5.43	2.48	3.17
Histidine	3.96	-	3.18
Ammonia	4.15	0.68	2.83
Arginine	9.32	Trace	6.23

FIG. 13

ELUTION DIAGRAM OF GUANIDINE HYDROCHLORIDE SOLUBLE EXTRACT



The water-soluble protein is formed from a few amino acids. It is possible that the protein may be a degraded product of the mantle or equally well it may be the glycoprotein portion of the mantle which is only released after all the disulphide bridges have been broken. Whether the glycoprotein may be the 'ovocapsin' as suggested by Candlish (1972) is not known. The aspartic acid content is high. This was represented as the N-terminal amino acid in all the proteins of the eggshell coverings. Moreover, cystine is also present. However, sugar analysis would have to be carried out before any firm conclusions can be drawn. Homogeniety tests would also be necessary.

Gel-filtration studies shown that the guanidine hydrochloride soluble extract is composed of 3 peaks, possibly 4 (see Fig 13).

The 3 peak pattern resembles that of the vitelline membrane run on SDS columns by Kido and coworkers (1975,1977).

It is doubtful whether all 3 of the peaks are glycoprotein in nature. However, each of the fraction peaks would need to be analysed to confirm their nature.

Since the protein concentrations of the extract was so low, any attempts at determining S-values were unsuccessful.

However, approximate molecular weights of the three peaks were calculated by using the equation:

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

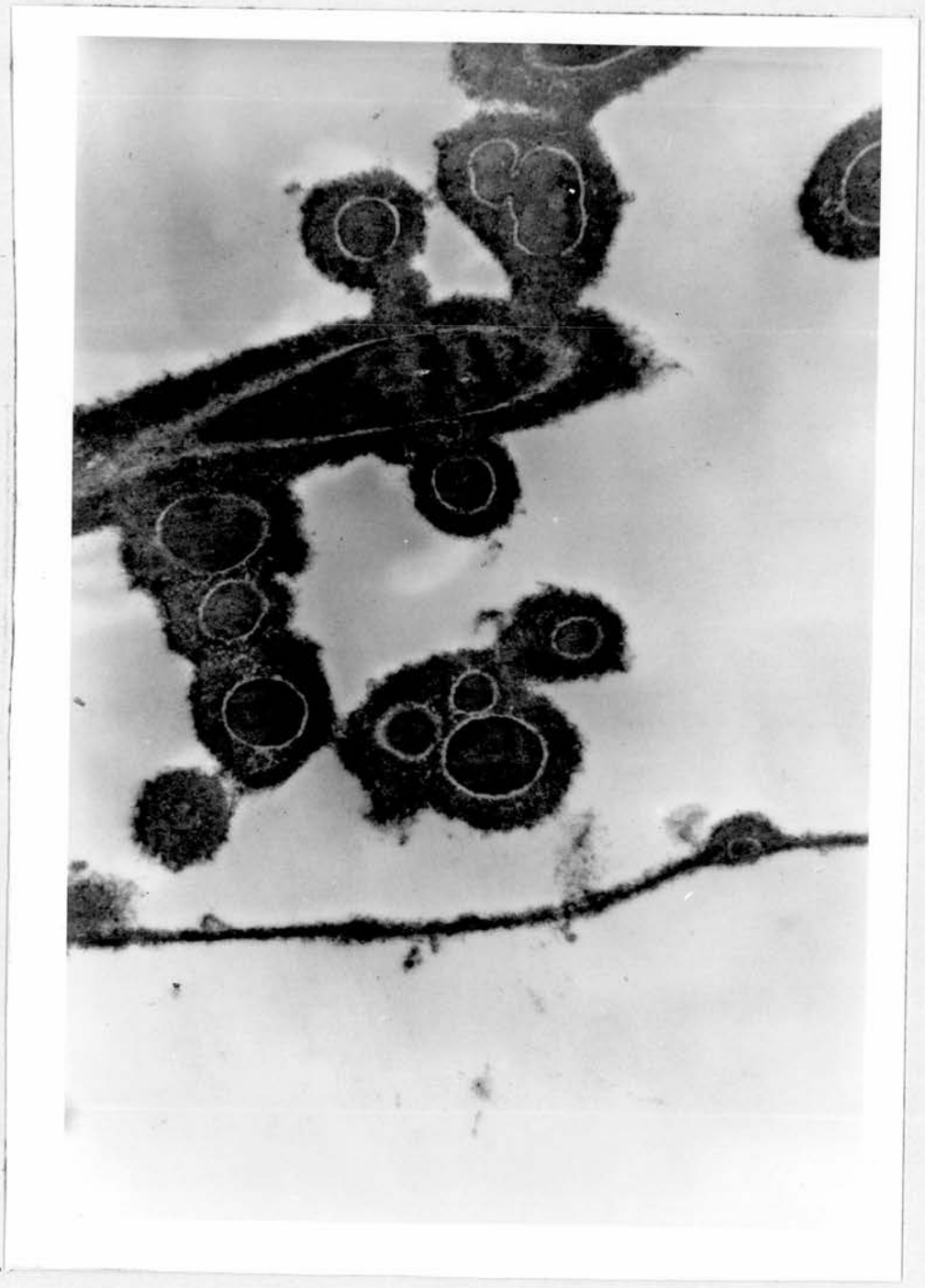
where K_{av} = fraction of stationary gel volume available for diffusion of a given solute species

V_e = elution volume of the respective peak under study

FIG. 14

MEMBRANE SHEET AT MAGNIFICATION

x 19, 600



V_o = void volume (40ml in this case)

and V_t = total bed volume (400ml in this case).

Since the sample volume was signficant it would need to be subtracted from the apparent elution volume to yield the actual elution volume.

The selectivity curve from Pharmacia (Pharmacia 1979) was used.

Therefore the approximate molecular weights are:

Peak I 8×10^6

Peak II 10^5

Peak III 5.5×10^4

According to Kido and coworkers (1975), their peaks II and III had approximate molecular weights of 2.6×10^5 and 3.2×10^4 respectively. These authors showed that peak II was glycoprotein in nature (Kido et al 1977).

Since peak II and III in Fig 13 have approximate molecular weights close to those obtained by Kido and coworkers (1975), it is possible that peak II at least is a glycoprotein.

Electron Microscope Studies

Besides the membrane powder, both treated with guanidine hydrochloride and otherwise, membrane sheets were also studied.

Fig 14 is an example of the membrane sheet at magnification x19,600. It shows the inner layer as a line running across the bottom half of the photograph. The small round cores (within the white boundaries) are believed to belong to the inner membrane, whilst the one large rather elliptical-looking core is believed to be part of the outer membrane (Simons 1971).

FIG. 15

POWDERED MEMBRANE AT MAGNIFICATION

x 57,350

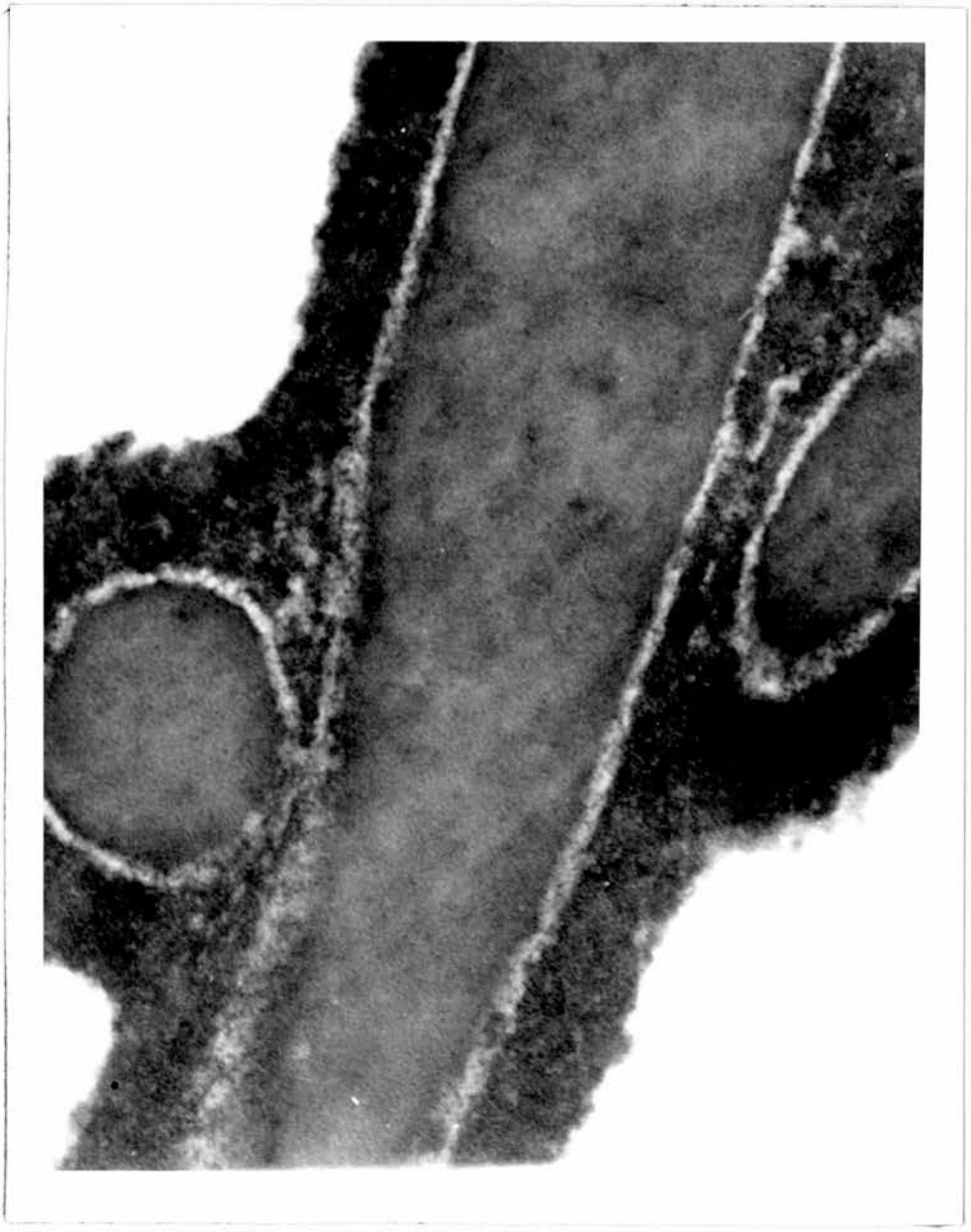


FIG. 16

GUANIDINE HYDROCHLORIDE TREATED MEMBRANE POWDER AT MAGNIFICATION X 20,250

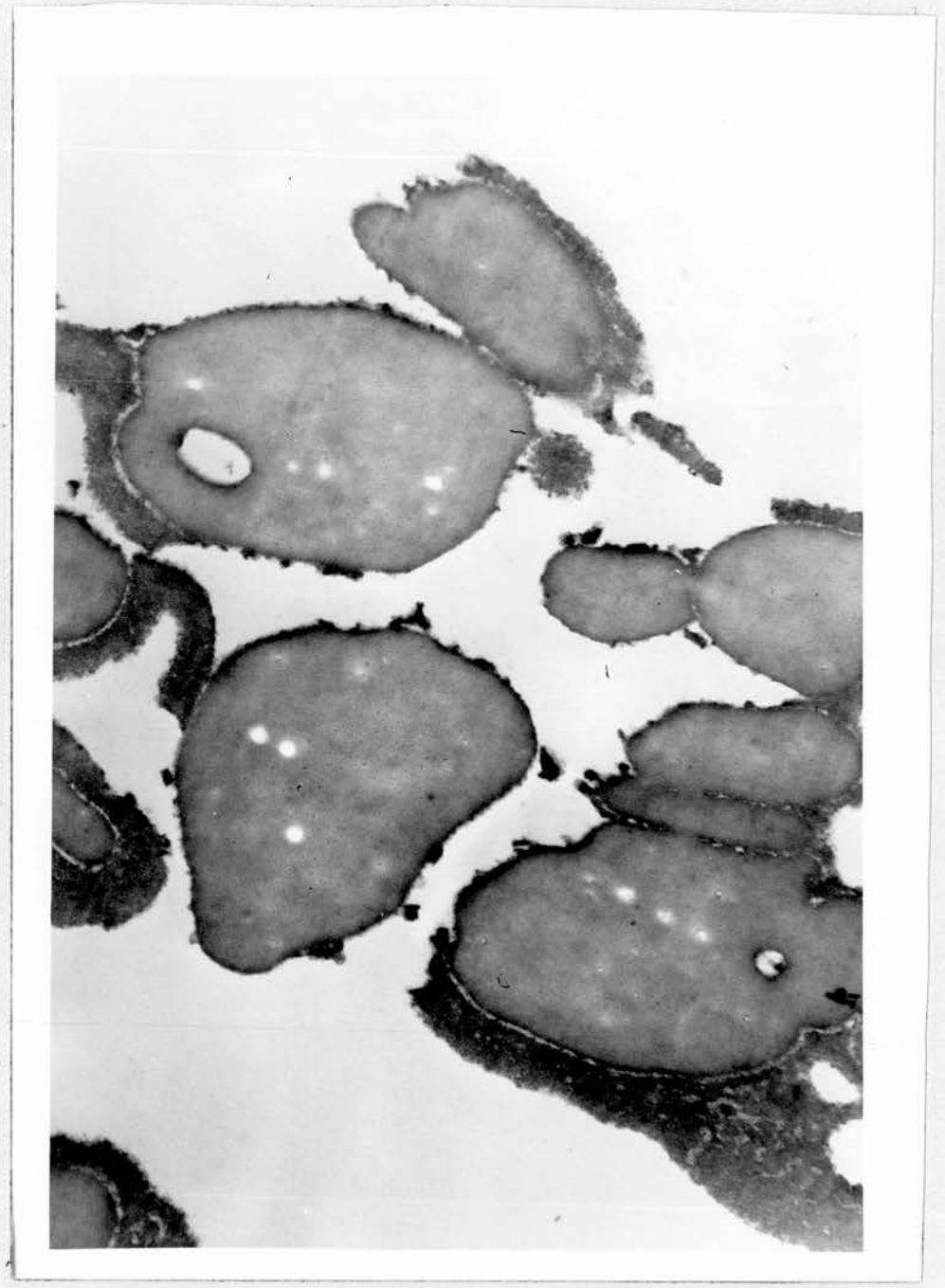


Fig 15 shows the powdered membrane at a higher magnification, x57,350. Obviously the membrane structure has not been affected by the grinding since the core and mantle appear exactly similar as in the membrane sheet.

The reason for the higher magnification was to examine the white boundary region. It appears rather diffuse and granular. It is possible that these boundaries surrounding the core (which have been described as artifacts by Candlish 1972), may well be a region where the mantle or cortex layer is much more granular and therefore stains rather badly. However, no further evidence is available to confirm this statement.

Fig 16 is a photograph of the guanidine hydrochloride treated powdered membrane. It shows that most of the mantle has been removed from the cores. No white boundary appears around such cores. It is obvious that this boundary forms part of the mantle.

The core appears to have 'holes' or spots, these are certainly known to be artifacts.

DISCUSSION

The membrane is a structural protein. However, it shows no resemblance to the other, more well-known fibrous proteins like the keratin, collagen and elastin. The membrane protein has been described to be unique (Leach and Rucker 1978).

Amino acid analyses show that indeed the membrane protein is different. It has a very high glutamic acid content. Proline and cystine are the next highest amino acids that are present. Aspartic acid, arginine, glycine, threonine, serine, valine and leucine then follow in that order. The glycine concentration is approximately half that of glutamic acid.

The keratin has a much higher cystine and hydrophobic amino acid content. In both collagen and elastin, the proportion of glycine and proline is far higher than in the membrane. Yet the membrane contains cystine, hydroxyproline, hydroxylysine, desmosine and isodesmosine, all of which relate the membrane to these fibrous proteins.

In association with the structural proteins, glycoproteins have been reported to be present (Wolff et al 1971). The membrane has been shown to consist of approximately 8% total carbohydrate, a value higher than that described by other groups of workers (see Table I).

The carbohydrate in the membrane is shown to consist of neutral sugars, the hexosamines and the sialic acid. The molar ratio of these sugars is approximately 4.5:2.5:1 respectively. The hexosamine content is higher than that described by Candlish (1972) but it agrees with values given by previous workers (Balch and Cooke 1970; Baker and Balch 1962).

The neutral sugars and the hexosamines have been analysed by many groups of workers. They have shown that these sugars all have the following composition. The neutral sugars are composed

of the hexoses, glucose and galactose and the methyl pentose, fucose. The hexosamines are the acetylated, N-acetylglucosamine and the N-acetylgalactosamine.

The structural glycoproteins, although containing only 5% total carbohydrate also consist of the same sugars as those described above for the membrane (John and Thomas 1972). The rates of release of these sugars have been observed in many structural proteins and all follow the same pattern (Conchie 1976). Moreover these glycoproteins are alkali-labile (John and Thomas 1972).

Alkali-labile glycoproteins have their carbohydrate groups linked to polypeptide chains through O-glycosidic bonds involving serine and/or threonine (Marshall and Neuberger 1972). The commonest structure for such glycoproteins consists of a backbone of hexose and hexosamine units with fucose and sialic acid as the terminal units.

The rates of release of the membrane sugars are in accordance with the structure just described above. Such rates of release have also been observed in the vitelline membrane (Kido and coworkers 1975,1977) and in the eggshell matrix protein (Balch and Cooke 1970). Whether these glycoproteins are all related, as described by Candlish (1972), is speculative, Certainly the matrix proteins contain large amounts of the uronic acid. This is not present in the membranes.

All the species that have been studied contain the same amino acids and in the same proportions as described above. The sugar content is also similar. Therefore the membranes are indistinct from one another. As probably noticed, these species are poultry or game birds and are fairly closely related. Such similarity was also observed by Cantoni and Beretta (1976) who studied the brown and white eggs of the hen and found no difference in the amino acid content of the membranes. Krampitz and coworkers (1975) studied a wide range of birds such as the Galliformes,

Gruiformes and the Anseriformes and still found no difference in their eggshell membranes.

Since all the species possess the same proportion of amino acids, the question to ask is: what is their purpose or function in the membrane? Britton and Hale (1977) answered this by dividing them into two groups of essential and non-essential amino acids. The non-essential amino acids were the serine, glutamic acid, aspartic acid and glycine. The essential amino acids were methionine, lysine, phenylalanine, histidine, threonine and arginine. These authors believed that changes in these two groups of amino acids were related to the decreased amounts of membrane in old hens and in eggs of poor shell quality. They proposed that these changes were due to an alteration in metabolism caused by dietary intake or a failure of the metabolic system.

Since the membrane protein is believed to be related to the fibrous proteins, attempts were made to show some such similarity with elastin. Elastin is believed to be unique in its content of the amino acids desmosine and isodesmosine. These two amino acids were recently reported to be present in the membrane (Leach et al 1978).

Amino acid analysis showed that very small quantities of the desmosine and isodesmosine were present. The proportions were not the same as in elastin. Moreover, UV and fluorescence spectroscopy studies demonstrated that although the membrane and elastin behaved very similarly, there was no definite proof that the elastin was a component of the membrane.

Extraction of the membrane protein in 6M guanidine hydrochloride was shown by e.m. studies to be entirely composed of the mantle region of the fibers. The fiber is made up of a central core surrounded by an outer layer of the mantle. This fiber structure closely resembles that of the elastin fiber. The latter is made up of a central amorphous region (the true elastin) and an outer layer of microfibrils (Ross and Bornstein 1969).

The microfibril layer is also soluble in guanidine hydrochloride. However the membrane and the microfibril are chemically distinct. The amino acid content of the mantle is entirely different from that of the microfibril.

The insoluble residue, which comprises the core region of the membrane fiber was also analysed. The amino acid content of this structure shows a striking similarity to the actual membrane. The proportion of the amino acids in both the structures are very much alike. The extracted protein of the mantle also shows a similar pattern. However, the actual content is higher. There is evidence to indicate that this difference is due to the presence of the glycoproteins. Even so, the core and mantle are subject to total sugar analysis.

Such glycoproteins associated with structural tissue have a high concentration of the hydrophilic amino acids, for example aspartic acid, glutamic acid and arginine. The hydrophobic amino acids such as glycine, alanine and valine are low. Cystine and methionine are present but hydroxyproline and hydroxylysine are absent. These proteins are highly insoluble due to extensive disulphide cross-linking.

This chemical composition of the glycoproteins is reflected in the mantle of the fibers. The aspartic acid, glutamic acid and arginine content are exceptionally high. The hydroxyproline cannot be detected, but it is obviously present. This value as mentioned earlier is subject to criticism. Moreover, the mantle has been extracted only after very strenuous efforts.

The extracted mantle protein on subjection to gel-filtration gave rise to three peaks of molecular weights 8×10^6, 10^5 and 5.5×10^4. The latter two peaks appear to correspond with the two glycoprotein peaks of the vitelline membrane. It is possible that more than one glycoprotein may be present in the mantle. However, it may be of interest to note that the membrane under study was the whole of the hen membrane. Therefore, it may be plausible to suggest that one glycoprotein may be from the inner

membrane and the other from the outer membrane.

Whether the glycoproteins in the eggshell membrane and the vitelline membrane are chemically alike can only be verified by analysing these proteins. The glycoproteins may resemble those determined by Paul-Gardais and coworkers (1973,1974). The sulphated glycopeptides A and B both have a slow turnover rate of about 1.5 days. It has been suggested that glycoproteins become chemically bound to the structural proteins since the two are in contact for very long periods.

If the inner and outer membrane were to contain separate glycoproteins that are structurally different, then this may verify the statement by Balch and Cooke (1970) that the two membranes are chemically distinct.

A second protein was extracted by 6M guanidine hydrochloride. Whether this water-soluble entity is a glycoprotein is not known. It is subject to verification.

Since the mantle and core composition have been described to be alike, it is obvious that the two must have a common protein backbone. Such a protein will be referred to as the parent protein. It is possible that the highest molecular weight protein peak in the mantle extract corresponds to this parent protein. The parent protein probably forms the entire structure of the core region. The question that may be asked now is why does not the core also dissolve in the guanidine hydrochloride? The answer is simple. In brief, it is heavily cross-linked. The possible explanation for this is given later.

The electron microscope stains, lead citrate and uranyl acetate are described to stain differentially in elastin and collagen. This was also observed in the membrane. Uranyl acetate which stains collagen and the microfibrils, stained both the core and the mantle. However, lead citrate stained only the mantle. The lead citrate also stains the elastin microfibrils. This differential staining in both collagen and elastin has been

explained as a difference in surface charge. Obviously the core and mantle must have separate surface charges and hence different chemical composition. The result indicates that in the mantle the glycoproteins must be staining with the lead citrate.

The mode of extraction of the membrane protein also indicates a difference in the chemical composition of the mantle and core regions of the fiber.

The mantle contains the parent protein which is probably interlinked by disulphide cross-bridges to the glycoprotein. Perhaps a few desmosine and isodesmosine cross linkages exist. Thus the mantle can only be extracted after almost all the cross-links are broken. Harsh conditions such as autoclaving and 6M guanidine hydrochloride at high temperatures can only break these bonds.

The core which has a higher desmosine, isodesmosine content and very little glycoprotein if any is much more compact. It is probably very tightly cross-linked and hence cannot be extracted even by such harsh conditions as have been described.

However, the core is not so highly cross-linked as in elastin and the hydrophobic amino acids which probably aid the insolubility of the elastin are much lower in the membrane.

IV. SUMMARY

IV SUMMARY

1) Both the amino acid analyses and the sugar analyses show that intrinsic differences do exist between the membranes of all species under study. However, these differences are so insignificant that the proportions of either the amino acids or the sugars are totally unaffected. It can be concluded that the membranes of all the species have an identical chemical composition.

2) Total sugar analyses reveal that the membrane is 8% polysaccharide in nature. The carbohydrates consist of the neutral sugars, the hexosamines and the sialic acid. The molar ratio is approximately 4.5:2.5:1 respectively. This pattern of the sugars resembles that found in the structural glycoproteins.

3) Amino acid analysis indicates that the membrane contains a high concentration of the hydrophilic amino acids. The concentration of the hydrophobic amino acids is rather low. Small concentrations of the hydroxyproline, hydroxylysine, desmosine and isodesmosine are to be found. This pattern of the amino acids shows no resemblance to any of the fibrous proteins.

4) E.M. studies demonstrate that the membrane is composed of a network of fibers which consist of a central core surrounded by an outer cortex or mantle. The two stain differently indicating a difference in surface charge and hence in chemical composition.

5) By use of the chemical denaturing agent 6M guanidine hydrochloride the core and mantle can be separated. Both the core and mantle are

characterised by a high concentration of the acidic amino acids. However, in the mantle the proportion of the relative amino acids is higher than in the core. Another protein which is water soluble can be separated from the mantle.

6) Gel-filtration studies of the mantle show the presence of three protein peaks of molecular weights $8x10^6$, 10^5, and $5.5x10^4$. It is believed that the lower two molecular weight peaks II and III are glycoprotein in nature. Peak I is probably the parent protein of the membrane and forms the entire central core region of the fibers.

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