University of Edinburgh

A STUDY OF BACTERIAL INFECTION OF THE HEN'S EGG

Ronald George Board, B.Sc. (Bristol)

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C O N T E N T S

INTRODUCTION

Although microbial infection of the hen's egg has been the subject of many investigations, it is only in the last decade that an understanding of the course of bacterial infection has begun to emerge. This development attended the adoption of a technique (Elliott, 1954; Brooks, 1960b) which allowed the process to be followed in intact eggs. The results obtained with this technique have also emphasized the caution which must be excercised when attempting to draw conclusions from experiments in which components of the egg are studied *in vitro*, an approach which is characteristic of the vast majority of investigations of the infection process.

The highly organized and complex physicochemical systems of an egg change during storage but the influence, if any, of these changes on the course of infection has not been investigated. An attempt was made in the present investigation and this called for an extensive review of the literature. A review is given at the beginning of the thesis.

Both Brooks (1960b) and Elliott (1954) used only pseudomonads in their investigations of infection of intact eggs. In the investigation about to be described a representative collection of commonly occurring contaminants of rotten eggs was assembled. The isolation and characterization of these organisms are discussed in the second part of the thesis. Representatives of the various

taxonomic groups were used in an investigation of: a) bacterial multiplication on the shell membranes *in vitro*, and b) the course of bacterial infection in eggs held at summer shade temperatures. The results are discussed in parts 3 & 4 of the thesis. Each of the experimental sections is self-contained in respect to introduction, methods and materials, results and discussion; the tables and figures are included at the end of each section. References and a general summary are given at the end of the thesis.

A REVIEW OF THE LITERATURE DEALING WITH THE MICROBIOLOGY OF THE HEN'S EGG

CONTENTS

CONTENTS (continued)

THE STRUCTURE AND CHEMICAL COMPOSITION OF THE HEN'S EGG

The material considered in this section has been chosen because of its pertinence in the understanding of the microbiology of the egg. The monograph of Romanoff & Romanoff (1949) and, to a lesser extent, the reviews of Brooks & Taylor (1955) and Brooks (1960a) have been the main sources of the information considered hereunder but reports appearing since their publication have been considered and these are referred to in the text.

The gross structure of the hen's egg is shown in Figure 1.

The shell. The structure of this is shown in Figure 2. The thickness of a shell is a property that varies from place to place in an individual egg; the thickness at the poles is generally greater than those in the intermediate latitudes (Tyler, 1961a). This investigator found that the mean thickness in 52 normal shells obtained from 15 birds of various breeds varied from 241 - 371μ. The chemical composition of the shell is shown in Table 1. The proteinaceous material consists of a collagen-like protein and an acid mucopolysaccharide, probably mucoitin sulphuric acid (Simkiss & Tyler, 1957). This material, whose concentration is greatest on the inner surface of the shell, occurs as delicate interwoven fibres the interstices of which are filled with inorganic substances. The shell is perforated with 7,000 - 17,000 pores (Tyler, 1953) whose distribution lies somewhere between randomness and uniformity (Tyler, 1955). The reader is referred to the paper of Tyler (1956) for a fuller consideration of the pores. The outer surface of the shell is covered with a proteinaceous deposit, the cuticle, which is thought to plug the outer orifice of many of the pores, a function that was investigated by

Marshall & Cruickshank (1938). The inner surface of the shell is composed of knob-like processes, the mammillae, the apices of which are rich in proteinaceous material similar to that found in the shell proper and, in addition, a Sudanophilic substance and a reducing substance neither of which is present in more than trace amounts elsewhere in the shell (Simkiss & Tyler, 1957). These apices are also the termini of many keratin fibres which have their origins in the shell membranes. The data concerning the physical strength of the shell have been reviewed by Tyler (1961b) and the genetic basis of shell quality has been reviewed by Baker (1960).

The shell membranes. The inside of the shell is lined with two membranes, the inner and outer shell membranes. The air cell provides the most easily obtained evidence of the existence of these. This cavity, which is normally located at the egg's blunt pole, is formed when the inner shell membrane is pulled away from the outer membrane. This occurs as a result of unequal reductions in volume of the egg shell and egg contents due to heat lost following laying. In addition, the air cell accommodates to the further reduction in the volume of the contents of the egg which results from evaporation during storage. In consequence, the size of the air cell serves as a useful index of an egg's freshness.

The outer shell membrane is, on average, 0·05 mm and the inner membrane 0·015 mm thick. Variation in the thickness of individual membranes has been examined by Tyler (1961c). Each membrane consists of numerous laminations which can be teased apart. Each lamination is composed of numerous net-like strata formed from anastomosing keratin fibres. The thicknesses of these fibres vary in the different strata, those of the innermost strata being

smaller than those in the strata nearest the shell. It is the fibres from the latter which terminate in the mammillae. It is thought that the networks are strengthened and their interstices blocked by either albuminous cementing material or mucin fibres or both.

Both membranes allow the passage of water, gases and salts. The outer membrane differs from the inner in that it also allows the passage of larger molecules such as Congo red, carboxyhaemoglobin and ovalbumin.

As far as can be ascertained direct evidence of changes in the chemical or physical properties of the membranes during storage of eggs has not been sought. Brooks (1960b) considered that such changes may have been responsible for the improved rate of growth which he observed with Pseudomonas spp. seeded onto the air cell membranes of aged eggs. An explanation which does not appear to be tenable in view of the results discussed on page 95 of this thesis. The increase in the rate of growth of a pseudomonad on the membranes in vitro which resulted from the storage of the membranes for three days prior to inoculation (Elliott & Brant, 1957) also implies some alteration of the properties of the membranes but the nature of such alterations has not been elucidated.

The white. This consists of 4 layers (the bracketed figures refer to the relative volume of each): outer liquid (23·2%), thick (57·3%), inner liquid (16·8%) and chalaziferous (2·7%). The latter is formed by a matted fibrous capsule embedded in dense albumen immediately surrounding the vitelline membrane. Fibres from this layer collect at the poles of the yolk to form the chalazae. These cord-like structures, some fibres from which terminate in the shell membranes

at the poles of the egg, help to maintain the yolk in a central position. They are assisted in this by the thick white. Brooks & Hale (1959) found that this "is a weak gel interpenetrated by a system of microscopic elastic fibres and not, as is usually believed, merely an entanglement network".

The percentage, definition and consistency of the thick white are of paramount importance in egg quality, a character which is not easily defined but whose standards are determined by consumer preferences (see Snyder, 1961). Broadly speaking, the market demands an egg with a high percentage of well defined thick white, and any departure from this is associated with staleness. Factors affecting egg quality have been reviewed by Sherwood (1958). This property appears to be determined by heritable factors (Baker, 1960) whose influence weakens with the ageing of the hen (Romanoff & Romanoff, 1949). The consumer has tended to associate poor quality with particular systems of management (e.g. batteries); the fallaciousness of this view has been shown by the investigations of Froning & Funk (1958), Walker & Offord (1958) and Bryce Jones, Houston & Harries (1961). The rate of deterioration of egg quality is determined both by the initial quality (May, Schmidt & Stadelman, 1957) and the method of storage and handling. Jordan, Barr & Wilson (1954), Funk (1955) and Baum, Stadelman, Walkup & Darroch (1960) all reported rapid deterioration in eggs stored at high temperatures. In addition, Dryden & Hale (1957) found that egg quality was seriously affected by transportation, particularly in the case of eggs whose air cells were not uppermost.

The yolk. The yolk membrane provides the physical boundary between the yolk and the white. This laminated structure consists of a layer

of collagen fibres coated with some as yet uncharacterized substance - the vitelline membrane - overlaid with a layer of mucin fibres - chalaziferous membrane - (Doran & Mueller, 1961). The egg yolk is characterized by its complexity. The principal organic constituents are proteins (32 - 33%), lipids (62 - 64%) and carbohydrates (2%). The colour of the yolk, which is mainly determined by the content of carotenoid pigments in the hen's diet, occurs as alternating concentric layers of light and dark coloured material, an arrangement which is easily seen in a transverse section of a hard-boiled egg. The centre of the yolk is occupied by light coloured material, the latebra, and this extends out to the blastodisc situated immediately below the vitelline membrane. This structural asymmetry is associated with the fact that the yolk comes to rest with the blastodisc uppermost.

In the newly laid egg there is a difference in osmotic pressure of the order of 1·8 atmospheres between the white (freezing point, 31·24°F) and the yolk (freezing point, 30·83°F). This difference is slowly reduced during storage, a phenomenon that has been reviewed by Brooks & Taylor (1955). It seems that a diffusion gradient existing inwards from the vitelline membrane is principally responsible for the slowness of this change and that the semipermeable vitelline membrane plays only a subordinate role. The specific gravity of the yolk of a newly laid egg is greater than that of the white, a situation which is reversed during storage. The specific gravity of the yolk decreases as a result of water being taken up; the specific gravity of the white increases due mainly to water lost by evaporation. In addition, the uptake of water by the yolk results in a decrease in the viscosity of the yolk material and a stretching and weakening of the vitelline membrane.

Profound changes in the appearance of the yolk and white follow either the storage of eggs laid by hens fed with substances obtained from plants of the order Malvales or by treating eggs in a certain way. In the former case, Sherwood (1928, 1931) and Lorenz, Almquist & Hendry (1933) appear to have been the first to associate the occurrence of "pink white" with constituents of the hen's diet. Schaible & Bandemer (1946) showed that the tinting was caused by iron diffusing from the yolk and forming a chromogenic complex with conalbumin. Shenstone & Vickery (1959) found that changes in the structure of the yolk and in the pH of the yolk and white occurred first and that migration of iron and amino acids from the yolk to the white and other substances from the white to the yolk occurred at a later stage. The active constituents of malvaceous plants have been identified as sterculic and malvalic acid (Masson, Vavich, Heywang & Kammerer, 1957; Shenstone & Vickery, 1956; Macfarlane, Shenstone & Vickery, 1957; Shenstone & Vickery, 1961). These acids, both of which give a positive Halphen reaction, are the only known fatty acids that contain a cyclo-propene ring in their structures. Shenstone & Vickery (1959) consider that this molecular arrangement is probably responsible for the disorganization of the contents of an egg. This disorganization was thought to be due to increased permeability of the vitelline membranes (Schaible & Bandemer, 1946) but Shenstone & Vickery (1959) consider that the changes are probably due to changes in the structures of emulsion and boundary layers of the yolk, an explanation which is supported by the observations that both the acids are secreted into the yolk and that both of them have high surface activity (Shenstone & Vickery, 1956). Changes similar to those described above have been reported by Hale (1950) in the

case of eggs that were frozen at temperatures above 21·2°F and then stored for 4 - 8 weeks at 32°F. The changes in the physico-chemical systems of the egg that are responsible for this fault have not been elucidated.

Chemical composition of the white. The general composition of the white has been reviewed by Romanoff & Romanoff (1949) and Brooks & Taylor (1955) and the egg white proteins have been reviewed by Fevold (1951) and Warner (1954). As will be seen from Table 2, the white is composed mainly of water plus proteins and a small amount of carbohydrate (glucose; Partridge, 1948). In addition, it contains small amounts of various vitamins (see Table 5), a large number of mineral elements - a concise review of which is given by Brooks & Taylor (1955) - and non-protein nitrogenous material, the amino acid fraction of which has been investigated by Ducay, Kline, & Mandeles (1960). The separate layers of the white differ in their chemical and physical properties, the greatest being in ovomucin the majority of which occurs in the thick white. Our knowledge of the protein composition of egg albumen is founded on data obtained from electrophoretic analyses, the results of one of which are given in Table 3. This knowledge may need to be revised in view of the results which Rhodes, Azari & Feeney (1958) and Mandeles (1960) have obtained from analyses using a form of paper chromatography. This method has indicated the presence of proteins in addition to those found by the first mentioned method. It would seem that an assessment of these differences must await studies in which both methods are used in parallel. The actual composition of the egg white is determined by heritable factors (Forsythe & Foster, 1950) and the composition of the hen's diet (Csonka & Jones, 1952). The influence of the first

mentioned is illustrated by the electrophoretograms of Lush (1961). Our knowledge of the biological activity of the various proteins is summarized in Table 3. When these properties were used as an index of a protein's distribution in the various layers of the white, it was found that ovomucin was the only one which was localized in one area, the thick white (Feeney, Ducay, Silva & MacDonnell, 1952), thus confirming the evidence obtained from other methods of analysis. Microbiological assay methods have also shown that the lysozyme content of the chalazae is 2 - 3 times greater than that of an equal amount of thick or thin white (Sugihara, MacDonnell, Knight & Feeney, 1957; Baker, Hartsell & Stadelman, 1959). Moreover, the lytic activity was found to be increased by incubating the chalazae with a "mucinase" produced by either Proteus vulgaris or Serratia marcescens or by holding them in distilled water at 37° for 24 hr (Baker, et al. 1959).

Changes occurring during the storage of an egg. In general the foregoing has been limited to a discussion of the state existing in a fresh egg. The following changes occur during storage: a) an alkaline reaction develops in the white, b) the loss of water from the white results in i) a decrease in the volume of the white which is associated with an increase in the size of the air cell and ii) an increase in its specific gravity, c) the various layers in the white disappear and d) the yolk becomes enlarged as a result of absorbing water (see page 7).

The pH of the white changes in the period immediately following laying (Healy & Peter, 1925). At the time of laying the white has a pH of about 7·6 but this rises, due to the loss of CO_2, to about 9·6 during the early storage period and does not drop appreciably

thereafter. This change normally occurs in the first 3 days in eggs stored at room temperature. The rate of change and/or the ultimate value can be modified by changes in the conditions of storage. For example, the rate of change can be accelerated by raising the storage temperature (Sharp & Powell, 1931) and the ultimate value can be held below the normal by storage in atmospheres rich in CO_2 (Moran, 1936).

The physical changes which occur in the white are illustrated by the data given in Table 4. The thick white contracts around the yolk and, in so doing, it appears to force the inner thin white out into the outer thin white. Brooks & Hale (1959) have discussed possible explanations of these changes, particularly in respect of the breakdown of the thick white, and they consider that it may be due to a non-enzymic interaction between the lysozyme and ovomucin (Hawthorne, 1950), an explanation favoured by a number of other investigators (Hawthorne, 1950; Feeney et al. 1952; Cotterill & Winter, 1955; Wilcox, 1955).

The yolk tends to settle in a fresh egg, but changes in its specific gravity result in the yolk becoming more buoyant (see page 7). This together with the breakdown of the thick white and the contraction of the chalazae results first, in a notable increase in the freedom of movement of the yolk and finally, in the yolk rising and making contact with the shell membrane.

The rates at which the changes discussed above occur are, broadly speaking, a function of the storage temperature, the rate of change is increased by an increase in temperature.

Romanoff & Romanoff (1949) have reviewed the early reports relating to chemical changes occurring in stored eggs. These reports were mainly concerned with eggs stored for periods of about a year and

are, therefore, of no immediate interest to the egg microbiologist who is mainly concerned with changes which might occur during the first 3 months storage. By paper electrophoretic analysis of the egg white proteins, Evans & Bandemer (1956) found a very slight reduction in the composition of the ovalbumin, a very slight reduction in the amount of lysozyme and a slight increase in the amount of both ovomucoid plus ovoglobulin and conalbumin in eggs stored for 3 months (the conditions of storage were not stated). Stute (1960), using methods similar to those of Evans & Bandemer, found that significant changes could only be established when eggs had been stored for more than either 1 year at 3° or 2 months at 22°. The investigation of Feeney et al. (1952) provides the most pertinent information on this aspect. They found that there was no significant reduction in the biological activity of lysozyme, conalbumin, ovomucin, ovomucoid or avidin during storage for either 15 days at 35° or 45 days at 22°. Evans and his collaborators have investigated the changes in the concentration and distribution of vitamins during storage. They did not examine the stored eggs for microbial sterility, an omission which must be remembered when attempting to assess the significance of the results summarized in Table 5. It will be noted that only vitamins B_6, B_{12} and riboflavin showed any marked reduction and that pantothenic acid was the only one to show any marked change in distribution. This latter observation indicates that one has to consider the migration of yolk material when discussing changes occurring during the storage of an egg. In this context, it has been found (Schaible, Davidson & Bandemer, 1946) that iron migrates from the yolk to the white; the albumen contained 74µg/100g albumen at 1 month and 118µg/100g albumen at 8·5 months. Similarly, Ducay, Kline & Mandeles (1960) considered that the increased concentration of free amino acids in the white of stored eggs was due

to a slow diffusion of these substances from the yolk. Lineweaver, Morris, Kline & Bean (1948) found that, apart from lysozyme, the white contained only traces of enzymes (catalase, a tributyrase and a peptidase), evidence which suggests that the principal changes occurring during storage are not mediated by enzyme action.

THE ANTI-MICROBIAL DEFENCE

A certain selection has been made in the choice of literature considered in this section. Reports of a technological nature (i.e. those dealing with the influence of washing on the incidence of rotting in stored eggs) have been omitted. Many of these do contain observations relevant to the present review but it is felt that this relevance is more clearly seen when these reports are considered against a background of data obtained from more fundamental investigations. In the interest of clarity the literature will be considered under three headings.

The shell

The pores, which number between 7,000 - 17,000 per shell (Tyler, 1953), the mean diameter of whose canals can be of the order of 25 - 35μ (Tyler, 1956), are obviously the potential avenues for the entrance of bacteria and fungi into eggs. This inference is supported by the demonstration that yeasts and bacteria can be drawn through shells from which the membranes have been removed (Haines & Moran, 1940; Garibaldi & Stokes, 1958).

Both Haines (1939) and Romanoff & Romanoff (1949) have reviewed the early literature dealing with microbial penetration of the shell of intact eggs. In the case of bacteria, the standard approach was to immerse the egg in a broth culture and, after a suitable period of incubation, test the contents of the egg for the

presence of viable bacteria. A great range of bacterial types have been found to pass through the shell under these conditions but these results are only of academic interest because of the unlikelihood of such conditions obtaining in practice. The penetration of fungal hyphae into the pores of eggs held in very moist chambers has been frequently demonstrated (e.g. Weston & Halnan, 1927) and in one case, Zagaevsky & Lutikova (1944), it was found that this penetration can result in the deposition of bacteria in the contents of the egg.

Haines (1938) was unable to recover viable bacteria from the contents of eggs which had been held for either 1 or 12 hr in a suspension of a pseudomonad and, moreover, he found that such treatment did not promote rotting in eggs stored for a fortnight at 20°. It must be pointed out that he did not attempt to isolate bacteria from the shell membranes (cf. Bean & MacLaury, 1959) nor did he take precautions to ensure that the medium was not rendered iron-deficient by the conalbumin present in the sample of albumen. There was a significant level of contamination of the contents and a concomitant increase in the incidence of rotting when eggs were brushed before being immersed in the bacterial suspension, evidence which suggests that some protective material, possibly the cuticle, had been removed from the outside of the shell. Both Stuart & McNally (1943) and Wilson (1945) have reported high incidences of contamination of the shell membranes following the wiping of the shell with a cloth moistened with pseudomonads. In the case of soiled second quality eggs, Haines (1938) found a 13% incidence of rotting in unwashed eggs and this incidence was increased to 19% when the eggs were wiped with a damp cloth and further increased to

31% when the shells were rubbed with steel-wool plus sand. Similarly, Fromm & Monroe (1960) found that the incidence of rotting could be increased by rubbing the shells of moist eggs with cheese cloth prior to their immersion in a broth culture of Pseudomonas aeruginosa. Haines & Moran (1940) re-investigated this problem but their results cannot be compared with those of Haines (1938) because of differences in both the temperature and relative humidity of the store as well as in the period of storage. In this latter study, using the incidence of fluorescence of the white of stored eggs as an index of the effective penetration of the shell with pseudomonads, they found an average incidence of 17·5% (0 - 41%) for eggs which had been immersed in a bacterial suspension when both of these were at about the same temperature. The incidence was increased to 100% when either eggs immediately they had been laid or eggs which had been re-heated to 98°F, were immersed in cold suspensions of pseudomonads. They concluded that the contraction of a warm egg resulted in bacteria being sucked through the pores of the shell. Bean & MacLaury (1959) recovered viable bacteria from the inner surface of 90 - 100% of shells immediately after warm eggs had been immersed for 15 min in cold suspensions of either Streptococcus faecalis, Proteus morganii, Proteus vulgaris or Pseudomonas aeruginosa. Moreover, 5 - 90% of the eggs had viable bacteria on the inner surfaces of their shell membranes and 0 - 30% of the albumens were contaminated. If the eggs, after being removed from the bacterial suspension, were stored for 7 days at room temperature, then there was an appreciable increase in the incidence of contamination of both the shell membranes and the albumen. It would seem reasonable

to expect a close parallelism between the incidence of contamination of the inner surface of the shell membranes and that of the whites. Such a parallelism is not apparent in the report under consideration and this may be due to the method of sampling. A cotton swab was dipped into the white and then streaked onto a nutrient agar slope (incubated for 24 hr at 37°) and no precautions were taken to ensure that the medium was not rendered iron-deficient by the conalbumin carried over on the swab.

A possible contribution by the shell to the anti-microbial defence of the egg was suggested by a theoretical consideration of the suitability of the shell as a medium for microbial growth, particularly in respect of available water (Gillespie & Scott, 1950). On the assumption that the water activity of the shell membranes is about 0·995 and that the water vapour pressure gradient through the shell is linear, they concluded that a level of available water (0·98) suitable for the growth of pseudomonads would extend outwards from the membranes for a distance of about 34μ in the case of eggs held at 0° and a relative humidity of 85%. This distance would vary with changes in any of the following: temperature, relative humidity and shell thickness. A value of 0·98 available water was chosen because of Scott's (1936) experience with pseudomonads growing on the surface of meat, a medium which can be considered to be more satisfactory than the egg shell. This being so, the figure of 34μ may need to be revised in view of our knowledge (Wodzinski & Frazier, 1960; 1961a, b, c and d) that an organism's requirement for moisture is determined by many environmental factors. Nevertheless, it would appear that an organism does have to be lodged in very close proximity to the membranes before it is in a position to infect the contents of the egg.

Gillespie & Scott (1950) found that eggs with white shells developed a significantly greater percentage of rots than brown shelled eggs produced on the same farm and subjected to the same conditions. Although the mean shell thickness and the shell density were very similar in both types of eggs, these authors were of the opinion that the observed differences were due to some inherent factor in the shell. The true nature of this phenomenon is not clear.

Shell membranes

The shell membranes appear to have two roles in the anti-microbial defence of the egg: a) mechanical and b) chemical. Mechanical. Hindrance of bacterial penetration into the egg was suggested by some of the earliest work (for references, see Haines, 1939). More recent investigators have concluded that, in the case of rot-producing bacteria, this is the primary function of the membranes (Gillespie & Scott, 1950; Stokes, Osborne & Bayne, 1956; Walden, Allen & Trussell, 1956; Garibaldi & Stokes, 1958; Kraft, Elliott & Brant, 1958). The most direct evidence in support of this conclusion has been presented by Walden et al. (1956) and Garibaldi & Stokes (1958). In the case of the latter investigators, who used the organisms which Florian & Trussell (1957) had isolated from rotten eggs, it was found that the membranes behaved as bacterial filters, a property which was lost during the exposure of the membranes to bacterial action. Similarly, using a pseudomonad which had been harvested from a medium containing $P_2{}^{32}O_5$ and the level of radio-activity in the filtrate as an index of the efficiency of the membranes as bacterial filters, Walden et al. (1956) found that the membranes restrained the organisms for periods of 15 - 20 hr. Whether the loss of restraint was due to bacterial action or whether

it was due to weaknesses created in the membranes during their exposure to rigorous methods of preparation and sterilization cannot be decided. In the case of both investigators, the organisms were forced through the shell and shell membranes in a direction opposite to that which occurs normally (i.e. from the inside of the shell outwards). In the latter case, Bean & MacLaury (1959) - see page 15 - gave results which indicate that the membranes are easily breached when a warm egg is allowed to contract in a cold bacterial suspension. It would appear, therefore, that the membranes offer only an imperfect barrier to bacterial penetration.

Chemical. Korotkova (1957) detected lysozyme in the shell membranes and considered that this substance, which remained in the membranes during the early stages of incubation, played an important role in the defence of the developing embryo against Gram-positive bacteria.

The presence in the membranes of substance(s) toxic to rot-producing bacteria was suggested by the work of Stuart & McNally (1943). They observed an 80% mortallity in the 4 hr following the addition of Pseudomonas aeruginosa to finely-ground membranes suspended in saline.

Alternative interpretations of this observation have been offered (Gillespie & Scott, 1950); namely, that the apparent reduction in viable numbers may have been due to either adhesion between bacteria and fragments of membrane or toxicity of the saline. Subsequent investigations have shown that the latter is the most probable interpretation. For instance, Stokes & Osborne (1956) noted a reduction in the number of viable cells of either Pseudomonas aeruginosa or Pseudomonas fluorescens when these were added to either

a suspension of membranes or the suspending medium (saline). After about 1 - 4 hr following inoculation, the die-off in the membrane suspension, which was less than that in the saline controls, stopped and multiplication followed. Results different from these were obtained with members of the coli-aerogenes group. In this case, the number of viable cells in both the membrane suspensions and the saline remained constant for upwards of 5 hr following inoculation, after which time bacterial multiplication began in the membrane suspension and a marked die-off occurred in the control. These authors made an unsuccessful search for a non-toxic suspending medium. Elliott & Brant (1957), using a strain of Pseudomonas, devised a suspending medium which did not exaggerate the lag-phase of growth.

The toxic agent(s) in these suspending media have not been identified but the available evidence does suggest that the toxicity might be due to metal ions. The chelation of these by proteins is a possible interpretation of the observed reduction in toxicity following the addition of membranes to the suspending media.

Garibaldi & Stokes (1958) reported significant multiplication of both Alcaligenes bookeri and Proteus melanovogenes (Aeromonas liquefaciens) in membrane suspensions. Five out of 6 strains of Alc. bookeri were the only bacteria of the many tested which liberated ninhydrin-reacting substances during growth in membrane suspensions.

The influence of the shell membranes in situ on micro-organisms has been infrequently studied (Elliott, 1954; Brooks, 1960b). Nevertheless, it has been established that strains of Pseudomonas can live in this situation. Both Elliott and Brooks noted that

significant numbers of viable organisms were not recoverable from the white until some considerable period following inoculation. This situation may be more apparent than real. For, as far as can be ascertained, neither of these investigators took precautions to ensure that nutrient media used for testing the white for the presence of viable organisms were not rendered iron-deficient as a result of the addition of conalbumin in the sample. This together with the difficulty of assessing the possible influence of the anti-microbial defence of the white means that the exact role of the membranes in the defence of the egg cannot be easily elucidated by this type of experiment.

Albumen

The most recent reviews of this aspect (Haines, 1939; Romanoff & Romanoff, 1949) were prepared before the more important work was done. In the following, therefore, the literature will be reconsidered in the light of modern knowledge. The nature of the anti-microbial defence of the albumen is most easily appreciated when it is considered as a medium for microbial growth, an approach which has been adopted in this review. In the present context, the term growth is used to cover multiplication and, in addition, the formation of enzymes whose synthesis may be influenced by the composition of the medium. This latter aspect has been recently reviewed by Pardee (1961).

Intrinsic factors

The concept that the albumen is an unfavourable medium for microbial growth arose from observations made at the turn of the 19th century (for references, see Haines, 1939). This has been confirmed in some recent investigations (Korotkova, 1955; 1956a and b;

Brooks, 1960b). Apart from the recognition that the white has a strongly alkaline reaction and that it contains lysozyme, the investigation of this defence did not make progress until methods were devised by which the principal proteins of the white could be isolated in relatively pure and undenatured form.

Availability of water. The water activity of the white is 0·995 (Smith, 1934), a value which is considered to be optimal for the commonly occurring micro-organisms (Mossel & Ingram, 1955).

Hydrogen-ion concentration and buffering power. The effect of the pH of the white on bacteria was investigated by Sharp & Whitaker (1927) using species of Bacillus and Pseudomonas and members of the coliform group. Standard volumes of egg white with pH values covering the range 5·0 - 10·5 were inoculated with 1 ml of a 3 hr broth culture and incubated at 37°. A colony count was made on the initial inoculum and on the egg white after 6 hr incubation. None or very few viable organisms were present when the pH was either 9·5 or 10·5; at pH values nearer to neutrality, the majority of organisms multiplied. The exception, Bacillus subtilis, was presumably killed by lysozyme. Multiplication occurred in thermally denatured egg white whose pH was 9·4, thus indicating that the pH alone was not responsible for the observed killing. Garibaldi (1960), using the organisms which Florian & Trussell (1957) had isolated from rotten eggs, found only one organism, a strain of Alcaligenes bookeri, which would grow in egg white whose pH was 9·1. Many more organisms grew when the pH was adjusted to 7·9. All the organisms grew when egg white, either at pH 7·9 or 9·1, was supplemented with iron in quantities sufficient to saturate the binding potential of the conalbumin. Brooks (1960b), also, has claimed that the growth rate of pseudomonads in egg white can be increased by the addition of iron; more iron was

required when the pH was 9·3 than was required when the pH had been adjusted to 6·0. The maximum response obtained in this way did not result in a rate of growth equal to that obtained in a chemically defined medium, evidence which led Brooks to conclude that some other factor, possibly the inadequate supply of suitable combined nitrogen, was also influencing the organisms.

It would appear that the pH of the white acts in two ways: a) as an inimical property, and b) as an adjunct to the biological activity of conalbumin, a review of which is given at a later stage.

The proteins of the white and the bicarbonate buffer system offer very little buffering capacity between pH 7·0 - 9·0 (Cotterill, Gardner, Cunningham & Funk, 1959). In consequence, many contemporary investigators are of the opinion that acids arising from microbial action on the glucose in the white of an infected egg lowers the pH to a value more optimal for the contaminants. Such a phenomenon is known to occur during the industrial fermentation of egg white (Brooks & Taylor, 1955), but its occurrence in infected eggs has not been demonstrated.

Major and minor mineral elements. A detailed summary (Brooks & Taylor, 1955) of many analyses shows that the white contains all the major and minor elements which are known to be essential for microbial growth. It would seem, however, that some of these may be rendered unavailable to micro-organisms as a result of their combination with substances (e.g. proteins and riboflavin) in the white. Of these, only one of the principal proteins, conalbumin, has been shown to play an important role in the egg's defence. Its action was first observed by Schade & Caroline (1944) and its identity was established by Alderton, Ward & Fevold (1946). The early investigations of the

physico-chemical properties of this substance have been reviewed by Fevold (1951) and Warner (1954). Pertinent material from these together with reports published since 1954 will be considered first so that the biological action of this substance may be seen in a proper perspective.

Conalbumin, an albuminous protein whose occurrence in egg white was first recorded by Osborne & Campbell (1900), is an organic ligand with properties similar to those of the serum albumen, siderophilin (transferrin). It accounts for about 10% of the total egg white solids (Longsworth, Cannan & MacInnes, 1940; Alderton et al. 1946) and is uniformly distributed in the thick and thin white (Feeney et al. 1952). Its sequestering power is not detectably reduced by short periods of storage (Feeney et al. 1952). Moreover, its sequestering potential would not appear to be exhausted by the iron diffusing from the yolk during the storage of eggs (Schaible, Davidson & Bandemer, 1946; Garibaldi, 1960).

Several methods have been devised for the separation of conalbumin from the other proteins of the white: precipitation with ammonium sulphate (Alderton et al. 1946), precipitation with ethanol (Bain & Deutsch, 1948), and absorption onto carboxymethylcellulose (Rhodes, Azari & Feeney, 1958; Woodworth & Schade, 1959). The iron-complex has been crystallized by Warner & Weber (1951). The molecular weight has been variously reported as 70,000 (Longsworth et al. 1940), 87,000 (Bain & Deutsch, 1948), 76,600 (Warner & Weber, 1951) and 85,000 ± 200 (Fuller & Briggs, 1956). The last mentioned value was obtained from a very comprehensive investigation and would seem to be the most probable value.

Conalbumin forms a chromogenic complex with ferric ions

(salmon-pink) and cupric ions (yellow) and a colourless complex with zinc (Fraenkel-Conrat & Feeney, 1950; Warner & Weber, 1951). The ferric ions are more firmly chelated than the other two. Although the active groups have not been identified, the hydroxyl groups of tyrosine have been implicated (Warner & Weber, 1953) The formation of the iron-complex requires 1 molecule of CO_2 as either $CO_3^{=}$ or HCO_3^{-} per atom of iron and 3 protons are released (Warner & Weber, 1953). The ability to form the complex is destroyed when the protein is denatured by any one of several methods (Fraenkel-Conrat & Feeney, 1950).

The properties of the iron-complex are different from those of conalbumin, the complex is less soluble and more resistant to enzymic digestion and thermal denaturation (Azari & Feeney, 1958; 1961). Other differences of a physico-chemical nature have been listed by Fuller & Briggs (1956).

Schade & Caroline (1944), working with Shigella dysenteriae, Staphylococcus aureus, Escherichia coli, and Saccharomyces cerevesiae, observed growth inhibition in nutrient broth containing egg white (0·02 ml / 1 ml broth). The degree of inhibition was influenced by the pH of the broth; at pH 5·8 and below, growth in the supplemented broth could not be distinguished from that in unsupplemented broth; between pH 5·8 - 6·4, there was a partial inhibition and at values greater than 7·4, the inhibition was complete for 48 hr at 37°. Of ten vitamins and 31 elements tested, iron alone overcame this inhibition.

The effect of pure preparations of this protein on bacterial growth has been examined (Fraenkel-Conrat & Feeney, 1950; Feeney & Nagy, 1952). When conalbumin was added to media in stoichiometric

excess of the total iron found in the media by chemical analysis, inhibition of growth of most of the organisms were observed. This inhibition expressed itself by an increase in the lag-phase of growth and decreased rates of growth once growth had begun. Schade (1958), using radio-active iron, has also claimed to have demonstrated the importance of conalbumin being in stoichiometric excess of iron. Feeney and his collaborators found that different organisms showed different degrees of inhibition which may be recorded thus: Micrococcus lysodeikticus (most sensitive) >Micrococcus pyogenes and Micrococcus conglomeratus >Bacillus subtilis >4 Gram-negative bacilli. Different degrees of inhibition have also been noted among rot-producing bacteria (Garibaldi 1960). These differences are presumably a reflection of different organisms' requirements for iron and, as such, are in concord with our knowledge of these requirements (Waring & Werkman, 1943). Increasing the pH, which decreases the dissociation of the iron-complex, increased further the inhibition of the organisms used by Feeney and his co-workers. Of great interest to the egg microbiologist was these authors' observation that inhibition occurred when the organisms were separated from the ligand by dialysis tubing. A similar situation could conceivably obtain on the outer surface of the shell membranes.

Feeney & Nagy (1952) also noted the stimulation of pigment production by pseudomonads growing in medium supplemented with conalbumin; the contamination of a supplemented medium with relatively large amounts of iron suppressed pigment production. Schade (1958) has claimed that the utilization of glucose and the production of catalase by Staphylococcus aureus is suppressed by conalbumin.

The biological actions of sequestering agents have been divided into a number of categories (Weinburg, 1957; Albert, 1958). Albert has listed the following: a) inactivation of a metal in its biological setting e.g. HCN poisoning, b) increasing the activity of the metal ion thereby making it either more available or toxic to an organism and c) making an essential ion unavailable to an organism. It is clear from the available evidence that conalbumin belongs to category c).

Several interpretations of the observed extension in the lag-phase of growth have been considered (Fraenkel-Conrat & Feeney, 1950; Feeney, 1951; Feeney & Nagy, 1952); namely, a) a slow release of iron from the complex, b) iron released by bacterial digestion of the iron-complex, c) microbial adaptation to growth in an iron-deficient environment, and d) toxicity due to other metal ions (e.g. Zn, Cu,Co) which assume toxic proportions because of a metal ion imbalance resulting from the removal of iron. The available evidence favours interpretation a) and is the situation which appears to obtain in eggs.

In this latter situation, Garibaldi (1960) has considered the possibility of rot-producing bacteria synthesizing chelating agents which would remove the iron from the conalbumin-complex. The production and/or utility of such systems in other environs have been demonstrated (e.g. Garibaldi & Neilands, 1956; Williams, Green & Rappoport, 1956; Fogg, 1956) and Garibaldi (1960) mentions some preliminary findings of a similar nature with rot-producing bacteria in vitro, but, as yet, the occurrence of this phenomenon in infected eggs has not been domonstrated.

The observation (Feeney & Nagy, 1952) that the addition of riboflavin to media supplemented with conalbumin further increased the inhibition of microbial growth lead them to suggest that this

substance might play a role in the egg's defence. These authors considered that riboflavin was acting as a supplementary binder of iron, an interpretation that was then tenable in view of the contemporary claims (Albert, 1950; 1953) that this substance combines with divalent metals. This interpretation has been placed in doubt because of observations made in more recent times. Riboflavin is not now considered to be a strong chelating agent (Hammerich & Fallab, 1958; Harkins & Freiser, 1959). Moreover, the iron in the egg occurs in the trivalent state (Halkett, Peters & Ross, 1958) and, finally, riboflavin has been found to occur in association with a protein in the egg white and the properties of riboflavin when combined with this protein are unknown (Rhodes, Azari & Feeney, 1958; Rhodes, Bennett & Feeney, 1959). A possible interpretation of the inhibition noted by Feeney & Nagy (1952) is suggested by the observation of Ramsey & Wilson (1957). These authors noted that the addition of riboflavin and Mn to a medium resulted in the inhibition of Micrococcus pyogenes. This inhibition, which was not reversed by Co, only occurred during aerobic incubation. Further work is necessary before a final conclusion can be made as to the role, if any, of riboflavin in the defence of the egg.

In the majority of experiments mentioned above, environmental factors other than the non-availability of iron were optimal; the exception is provided by the investigation of Garibaldi (1960) who used nutritionally non-exacting Gram-negative bacteria. Other factors can be expected to be sub-optimal in the egg white and, in this case, the binding of metals by proteins other than conalbumin might assume practical significance. A recent report (Tanford, 1952) has listed the complexing strength of serum albumen as Cu > Zn > Cd > Pb; a complex was

strongest when the protein was held at an alkaline reaction. The unavailability of metals other than iron might conceivably either prevent the growth of organisms more nutritionally demanding than those discussed above or modify the metabolism of contaminants in the white. In the latter context, it would be of interest to know whether or not the binding of Ca by albumen, as claimed by Abels (1936), prevents the formation of proteolytic enzymes by those organisms which require Ca for this synthesis (Haines, 1931; 1932). A first step in the elucidation of this problem would be a re-assessment, based on modern methods, of the claim made by Abels.

In the foregoing review, the discussion has been limited to the situation which could be expected to obtain with normal eggs, either freshly laid or stored for a short period. A different situation might be present in certain types of abnormal eggs. For example, it is conceivable that micro-organisms would be able to satisfy their iron requirements more easily in egg whites containing blood ("blood spots", "diffuse blood" or "meat spots"). This consideration is mainly academic, however, because abnormalities of this type are normally discarded during the primary grading of eggs in countries whose marketing procedures are highly organized. The occurrence of eggs whose white contain abnormally high concentrations of iron has been discussed on page 8. The available evidence indicates that such eggs do not contain micro-organisms. It is noteworthy however, that McKenzie, Thom & Getty (1955) found significant bacterial contamination (more than 6 organisms/ml) in 25% of the 46 examples of "yellow rots" which they examined. This abnormality appears to be due to faults in the vitelline membrane and/or the diffusion gradient in the yolk. As such, it resembles the faults discussed on page 8

but it differs from these in that only a relatively short period of storage is required before the abnormality is manifested. It may be that the high incidence of contamination observed by McKenzie et al. (1955) is associated with this speed of manifestation. The contamination of the shell membranes and/or the albumen with iron during the washing of eggs may be of more practical importance. Such a possibility has been the subject of much discussion in American poultry journals following the report of Garibaldi (1960). At the moment, however, this aspect does not appear to have been the subject of systematic investigation.

Energy sources. The carbohydrates and proteins are the two main sources of energy available to contaminants in the white. At the present time, consideration will be reserved to the first mentioned; the utilization of proteins is considered at a later stage.

Romanoff & Romanoff (1949) have reviewed the literature dealing with the carbohydrates of the white. Glucose is the only free carbohydrate in the white (Partridge, 1948), the others, which are residues consisting of mannose, galactose and glucosamine (Stacey & Woolley, 1940; Johansen, Marshall & Neuberger, 1960), are combined with ovalbumin and the glycoproteins, ovomucoid and ovomucin.

The discovery that glucose was responsible for deleterious changes in the quality of dried egg albumen stimulated research into methods for the removal of this substance. This work, which has been reviewed by Brooks & Taylor (1955), showed that it could be removed by either microbial fermentation or glucose oxidase. The ease with which the fermentation could be started led to the belief among egg microbiologists that in an infected egg the glucose is readily available to the contaminants. This belief has not been tested experimentally.

As far as can be ascertained, there are no reports dealing with microbial action on the carbohydrate moities of the egg white proteins.

Combined nitrogen. The egg white contains two forms of combined nitrogen: protein nitrogen and non-protein nitrogen. The former comprises the major fraction of the egg white solids whereas the latter ammounts, on average, to only 12 mg/egg (Romanoff & Romanoff, 1949). Ducay, Kline & Mandeles (1960) have investigated the amino acid composition of the latter. They found at least 16 free amino acids whose total concentration was approximately 0·14 - 0·54 μmoles/ml in the albumen of fresh eggs and up to 2·3 μmoles in albumen from eggs held under various conditions of storage It is tacitly assumed by egg microbiologists that, because the majority of rot-producing bacteria are proteolytic, they satisfy their nitrogen requirements at the expense of the egg white proteins. This assumption persists in spite of the review by Haines (1939) in which he outlined the difficulties confronting the organisms during the early stages of an egg's infection. No attempts have been made to elucidate the problems discussed by Haines, a situation no doubt resulting from the lack of knowledge concerning the conditions required for the production and functioning of proteolytic enzymes of Gram-negative bacteria. This aspect has been reviewed by Haines (1934) and the few generalizations which can be drawn from this together with information contained in more recent publications will be cited in the following discussion.

The early workers established that proteolytic bacteria can synthesize filterable proteases in a chemically defined medium without the enzyme's substrate being present provided that Ca is

available. It would be important to know whether or not the binding of this element by albumen, as claimed by Abels (1936), prevents the formation of proteases by contaminants in the egg white. Likewise, it would be desirable to know whether or not the binding of other metal ions (Mg, Mn, Co and Zn), the importance of which in the activation of dipeptidases of Pseudomonas aeruginosa was mentioned by Gale (1951), has any influence on the functioning of such enzymes if and when they are produced in contaminated egg white. The early work also revealed that organisms would not grow and produce proteases in a mineral medium containing a well purified protein unless some other more easily assimilable form of combined nitrogen was also added to the medium. In this context, it would seem desirable to establish whether or not the non-protein nitrogen of the white is sufficient in amount and suitable in composition for the rot-producing bacteria to synthesize proteases. In the case of pseudomonads, Brooks (1960b) has stated that their rate of growth in egg white cannot be increased by the addition of either glycine or ammonium salts. He concluded that the non-availability of suitable combined nitrogen was of less importance than the non-availability of iron in the defence of the egg. The early observers also recorded the fact that the addition of glucose to a medium containing protein protected the protein from digestion. A similar situation was noted by physiologists and they hypothesized that glucose had a "protein-sparing" action. In the case of bacteria, Salle (1961) has stated that it is the fall in pH resulting from the breakdown of glucose which inhibits the digestion of the protein. As has been mentioned previously, nothing is known concerning the fate of glucose in an infected egg

so it is not known if glucose has a "protein-sparing" action in this environment. Finally, it was noted that microbial proteases digested denatured protein more easily than they did native proteins. Ingram (1956) reported that a fair proportion of yeasts slowly attacked coagulated egg white but very few attacked the native protein. Similarly, Mills & Wilkins (1958), working with a partially purified protease of Proteus vulgaris, found that the denatured protein was more easily attacked than the native protein. This aspect has been the subject of a number of recent publications (for references, see Schlamowitz & Peterson, 1959).

Several investigators at the turn of the century reported that the white inhibits the action of trypsin and Lineweaver & Murray (1947) have identified the anti-tryptic factor as ovomucoid. This substance does not affect bacterial multiplication (Garibaldi, 1960) and has no action on a protease of an aspergillus (Masushima, 1958). The latter author isolated another and previously unrecognized protein from the egg white which was more effective than ovomucoid in the inhibition of trypsin. Moreover, it inhibited the action of a fungal protease. This observation, which is as yet unconfirmed, suggests that the white may contain a potent inhibitor of microbial proteases.

The paucity of our knowledge of this aspect of the egg's defence has been emphasized by the above discussion. Nevertheless, the available evidence does suggest that the form and availability of the combined nitrogen in the white will preferentially select those organisms whose requirements, at least in the initial phases of infection, are satisfied by the small amount of non-protein nitrogen. This selection, which may be of very great importance in the defence of the developing embryo, would appear to be reflected

in the types of organisms present in rotten eggs of commercial origin. These, Gram negative bacteria many of which are proteolytic and many of which grow in a Koser's type medium, are the type of organism which one would expect to be best adapted for the exploitation of the environment under discussion.

The possibility of the situation discussed above being modified by methods of poultry husbandry is suggested by the reports of Cotterill & Nordskog (1954), Rhodes & Feeney (1957) and Gunther, Blinn, Kolbezen, Conkin & Wilson (1959), all of whom have presented indirect evidence which suggests that eggs can absorb ammonia. It would be interesting to know whether or not eggs do absorb appreciable quantities of this substance during exposure to the atmosphere of laying houses, particularly those in which the deep litter method is used. And, if so, whether or not this contamination of the white with ammonia would assist bacteria in the initial stages of infection.

Growth factors. The occurrence of these in the egg has been reviewed by Romanoff & Romanoff (1949). The actual concentration of these in an egg is determined by the breed of the hen and, more important, by the amount of the factor present in the hen's diet. The average concentrations of growth factors in materials commonly used in the preparation of poultry foods have been listed by Bolton (1959). The fat soluble growth factors occur only in the yolk whilst the water soluble ones are present both in the yolk and the white. The latter are listed in Table 5.

It should not be inferred that because these vitamins are present that they are available to organisms which may require them. Romanoff & Romanoff (1949) have reviewed the observations which lead to the recognition that biotin in raw egg white is unavailable to

laboratory animals which require this vitamin. Subsequent work (Eakin, McKinley & Williams, 1940; Eakin, Snell & Williams, 1940; György, Rose, Eakin, Snell & Williams, 1941) terminated in the isolation of a previously unknown protein of the white to which biotin became bound. The addition of this protein to a medium prevented the growth of a biotin-requiring yeast. Other workers (Woolley & Longsworth, 1942) also isolated this protein and found that its addition to a medium prevented the growth of Clostridium butylicum. This protein is generally known as avidin. The demonstration of the biological activity of avidin in animal feeding experiments as well as with microbiological assay methods has led to the assumption that this substance may play a part in the egg's defence. This assumption has not been tested experimentally and the claim (Baumgärtner, 1957) that avidin was responsible for the inhibition of Bacillus mesentericus was founded on results obtained from very uncritical experiments.

Some recent studies (Rhodes et al. 1958 Rhodes et al. 1959) have shown that riboflavin is attached to a protein, "apoprotein", in the egg white. Moreover, the combining potential of this protein could not be saturated by feeding hens a diet rich in riboflavin. The observation that the addition of the "apoprotein" to media prevented the growth of riboflavin-requiring organisms (Streptococcus zymogenes and Lactobacillus casei) lead these authors to suggest that the protein may play some part in the egg's defence. The practical importance of this has not been tested.

There is some uncertainty as to the actual concentration of vitamin B6 in the egg, particularly in the white. Evans, Butts & Davidson (1951a), who reported some of the highest values for this

vitamin in the white, considered that the vitamin through combination with some substance in the white was rendered unavailable to the micro-organisms being used for its assay. It was their opinion that their high values resulted from the use of a hydrolyzing procedure which was more rigorous than those used by earlier investigators. As far as can be ascertained, this problem has not been re-investigated.

The work discussed above contains a warning against the assumption that, because there is no evidence to the contrary, the other vitamins of the white are available to contaminants. Neither avidin nor the "apoprotein" were isolated by the older methods of protein separation. The isolation of avidin followed the failure to associate the binding of biotin with any of the principal proteins of the white, and the "apoprotein" was not recognized until the cationic exchange method of protein separation (Peterson & Sober, 1956) had been applied to egg white (Rhodes et al, 1958). The last mentioned authors also reported the presence of several other proteins which occurred in very small amounts and which had not been previously recognized, an observation confirmed by Mandeles (1960). Nothing is known about these and it could be that they have important biological functions some of which may be concerned with the egg's defence. This warning is underlined by the observations made in another discipline. Gregory & Holdsworth (1959) found a minor (less than 0·1% of the total proteins) and previously unrecognized protein of chick serum combined with vitamin B_{12} thereby reducing the availability of this vitamin to B_{12}-requiring organisms.

Redox potential. The redox potential of the white is such that anaerobic bacteria would not be expected to grow. The finding that

such bacteria are very rare contaminants of rotten eggs appears to be the only evidence in support of this hypothesis.

Anti-microbial constituents. Laschtschenko (1909) observed the lysis of Bacillus subtilis seeded into egg white and recognized the enzymic nature of this phenomenon. Similar observations were made by Fleming (1922) and he coined the term lysozyme for the enzyme responsible for this lysis. This enzyme has been the subject of a great number of investigations, the results of which have been frequently reviewed; the references to the earlier reviews are given in that of Salton (1957). Lysozyme, a basic protein of low molecular weight, affects sensitive bacteria by hydrolyzing a mucopolysaccharide component of the cell wall. Some of the more easily lysed bacteria belong to the following: Micrococcus, Sarcina, Staphylococcus and Bacillus. Resistant variants can be obtained by successive subculturing of a sensitive organism through media containing increasing concentrations of lysozyme (Fleming & Allison, 1927). Gram-negative bacteria are generally more resistant but some of the resistant ones can be rendered sensitive by suitable manipulations. For example, Noller & Hartsell (1961a and b) found that many strains of the coli-aerogenes group can be rendered sensitive by either pre-treatment with heat, low pH or butanol, or co-treatment with butanol or versene. They concluded that these treatments degraded some lipoprotein complex which, in the normal cell, prevents lysozyme reaching its substrate.

From this account it will be appreciated that lysozyme is another selective factor in the defence of the egg. The majority of work has been done with crystallized lysozyme and organisms grown

under optimal conditions. It is conceivable that in eggs the conditions of growth are sub-optimal and that organisms which are normally resistant to lysozyme are rendered sensitive to its action. Another possible role of lysozyme was suggested by the work of Friedberger & Hoder (1932) who noted that organisms not lysed by high dilutions of lysozyme were flocculated. This phenomenon, which has been observed with both sensitive and resistant organisms (for references, see Salton, 1957), is due to the basic properties of the protein which attracts it to the negatively charged groups on the bacterial cell. This phenomenon may possibly prevent rapid dissemination of contaminants throughout the white.

Extrinsic factors

The temperature at which the egg is kept would appear to be the most important extrinsic factor in the egg's defence. In the case of the incubating egg, this would fluctuate between ambient and that of the hen's body, the actual rhythm being determined by the nesting habits of the brooding hen. This might be yet another selective factor in the defence of the egg. In contemporary commerce, the control of storage temperature has been the most favoured method of preserving the egg's freshness. Moreover, this form of storage has been improved by the addition of CO_2 (or ozone) to the storage atmosphere. This aspect has been reviewed by Sharp (1937), Haines (1939), Romanoff & Romanoff (1949) and Brooks & Taylor (1955).

Conclusions

The available evidence (Haines & Moran, 1940; Garibaldi & Stokes, 1958; Bean & MacLaury, 1959) does suggest that the shell and shell membranes offer only an imperfect barrier to bacterial penetration The principal anti-microbial defence appears to reside

in the albumen. The highly selective nature of this defence has been emphasized in the foregoing. In all cases the many components of this defence have been recognized as a result of experiments conducted *in vitro* during the investigation of problems which, in many cases, were not directly concerned with the microbiology of the egg. Consequently, although there is much circumstantial evidence relating to the individual components, there is neither direct evidence concerning their coordination in the whole egg, nor data that can be used to assess their relative importance. It would seem that the technique introduced by Elliott (1954) and used by Brooks (1960b) offers a possible method for the elucidation of some of the problems discussed above. In the meantime, it would seem reasonable to conclude that lysozyme together with the nutritional inadequacies of the white are most probably responsible for the low incidence of Gram-positive bacteria in rotten eggs. Moreover, these two factors together with the oxidized state of the white may account for the absence of obligate anaerobes. In the case of the Gram-negative bacteria, particularly those that are common contaminants of rotten eggs, the available evidence (Brooks, 1960b; Garibaldi, 1960) does implicate conalbumin as being the most important component.

In practice, our knowledge of the selective nature of the egg's defence derives indirectly from investigations of the microbiology of rotten eggs. As a result, any conclusion must be carefully drawn. This is because, if the terms of ecology are used in an exposition of this problem, we are dealing with a 'climax population'. In rotten eggs of commercial origin, this population is composed almost exclusively of Gram-negative bacteria. In the case of nest clean eggs, Haines (1938) found that these bacteria comprised 38%

of the micro-organisms residing on the shell. It is very tempting to conclude that these bacteria, because of their simple nutritional requirements, their resistance to lysozyme and their tolerance of alkaline conditions, are preferentially selected by the co-ordinated actions of the egg's defence. Such a conclusion would obviate the need to give further thought to the possibility that, in fact, we may be dealing with something like the following: a 'dominant' organism which pioneers for species that are unable to exploit the environment themselves; a succession of these latter organisms terminating in the 'climax population'.

It would appear that this conclusion was tacitly accepted by both Elliott (1954) and Brooks (1960b) at the outset of their investigations of the course of infection in eggs whose air cells had been inoculated with pseudomonads. This departure from *in vitro* experiments has given promising results, as could be expected in view of the *in vivo* experiments which Smith and his collaborators (Smith, 1958) have used successfully in the elucidation of bacterial pathogenicity. A more extensive application of this technique together with a more liberal selection of organisms could well provide answers to some of the problems discussed above. In particular, the possibility of a 'pioneering' organism being involved in the infection process should not be overlooked. In this context, it would be interesting to use *Alcaligenes bookeri*. Although this organism is frequently present together with others in rotten eggs (Florian & Trussell, 1957) it is not normally regarded as an important rot-producer. It was the only one which Garibaldi & Stokes (1958) found to be capable of digesting shell membranes *in vitro* and it was one of the few organisms which Garibaldi (1960) found to be capable of growing in normal egg white. Both these attributes suggest that this organism may play an important

'pioneering' role and the fact that it is a slow growing organism may account for its failure to become dominant in the 'climax population'.

THE MICROBIOLOGY OF INFECTED EGGS

In this section the emphasis is placed on the microbiology of two types of infected eggs which, for convenience, are termed: a) rotten eggs, and b) tainted eggs. The microbiology of eggs at the time of laying is discussed at a later stage.

Rotten eggs

The earliest work on this type of infection, reviewed by Pennington, Jenkins, St. John & Hicks (1914), was mainly concerned with the characterization of the organisms, an approach which persisted until re-infection experiments were introduced by Miles & Halnan (1937) and Haines (1938). These revealed that each characteristic rot was produced by a specific type of organism, a finding that was in opposition to the veiw of Pennington *et al*. (1914) who considered that these various rots were stages in an egg's decomposition. The types of rot together with the causative agents are listed in Table 6. Both Miles & Halnan (1937) and Haines (1938) found that bacterial rots were dominated by Gram-negative bacteria. This situation has been found in all subsequent investigations (Alford, Holmes, Scott & Vickery, 1950; Richard & Mohler, 1950; Miller & Crawford, 1953; Florian & Trussell, 1957). Furthermore, it has been a common finding that an infected egg normally contains organisms other than those that are responsible for the distinctive features of the rot. These have been regarded as either secondary invaders (Florian & Trussell, 1957) or the causative agents of "colourless rots" (Alford *et. al*. 1950; Florian & Trussell, 1957). The importance of these

organisms in the infection process is not known and it would seem desirable to hold the term "secondary invaders" in abeyance until the nature of the infection process is known in more detail. Moreover, many of these organisms are similar to those that are associated with tainted eggs (see Table 7) and the application of organoleptic test to the organisms isolated from rotten eggs might well show that the same problem is being considered under two headings.

The absence of adequate data prevents an assessment of the relative importance of the various organisms listed in Table 6 in the infection of commercial eggs. It would seem that such an assessment would be possible if large numbers of organisms isolated from rotten eggs whose histories were well documented could be identified by a few tests. At the moment, however, there does not appear to be satisfactory evidence in support of the widely accepted belief that pseudomonads are the most important contaminants of commercial eggs. Alford et al. (1950) found that only 38% of the 3,783 rots examined in stored Australian eggs manifested features which are known to be produced by these organisms. Table 8 contains data that have been compiled from the report of Knowles (1957) who examined stored Irish eggs. It will be seen that even under conditions (cold storage) which could be expected to be selective for pseudomonads, this type of organism did not show a marked dominance. Moreover, the high incidence of infection with pseudomonads in eggs washed in machine C may have resulted from a heavy contamination of the machine, an interpretation that is suggested by the report of Gillespie & Scott (1950). In Canada, Florian & Trussell (1957) found that 40 out of 81 eggs were infected with these organisms. The organisms' production of an easily detected

pigment and the possible enhancement of this property by the iron-deficient state of the white may account for what appears to be a biased view. This situation should discourage the existing practice of using only fluorescent pseudomonads in investigations of the infection process.

The literature dealing with eggs infected with fungi has been reviewed by Haines (1939). This type of spoilage is rare in commercial eggs and only occurs during storage in relative humidities of 90% and upwards (Sharp & Stewart, 1936). These authors found that during short periods of storage the infection is superficial and remains confined to the shell, a condition which is known as "whiskers" in the industry. During prolonged storage, the infection becomes disseminated through the egg, the speed and incidence of this being determined by the relative humidity. Sporotrichum carnis and Cladosporium herbarum, both of which produce very characteristic changes in the egg, are common contaminants (Moran & Piqué, 1926; Weston & Halnan, 1927).

Tainted eggs

There are a number of publications dealing with the microbiology of eggs having either off-odours or off-flavours which, for the sake of convenience, are termed tainted eggs in this review. The authors together with the causative organisms are listed in Table 7. Several of these organisms have been the subject of a recent taxonomic investigation (Szybalski, 1950). The ability of these bacteria to produce taints in fresh eggs was investigated by Brooks & Hale (1954). The type of taint under discussion is transferable by inoculation of the causative organism into fresh eggs and, as such, it differs from the taints which arise from storage of eggs in cartons contaminated with odoriferous chemicals or substances produced by

micro-organisms eg. actinomycetes. It will be noted that these taints are produced by Gram-negative bacteria many of which are similar to those included under "colourless rots" in Table 6. As has already been mentioned, the separation of tainted eggs from rotten eggs may be an artificial division.

THE COURSE OF BACTERIAL INFECTION OF THE HEN'S EGG

Information derived from a) fundamental investigations and b) empirical observations is available for discussion in this section. That coming from a) will be considered in the first instance.

Fundamental investigations

Gillespie & Scott (1950), using information obtained mainly from empirical observations, considered the following to be possible stages in the infection of an egg: a) contamination of the shell, b) penetration of the shell, c) colonization of the shell membranes and d) infection of the contents of the egg. These stages are used in the following discussion.

Contamination of the shell and contents of the egg

Congenital. The literature on the microbiology of the oviduct has been reviewed by Romanoff & Romanoff (1949). It appears that in the normal hen the oviduct is sterile and that it has mechanisms which frees it from chance contamination. In some hens, the ovaries become infected with *Salmonella* and these organisms can be included in the yolk. It is interesting to note that Miles & Halnan (1937) were unable to infect eggs by feeding hens with cultures of *Proteus melanovogenes* (*Aeromonas liquefaciens*). Platt (1936), on the other hand, has claimed to have produced "floating yolks" by feeding hens with the spore forming bacillus which he had isolated from eggs suffering from this fault. This problem does not appear to have been investigated further.

Rettger (1914), Hadley & Caldwell (1916) and Haines (1939) have reviewed the early literature dealing with the microbiology of freshly laid eggs. All three are of the opinion that the contents of roughly 90% of such eggs are free from micro-organisms. More recently, Wolk, McNally & Spicknall (1950) reported an average rate of contamination of 12·0% and Forsythe, Ayres & Radlo (1953) found the highest incidence in eggs that had been washed in cold water. Moreover, Kraft, McNally & Brant (1958) found a linear relationship when the incidence of contamination was plotted against the porosity of the shells, the highest incidence of contamination occurred with the most porous eggs. The existence of an approximate instead of an absolute value of contamination can be attributed to two difficulties associated with the sampling of eggs under strictly aseptic conditions: a) sterilization of the shell, and b) removal of the contents of the egg and sampling of the white. Many of the isolates have been found to grow poorly, if at all, at the hen's body temperature and the majority would not be regarded as typical representatives of the organisms normally associated with the hen's alimentary canal, evidence which suggests that either many of these bacteria were included in the egg after it had been laid or that they were contaminants procured during sampling.

Extragenital. The shell, which is considered to be sterile at the time of laying, acquires micro-organisms from all surfaces with which it makes contact. Haines (1938) gave figures ranging from $1{\cdot}35 \times 10^4$ - $8{\cdot}0 \times 10^6$ (incubation, 20°) and $3{\cdot}5 \times 10^4$ - $1{\cdot}6 \times 10^6$ (incubation, 37°) for the number of viable organisms present on the shells of naturally clean eggs. Forsythe *et al.* (1953) reported a mean count of $6{\cdot}3 \times 10^4$

($1{\cdot}0 \times 10^4 - 1{\cdot}0 \times 10^6$) for micro-organism, both bacteria and moulds, on the shells of soiled eggs. Tomkins (1937) found between 200 - 500 mould spores per shell. Neither Haines (1938) nor Forsythe et al. (1953) found any definite or characteristic changes in the numbers of bacteria on the shells of eggs held in stores controlled for temperature, relative humidity and, in certain cases, concentration of CO_2.

Haines (1938) reported the following composition for the isolates obtained from naturally clean eggs: sporing rods (30%), cocci (25%), Gram-negative bacteria (38%), yeasts (4%) and actinomycetes (3%).

Penetration of the shell

This topic has been considered on page 13 and it will suffice to list the known factors: a) sucking in of bacteria during the contraction of a warm egg, b) flooding of the pores with contaminated water drawn in by capillary attraction and c) deposition of bacteria in the egg's contents by fungal hyphae that have grown through the shell. In addition, more effective penetration by method b) can be procured by rubbing the surface of the shell with an abrasive.

Colonization of the shell membranes

The fate of organisms placed on the inner membrane of the air cell of whole eggs has been infrequently studied (Elliott, 1954; Brooks, 1960b). Elliott (1954), who inoculated the air cell with 0·5 ml of either a washed cell suspension or a broth culture of Pseudomonas ovalis, found that the organisms remained viable in the membranes of the majority of eggs. He was unable to recover viable organisms from the white until 8 days following inoculation and it took a further 2 days before all the albumens were infected. After 30 days incubation, there were about $3{\cdot}0 \times 10^9$ viable organisms /g egg white. A possible

explanation of the non-recovery of viable organisms from the white during the early stages of infection has been discussed on page 15. A similar technique was used by Brooks (1960b) but, owing to his untimely death, his results have been inadequately documented in a preliminary report. Nevertheless, in the case of fresh eggs he found that the organisms multiplied slowly in the shell membrane of the air cell for the first 12 days, at which time a marked increase in the number of organisms both in the membrane and in the white occurred. He was of the opinion that this increase was due to some age-induced change in the membranes, an aspect which has been discussed on page 5. The number of viable organisms in the white showed a certain parallelism with those in the membrane, evidence which led Brooks to suggest that these organisms were derived from those present in the membrane and had not arisen from the few which had invaded the albumen during the early stages of the infection process.

Infection of the egg's contents

It would appear from the above that the membranes play an important part in the contamination of the albumen Ayres & Taylor (1956) have studied the fate of several Gram-negative bacteria inoculated in the albumen of either whole or broken-out eggs. The rate and extent of multiplication in whole eggs was not appreciably different from that in the broken-out eggs. Both characters were markedly influenced by the incubation temperature viz. (the bracketed figures refer to the temperature at which multiplication occurred): Pseudomonas fluorescens (2, 10 and 20°), Serratia marcescens and Proteus vulgaris (10 and 20°) and Salmonella pullorum (20°). This report does not allow satisfactory generalizations to be made. This is due to: a) the use of only one strain of each organism;

Brooks (1960b) has shown that the rate and extent of multiplication of pseudomonads in albumen *in vitro* varies appreciably among strains and that the pattern of a particular strain can be modified by changing the methods used for preserving the culture, and b) no attempt was made to assess the influence, if any, of extraneous substances derived from the medium on the growth of the organisms in the albumen.

Empirical observations

Following the observation of Haines & Moran (1940), many investigators have used the immersion of a warm egg in a cold suspension of an organism as the first step in the study of the infection process. In general a fluorescent pseudomonad has been used and the incidence of fluorescence of the white following storage has been used as the index of infection. It appears that if another index is used, e.g. isolation of organisms from the white, (Stokes, Osborne & Bayne, 1956), this technique can be used with organisms other than pseudomonads (Trussell, 1955; Bean & MacLaury, 1959). The assessment of the information arising from this approach was difficult until the publications of Brooks (1960b), Stokes *et al.* (1956) and Fromm & Monroe (1960).

Stokes *et al.* (1956) used strains of *Salmonella* and traced the course of infection by determining the number of viable organisms present in the albumen. In the case of eggs infected with *Salmonella oranienburg* and incubated at 29°, there was a slow penetration of the shell membranes during the first week of incubation, followed in the second week by a slow increase in the number of organisms in the white and culminating in the third week with a marked increase in the number of organisms in the white. Fromm & Monroe (1960) immersed eggs in a broth culture of *Pseudomonas aeruginosa* (both of which were at the

same temperature) and followed the course of infection by examination of the eggs in a candling lamp fitted with a source of ultraviolet irradiation. Less than 5% of the eggs manifested signs of infection during the first 10 days incubation but between 10 and 15 days there was a very marked increase in the incidence of infection, a trend whose strength and duration during the subsequent 10 - 15 days incubation was influenced by the treatment that the eggs had received prior to infection. The patterns in both these investigations show certain similarities to those reported by Brooks (1960b), particularly in respect of the slow rate of infection during the first 10 - 15 days following inoculation. This evidence suggests that the method of Brooks might provide a means of elucidating some of the problems discussed on page 53.

Attention will now be given to the other investigations in which the method of Haines & Moran (1940) was used. Lorenz, Starr, Starr & Ogasawara (1952) found that the incidence of fluorescence was influenced by: a) the concentration of the organisms in the suspension - an observation also made by Stokes et al. (1956), and b) the temperature differential between the egg and the suspension; the incidence of fluorescence was directly proportional to the temperature differential over the range 6 - 21°. They found that the thickness of the shell did not influence the incidence of rotting. Orel (1959a), on the other hand, found that there was a statistically satisfactory correlation between the rate of development of fluorescence and the eggs' specific gravity, a character which is considered to be a reflection of the quality of the shell, particularly its thickness (Romanoff & Romanoff, 1949). Eggs with a specific gravity greater than 1·082 did not show fluorescence until 8 days after its appearance

in eggs whose specific gravity was less than 1·082. Elliott (1954) found that the incidence of fluorescence and the rate of its development could be increased by storing the eggs for several weeks prior to their immersion in a bacterial suspension. Fromm & Monroe (1960) increased the incidence of fluorescence (but not its speed of development) by washing and/or rubbing the shells prior to immersing the eggs in a bacterial suspension. Stokes et al. (1956) and Orel (1959b) found that the rate of infection could be retarded by storing the eggs at low temperatures. In addition, Orel (1959b) reported that the onset of fluorescence could be delayed by either oiling or thermostabilizing (10 min at 58 - 60°) the eggs after they had been infected.

MICROBIOLOGY IN THE PRESERVATION OF THE HEN'S EGG

This subject has been reviewed by Sharp (1937), Haines (1939), Romanoff & Romanoff (1949) and Brooks & Taylor (1955). It appears that seasonal variation in egg production was the main reason in the past for egg preservation. Today, the extent of this variation has been reduced as a result of changes in the methods of poultry husbandry. Hence the preservation of shell eggs for use in the country of their origin has declined in importance, a decline that has been further accentuated by an increase in the preservation of egg products e.g. frozen eggs and dried egg powder. Nevertheless, significant numbers of eggs are still imported into those countries whose indigenous supplies are not sufficient. The needs of transportation have resulted in changes in the methods of preservation of shell eggs. The introduction of refrigerated stores whose atmospheres are controlled in respect of humidity and/or concentration of CO_2 is evidence of the

efforts made by applied scientists.

Similarly, fundamental principles have been applied in efforts to retard the loss of quality during the storage of shell eggs. It is surprising therefore, that, unlike other foods, microbiology has had only a limited impact on the theory and practice of preservation of the shell egg. This situation is emphasized in the reviews dealing with the influence of washing dirty eggs on the incidence of rotting in such eggs during subsequent storage (Haines, 1939; Winter, Burhart, & Wettling, 1952: Brooks & Taylor, 1955). Although these authors are of the opinion that the incidence of rotting is generally increased by washing, their reviews contain references to numerous investigations in which no detrimental effects attended this practice. It is noteworthy however that this aspect remains a controversial issue only in those countries whose egg producers are either subsidized (e.g. Britain) or not dependent upon an export market for the disposal of their products. Two major exporting countries (Denmark and Australia) have long since introduced legislation whereby the producer is paid a premium for naturally clean eggs.

The following is not intended to be a comprehensive review of the literature dealing with microbial wastage in stored eggs. Its primary purpose is to outline the problem in such a manner so that emphasis is given to the many gaps in our knowledge.

It is known (Winter, Burkart, Clements & MacDonald, 1955) that soiled eggs harbour more organisms per shell than do naturally clean eggs. The available evidence (page 40) indicates that bacteria other than those which are normally associated with the hen's alimentary canal are the most important rot-producers. Since there appears to be no information concerning the relative importance of faeces, nesting

material, deep litter material and/or soil as soiling agents, it would seem that a tacit assumption of a correlation between heavy soiling and the egg's contamination with potential rot-producing bacteria is unwarranted. It has been shown (Lorenz et al. 1952) that the extent and location of the soiling of the shell is of some importance. They studied the incidence of rotting in eggs to whose surface had been applied sterilized soil containing a culture of a pseudomonad. The extent and location together with the incidence of rotting (bracketed figures) were as follows: shell completely covered (66·7%); half the shell, large end (28·6%); half the shell, small end (18·2%); 1 cm^2, large end (28·6%) and 1 cm^2, small end (17·4%). It is interesting to note that they could induce a high incidence of rotting during the storage of unwashed naturally clean eggs by the simple expedient of contaminating the nesting material with a pseudomonad. A similar claim has been made by Dr. J. Patton (pers. comm.).

It would seem that this form of contamination is of little importance in practice (Alford et al. 1950; Trussell, Fulton & Cameron, 1955; Trussell, Triggs & Greer, 1955). These authors, who attempted to assess the influence of various farm practices on the incidence of rotting in stored eggs, found that: a) an egg's resistance to rotting has a genetic basis, and b) the highest incidences of rotting are generally associated with eggs that have been cleaned in mechanical washers. The true nature of a) is not understood. It is impossible to draw satisfactory conclusions from the many investigations of b), investigations which have been reviewed by Haines (1939), Winter et al. (1952) and Brooks & Taylor (1955). This is due to: a) the use of many different types of washing machine, b) the use of different periods and conditions of storage together with the use of different

criteria for the assessment of the incidence of rotting, and c) the almost complete absence of information concerning the numbers of potential rot-producing bacteria present on the eggs and/or the machines. Moreover, the limited information derived from more fundamental investigations (Elliott, 1954; Brooks, 1960b) does not permit an interpretation of the information obtained from these empirical observations.

The negative correlation between the number of organisms per shell and the incidence of rotting which is evident in the data given in Table 9 suggests that the location of the organism in the egg is of more importance than the number present on the shell. This implies that washing machines assist bacterial penetration of the shell Although our knowledge (page 13) allows us to suggest methods whereby the machines could assist this penetration, the information derived from empirical observations does not permit an assessment of the relative importance of these methods in commercial practice.

Attempts to use disinfectants (or antibiotics) for the control of rotting in washed eggs have given indirect evidence in support of the conclusion that washing machines can play an important part in bacterial penetration of the shell. The data summerized by Winter et al. (1955) show that a significant reduction in the number of viable bacteria per shell can be affected by washing eggs in water containing any one of a number of commonly used disinfectants. Both Gillespie & Scott (1950) and Ayres & Taylor (1956) have shown, however, that even a rigorously controlled disinfecting procedure does not result in the sterilization of the shell of all eggs. In commercial practice, Gillespie, Salton & Scott (1950) found that bacterial rotting in washed eggs could be significantly reduced if relatively

high concentrations (5,000 ppm) of hypochlorite was added to the water in a washing machine. Moreover, they found that a marked reduction in the number of organisms per shell was not always associated with freedom from rotting when such eggs were stored. Gillespie & Scott (1950) found that the treatment of the shells of washed eggs with disinfectants did not reduce the incidence of rotting during the subsequent storage of such eggs. Attempts to use antibiotic for the control of bacterial rotting of eggs have not proved successful (Schmidt & Stadelman, 1957; Bean & MacLaury, 1959) and it would seem that this failure is due to the contaminants in the egg being protected from the antibiotic through the latter's combination with proteins of the albumen (Elliott & Romoser, 1957).

The observation (Gillespie & Scott, 1950) that viable bacteria were not recoverable from the contents of washed eggs until the 14th day following storage at 18° does suggest that the course of bacterial infection in commercially washed eggs is similar to that in eggs infected in the laboratory (Elliott, 1954; Stokes *et al*. 1956; Brooks, 1960b; Fromm & Monroe, 1960). It would seem, therefore, that some of the problems discussed above might be resolved if a more liberal selection of organisms were used in experiments similar to those of Elliott (1954) and Brooks (1960b).

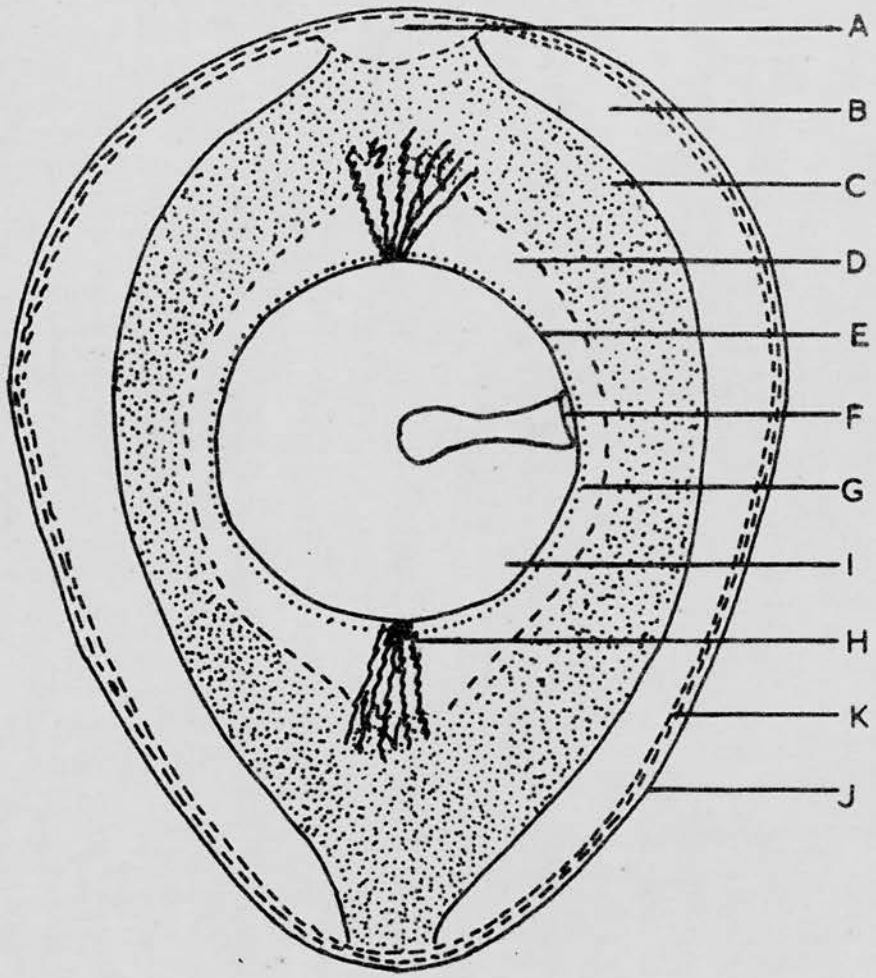


FIG. I. THE STRUCTURE OF THE HEN'S EGG AS SHOWN BY A SECTION THROUGH THE LONG AXIS

A: AIR CELL. B: OUTER THIN ALBUMEN. C: THICK ALBUMEN. D: INNER THIN ALBUMEN. E: VITELLINE MEMBRANE. F: BLASTODERM. G: CHALAZIFEROUS MEMBRANE. H: CHALAZA. I: YOLK. J: SHELL. K: SHELL MEMBRANES.

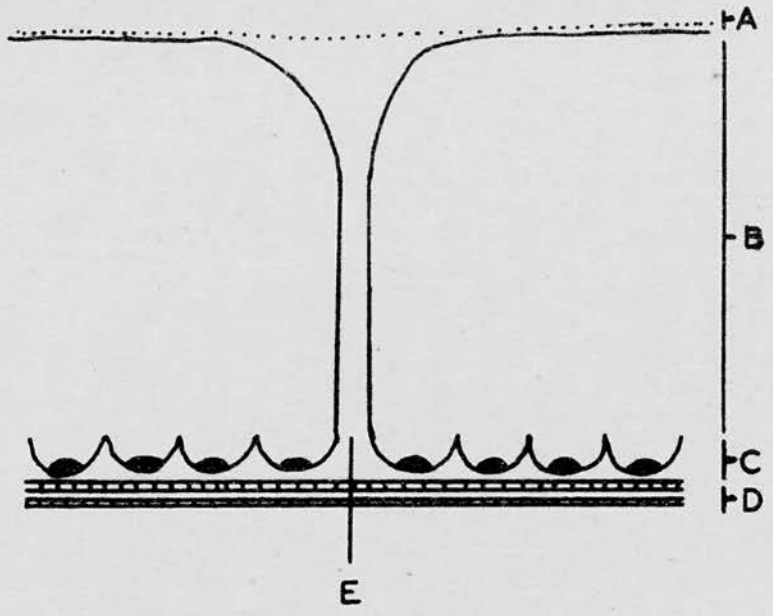


FIG.2. A CONVENTIONAL DRAWING OF A RADIAL SECTION OF AN EGG SHELL

A: CUTICLE. B: SPONGY LAYER. C: MAMMILLARY LAYER. D: SHELL MEMBRANES. E: PORE.

Table 1.

Average composition of the egg shell*

	Weight g	Composition %
Inorganic matter	5·8	95·1
Calcium carbonate (calcite) (98·43%), Magnesium carbonate (0·84%), Tricalcium phosphate (0·73%)		
Protein	0·2	3·3
Lipids	Trace	Trace
Total solids	6·0	98·4
Water	0·1	1·6
TOTAL	6·1	100·0

*A modification of the Table given by Brooks & Taylor (1955)

Table 2.

Average composition of egg albumen

	Amount g
Water	28·9
Solids	
Organic	
Proteins	3·5
Lipids	trace
Carbohydrates	0·3
Inorganic	0·2
TOTAL	32·9

Table 3.

Protein composition of egg albumen

	Percentage of total protein	Biological activity
Ovalbumin	60·0[a]	-
Conalbumin	13·8	Iron-binding (Schade & Caroline, 1944)
Ovomucoid	14·0	Anti-tryptic factor (Lineweaver & Murray, 1947)
Globulins		
G_1	2·8	Bacterial lytic agent (Fleming, 1922)
G_2	4·6	-
G_3	4·3	-
Avidin	0·06[b]	Biotin-binding (Eakin *et al.* 1940)
Ovomucin	2·0 [c]	Haemagglutination inhibitor (Lanni *et al.* 1949)

a: the figures listed below the letter a are taken from Longsworth, Cannan & MacInnes (1940).

b: figure taken from Woolley & Longsworth (1942)

c: figure taken from Sørensen (1934)

Table 4.

The effect of keeping oiled eggs at 0° and 80% relative humidity*

Time (weeks)	Average weight of total white g	Average percentage of total white as:		
		Outer thin white	Thick white	Inner thin white
0	33·0	22·7	46·4	30·9
12	31·9	45·8	37·4	16·8
24	30·6	52·9	31·5	15·6

*Brooks & Hale (1959)

Table 5.

Changes in the vitamin content of eggs stored at 0°

Vitamin	Unit/egg		Fresh eggs	Eggs stored for: 3 months	12 months
Folic acid[1]	WE	µg	4·59	4·59	3·37
	EW		0·51	0·65	0·62
	EY		4·09	3·93	2·75
Choline[2]	WE	mg	252	255	251
	EW		0·4	0·5	3·3
	EY		251	254	248
B_6[3]	WE	µg	123·6	98·6	60·2
	EW		68·1	49·3	31·0
	EY		54·6	49·3	29·3
Biotin[4]	WE	mµg/g	225	244	228
	EW		70	65	71
	EY		520	561	469
Niacin[5]	WE	µg	31·3	28·9	32·1
	EW		28·5	25·5	22·5
	EY		2·8	3·4	9·6
Pantothenic acid[6]	WE	µg	608	570	560
	EW		97	97	213
	EY		511	473	347
Riboflavin[7]	WE	µg	169·7	162·5	147·0
	EW		100·6	100·1	92·7
	EY		69·1	62·4	54·3
B_{12}[8]	WE	mµg	387	341	260
	EW		25	22	23
	EY		362	319	237

WE, whole egg. EW, egg white EY, egg yolk

1, Evans, Davidson, Bauer & Butts (1953). 2, Evans & Davidson (1951). 3, Evans, Butts & Davidson (1951a). 4, Evans, Davidson, Bauer & Butts (1953). 5, Evans, Butts & Davidson (1951b). 6, Evans, Davidson & Butts (1952). 7, Evans, Butts & Davidson (1952). 8, Evans, Bandemer, Bauer & Davidson (1955).

Table 6.

Types of rot produced in eggs by various bacteria

Rot	Causative organism	Investigator
Black rot, type 1	*Aeromonas liquefaciens*	Miles & Halnan (1937)
Black rot, type 2	*Proteus vulgaris* (pseudomonas*)	Haines (1939)
Fluorescent green rot	*Pseudomonas fluorescens*	Haines (1939)
Pink rot	*Pseudomonas fluorescens*	Haines (1939)
Red rot	*Serratia marcescens*	Alford *et al.* (1950)
Green rot, Yellow rot	*Alcaligenes bookeri*	Florian & Trussell (1957)
Custard rot	*Paracolobactrum intermedium*	Florian & Trussell (1957)
Colourless rot	*Alcaligenes metacaligenes*, *Alcaligenes faecalis*	Florian & Trussell (1957)
	pseudomonas@, achromobacter, coliform	Alford *et al.* (1950)

*It would seem that this type of rot can be produced by certain strains of pseudomonads (Haines, 1939; Florian & Trussell, 1957).

@In the publication of Alford *et al.* (1950) *Ps. achromobacter* is given as a causative agent of this rot. This was an editorial error and should read "strains of *Pseudomonas*, *Achromobacter* etc." (Scott, pers. comm.).

Table 7.

Types of organism isolated from eggs having either an off-flavour or an off-odour

Organism	Investigator
Off-odours	
Achromobacter perolens	Turner (1927); Spanswick (1932)
Pseudomonas graveolens; *Pseudomonas mucidolens*; *Pseudomonas mucidolens* var. *tarda*	Levine & Anderson (1932)
Pseudomonas putida; *Pseudomonas ovalis*; *Pseudomonas schuylkillensis*; *Pseudomonas* spp.; *Aerobacter cloacae*; *Alcaligenes* spp.	Richard & Mohler (1950)
Off-flavours	
Escerichia coli	Haines (1938)
Pseudomonas spp.	Haines (1939)

Table 8.

Types of rot found in naturally clean, dry-cleaned and machine washed eggs during cold storage for 6 months

Treatment	No. of eggs tested	Types of rot	
		Green	Sour and black
Naturally clean eggs	1,756	6	16
Dry-cleaned eggs	1,796	12	21
Machine-washed eggs 1*	1,731	108	66
Machine-washed eggs 2	1,664	120	79
Machine-washed eggs 3	1,559	808	309

* 1, rotating drum washer; 2, agitating washer; 3, brush washer

Table compiled from the data given in the report of Knowles (1957)

Table 9.

The effect on wastage of double cleaning on both roller and tape machines

Treatment	Rots (%)	Number of organisms/ shell*
Uncleaned	1·7	60,000
Cleaned on roller machine once	26·7	2,100
Cleaned on roller machine twice	56·7	2,700
Cleaned on roller machine followed by tape machine	30·5	9,100
Cleaned on tape machine followed by roller machine	45·0	4,800
Cleaned on tape machine twice	8·9	15,000
Cleaned on tape machine once	7·2	21,000

*Counts made on nutrient agar containing 0·5 p.p.m. crystal violet

A modified form of the table given in the report of Gillespie & Scott (1950).

THE ISOLATION AND CHARACTERIZATION OF MICRO-ORGANISMS FROM ROTTEN EGGS

CONTENTS

INTRODUCTION

The literature dealing with the microbiology of infected eggs has been considered on page 40.

The object of the work reported hereunder was to assemble a comprehensive collection of typical contaminants of rotten eggs so that an examination of the bacterial infection process could be initiated. The characteristics of representatives of each major group of isolates were examined in detail. The results obtained were used a) to identify the organisms, and b) in an attempt to ascribe the appearances of infected eggs to the metabolic attributes of the causative agents.

METHODS AND MATERIALS

Rotten eggs. These were obtained during the autumn of 1958 from 2 egg collecting centres in the South East of Scotland and 1 in the South of England. These eggs had rotted on the premises of the producers and were rejected during candling immediately on receipt at the collecting centres. In addition, eggs induced to rot under controlled conditions were examined. First quality eggs (laid by White Leghorns), which had been badly soiled with faeces and/or deep litter material, were washed in tap water and then held for several hours at 37° before being returned to the wash water (c. 18 - 20°). Bacterial penetration of the shell is known to be assisted by the contraction of a warm egg on cooling (Haines & Moran, 1940). The eggs were allowed to soak for 20 min. They were then placed in screw-capped glass jars, the bottoms of which were lined with moist cotton wool, and these containers were stored at one of three temperatures, 30, 20 and c.10°. Three separate batches of eggs were treated in this manner. The eggs were

frequently candled and rotten eggs were removed for examination

Isolation of micro-organisms from rotten eggs. The eggs were wiped with alcohol and placed on a pipe-clay triangle the arms of which had been bent through 90° so that the triangle now stood upon three legs. The surface of the shell was flamed before being pierced with a sterile scalpel and the contents were poured into a Petri dish. Recordings were made of the appearance of the contents and the material was examined under ultra-violet light. A loopful of the egg was streaked onto nutrient agar (composition given in appendix) containing 0·3% (w/v) yeast extract (Yeastrel) and filter-sterilized glucose in a final concentration of 0·1% (w/v). Two streak plates were prepared from each rot. One plate was incubated aerobically at 27° and the other, after having its surface covered with 5 ml of the following, was incubated in a MacIntosh & Fildes jar containing an atmosphere of CO_2 (5 parts) and H_2 (95 parts): (g/l.) Cysteine-HCl, 0·5; thioglycollic acid, 0·5; agar (Davis, New Zealand), 15; resazurin, 0·5 mg, pH 7·2. The plates incubated aerobically were inspected daily for seven days at which time representatives of each morphological colony type were transferred to nutrient broth. The purity of these isolates was ensured by re-plating. Selected colonies developing during anaerobic incubation were stab-inoculated into glucose nutrient agar and the donor colony was tested for catalase.

Characterization of the isolates. An 18 hr culture in nutrient broth was used as the inoculum in all experiments. All tests were done at 27° except in the case of nutrient gelatin which was incubated at 22°. Recordings were made at frequent intervals during the first 14 days incubation and finally on the 28th day following inoculation.

Maintenance of stock cultures. One set was maintained under mineral oil on nutrient agar slopes stored at room temperature. Another set was stored in nutrient broth at 4°. This latter set was sub-cultured at 6 monthly intervals, at which time the purity of the culture was checked and fresh broth cultures were prepared for laboratory use.

Flagella staining (Rhodes, 1958). Preparations were made from nutrient agar slope cultures after 12, 24, 48 and 96 hr incubation.

Fermentation tests. The medium of Hugh & Leifson (1953) was used routinely but bacteria which produced either no change in the pH or an alkaline reaction in this medium were re-tested in the medium of Board & Holding (1960). Both inoculated and uninoculated tubes of basal medium were incubated along with basal medium containing the fermentation substrate. The latter were sterilized by Seitz-filtration, except in the case of the primary alcohols which were held for 15 min at 100°, and added to the liquefied medium when this had cooled to 45°. The substrates were normally used in a final concentration of 0·5% (w/v) or, in the case of the primary alcohols, 0·5% (v/v). Exceptions to this are noted in the text. Stab-inoculations were made into 10 ml of medium in 15 x 180 mm test tubes.

Acetoin production. Cultures in peptone (1·0%; w/v) containing glucose (0·5%; w/v) were tested with Barritt's (1936) method and the pH of the medium was tested with methyl red.

Starch hydrolysis Plates of nutrient agar containing 1·0% (w/v) soluble starch were inoculated on the surface and after incubation they were flooded with a solution of Lugol's iodine.

Aesculin. Spot inoculations were made on the surface of nutrient agar containing aesculin (0·1%; w/v) and iron citrate (0·005%; w/v), the blackening of the colony and/or the medium in the vicinity of the colony was taken to indicate aesculin breakdown.

Protein digestion. Nutrient gelatin was stabbed with a straight wire and incubated at 22°.

H_2S production. Filter paper soaked in lead acetate was suspended over peptone water containing a piece of heat-coagulated egg white.

Organic acid utilization. Slopes of the medium of Board & Holding (1960) containing the sodium salt of the acid (final concentration, 0·25%; w/v) were inoculated with a cell suspension prepared by adding a drop of an 18 hr nutrient broth culture to 10 ml of water. The growth on and pH of the supplemented medium were compared with those on the basal medium.

Arginine test. Medium 2A of Thornley (1960) was dispensed in 3 ml amounts in screw-capped bottles whose volumes were about 5 ml. After inoculation the remaining free air-space was filled with sterile vaseline. To eliminate the possibility of false negatives which might be expected to arise through the decolorization of the acid-base indicator, freshly prepared indicator was injected into the medium at the end of the incubation period.

Production of phenylpyruvic acid. $FeCl_3$ was used to test for the presence of phenylpyruvic acid (bottle-green colour) in the water of syneresis collecting at the base of slopes of nutrient agar containing phenylalanine. In the case of doubtful reactions, a cell suspension prepared from a nutrient agar slope culture (Henriksen & Closs, 1938) was tested by the method of Stewart (1959).

Indole. Cultures in tryptone (Difco) broth (1·0%; w/v) were tested by the method of Roessler & McClung (1943).

Urea hydrolysis. Those bacteria which produced an alkaline reaction in the medium of Christensen (1946) within 48 hr were recorded as urease positive.

Oxidase test. A loopful of cells from a nutrient agar slope culture was placed on filter paper containing a 1·0% (w/v) aqueous solution of para-amino-N:N-dimethyl aniline (B.D.H., Laboratory reagent). The development of a blue colour within 1 min was recorded as a positive reaction.

Production of lecithinase. Egg yolk was prepared by the method given in the Manual of Methods (1957). Lecithinase activity was tested by inoculating either nutrient agar (10 parts) containing egg yolk (1 part) or nutrient broth containing egg yolk (proportions same as above). Known positive strains (Serratia marcescens and Bacillus cereus) were used as the controls.

Chelated gluconate medium. This medium was prepared and used according to the instructions given by Paton (1959).

Nitrate reduction. Cultures in peptone water (1·0%; w/v) and KNO_3 (0·1%; w/v) were tested for nitrite and, if negative, nitrate.

Reaction in skim milk containing an acid-base indicator. Skim milk containing either litmus or bromthymol blue was sterilized by steaming for 1 hr on three successive days.

Growth temperatures. Inoculated nutrient agar slopes were incubated at the following temperatures 4 (in a beaker of water held in a cold room), 10, 20, 30 and 40° (in thermostatically controlled water baths).

RESULTS

Of the 133 eggs examined, 70 were obtained from commercial

channels and the remainder were laboratory induced rots. A total of 226 isolates were obtained from these eggs, the majority of which contained a mixed infection. No obligate anaerobic bacteria, moulds or yeasts were isolated, the absence of the latter two were confirmed by microscopic examination of infected material which had been stained with polychrome methylene blue. Subsequent examinations of small numbers of rotten eggs originating from divers sources indicated that a normal and representative range of both bacterial types and egg rots were encountered in the Autumn of 1958.

The preliminary separation of the isolates was based on the following tests: Gram's staining reaction, shape and motility; reaction in litmus milk; action on glucose, lactose and urea; oxygen requirements and examination of cultures on nutrient agar or in litmus milk under ultra-violet irradiation. The grouping of the organisms is given in Table 10. It will be seen that the egg infections were dominated by Gram-negative bacteria. The number of isolates of alcaligenes and non-fluorescent pseudomonads was possibly exaggerated because of colonial variations which were not recognized as such when the plates were sampled.

The occurrence of the different types of Gram-negative bacteria in the various groups of eggs is shown in Table 11. It is considered that no definite conclusions should be drawn from this data because of the small number of eggs examined and the method (streak plate) used in the isolations from the rotten eggs, a method which could be expected to result in the isolation of only the numerically dominant contaminants. Nevertheless, there appear to be three points worthy of comment. Firstly, the same types of bacteria were found in the

commercial rejects as were found in the rots produced in the laboratory. Secondly, the storage temperature appeared to have had some selective effect in the induced rots. The majority of alcaligenes were isolated from eggs stored at 20°. Fluorescent pseudomonads were isolated from eggs stored at 10 and 20° but not from eggs stored at 30°. The majority of proteus-type bacteria (8/9) were isolated from eggs held at 30°. Thirdly, the number of fluorescent pseudomonads was surprisingly low in view of the widely accepted belief that these are the most important organisms in commercial eggs.

Representatives of the various groups listed in Table 11 were selected for detailed examination. In making this selection, care was exercised to ensure that the collection was composed of organisms which had been isolated from the various sources listed in Table 11. In all cases the properties of these organisms were tested on at least two occasions during the two years following isolation. Some of the results are given in Table 12. Additional details and the identification of the groups are discussed in the following section.

Alcaligenes

A homogeneous group was formed by the 9 representatives selected for detailed examination. These bacteria were inert in the more commonly used biochemical tests and some of the following characters proved useful adjuncts in recognizing strains of this group. Skim milk containing either litmus or bromthymol blue became alkaline within 72 hr and remained so for 28 days. Respirometer studies and chemical analysis of growing cultures failed to demonstrate glucose utilization, results which confirmed

those obtained with conventional fermentation tests. Acidic substances were formed from an oxidative breakdown of the primary alcohols ethanol, propanol and butanol (tested in the medium of Board & Holding, 1960). No clearing was seen on the ethanol-$CaCO_3$ medium of Shimwell, Carr & Rhodes (1960). Table 13 summarizes the results obtained from an investigation of organic acid utilization by these bacteria.

In media, in which ammonia was the sole source of combined nitrogen, growth occurred when the carbon source was either gluconate or ethanol but not when it was glucose or citrate. No growth occurred when either ethylamine or methylamine was the sole source of carbon and nitrogen.

These organisms had no detectable action on: egg yolk, potato slopes or glycerol and no growth occurred in nutrient broth at pH 4·5 (adjusted with HCl).

A characteristic variation of colonial morphology was noted and this is illustrated in Plate 1. It is considered that this property may have been responsible for the isolation of such large numbers of these organisms.

Cook (1961) observed that these organisms grew anaerobically in media containing NO_2, but not NO_3, with the production of gas.

The bacteria isolated in this study have been identified with Alcaligenes faecalis, a conclusion supported by comparison with Conn's culture of Alcaligenes faecalis (NCIB 8156).

Fluorescent pseudomonads

Table 14 shows the biochemical differences that existed among the 9 isolates chosen for detailed examination. These organisms have

been identified with Pseudomonas fluorescens as defined by Rhodes (1959).

Proteus

The six representatives of this group had the following properties in addition to those given in Table 12. All the strains fermented sucrose, 5 out of the 6 strains fermented mannitol, dulcitol and inositol. None of the strains produced sub-culturable growth in a mineral medium containing either glucose or citrate. They had no detectable action on egg yolk. A brown pigment was formed by cultures growing on nutrient agar containing tyrosine after prolonged incubation at room temperature. All the cultures produced proteus-type growth on nutrient agar. The six strains are considered to be Proteus vulgaris Hauser.

Coli-aerogenes group

The results obtained from test for acetoin formation and methyl red reaction were used to separate the strains of this group into A and B of Table 12. Group A has been identified with Cloaca (Report, 1958) and two main types were recognized: i) a non-lactose fermenting, proteolytic group which produced a heavy scum in nutrient broth containing egg yolk and a soft clot, slight acidity and marked digestion in litmus milk, and ii) a lactose fermenting group which had no detectable action on egg yolk and produced only an acid reaction in litmus milk.

Strains of Group B have been identified with Citrobacter (Report, 1958).

Aeromonads

Strains of this group were not recognized in the preliminary work and it was not until flagella stains and re-infection experiments

had been done that the present collection was obtained. The identification of these with Aeromonas was supported by studies in which they were compared with Pseudomonas fermentans L 417 (von Wolzogen Kühr, 1932) and Aeromonas liquefaciens (NCTC 7810). The strains isolated in the present study have been identified with Aeromonas liquefaciens as defined by Eddy (1960). This species has been called Aeromonas hydrophila in another recent investigation of this group (Ewing, Hugh & Johnson, 1961). Three of our isolates have been examined by Dr. Eddy who is in agreement with the above identification.

The first adequately documented account of the occurrence of this organism in rotten eggs was that of Miles & Halnan (1937). These authors were of the opinion that it was a proteus to which they gave the name Proteus melanovogenes. This name is still used in current reports (e.g. Florian & Trussell, 1957; Frazier, 1958) although the organisms were later identified with Aeromonas (Miles & Miles, 1951). Strains from this latter study were included among those examined by Eddy (1960).

Non-fluorescent pseudomonads (pseudomonad E)

A homogeneous group was formed by the 9 isolates selected for detailed examination. The organisms are obligate aerobes and have from 1 - 3 flagella attached to one pole of the cell, an arrangement which did not change during a 96 hr observation period.

On nutrient agar, a smooth circular raised colony of butter-like consistency was generally formed, occasionally an irregularly outlined rugose colony developed. An unsuccessful attempt was made to find the reason for this variation. Young cultures on nutrient agar were colourless but a light yellow colour developed after pro-

longed incubation at room temperature. This pigment could be most easily seen on media containing $CaCO_3$ or some other white insoluble material. This pale yellow colour was also evident in the packed cells produced by centrifugation of an 18 hr nutrient broth culture. No pigment was formed in the gluconate medium of Paton (1959) - this medium did not support growth unless supplemented with yeast extract

Good growth was obtained with all the commonly used laboratory media. In media in which ammonia was the sole source of combined nitrogen no growth occurred with the following carbon sources: glucose, gluconate, citrate or ethanol. Moreover, none of the following could serve as a source of both carbon and nitrogen: ammonium lactate, methylamine or ethylamine. Growth was obtained when a glucose mineral medium was supplemented with yeast extract. The amount of growth (visual estimation) appeared to be determined by the concentration of yeast extract.

Viability was quickly lost during storage at room temperature of cultures on nutrient agar which had been covered with mineral oil within 48 hr of inoculation. Satisfactory maintenance of viability was obtained with nutrient broth cultures stored either at 4° or room temperature and sub-cultured at 6-monthly intervals.

The utilization of glucose by strains of this organism was demonstrated by standard manometric techniques. The results of one such experiment are given in Figure 3 from which it will be seen that this is a constitutive property. The disappearance of glucose from a growing culture was demonstrated by chemical methods. Dubious results were obtained from early attempts to demonstrate acid production by cultures growing in a medium containing glucose and an acid-base indicator. It will be seen from Table 15 that with

either the medium of Hugh & Leifson (1953) or that of Hugh & Ryschenkow (1961) there was no clear-cut reactions other than a pronounced alkaline reaction in the basal medium. It was inferred that this reaction was masking any acid which might arise from glucose utilization. A medium was devised (Board & Holding, 1960) in which a pronounced alkaline reaction did not develop in the basal medium and it will be seen from the results given in Table 15 that when this medium contained glucose it registered an acid reaction on the 7th day of incubation.

Acid reactions detectable only in the medium of Board & Holding (1960) were obtained with the following substrates: fructose, xylose, lactose, ethanol, propanol or butanol. No clearing occurred during growth on the ethanol-$CaCO_3$ medium of Shimwell *et al.* (1960). A strong acid reaction developed in the media of Hugh & Leifson (1953) and Hugh & Ryschenkow (1961) containing either arabinose or maltose.

No change in the pH was seen in skim milk containing either litmus or bromthymol blue but the milk was slowly digested from the top downwards and a soft clot formed at the bottom of the tube. A very characteristic almond-like (nutty) odour was produced in these cultures, an odour that was particularly noticeable immediately upon opening an incubator containing a number of these cultures. Moreover, it was also detected in infected eggs and on a number of media containing proteins particularly those containing native proteins derived from eggs. This property has not declined during a 3 year period in which these organisms have been maintained on laboratory media.

These organisms digested the following proteins: gelatin, casein and heat-coagulated egg white. The rate of digestion was slow when

judged by the speed of liquefaction in nutrient gelatin at 22°. In this case the following sequence was observed (bracketed figures refer to days incubation): napiform (2-3), stratiform (7), the latter progressed slowly until about a third of the gelatin was liquefied on the 28th day following inoculation. A similar sequence was seen in 12% gelatin or nutrient gelatin containing glucose (final concentration, 0·5%, w/v). No liquefaction occurred when the surface of nutrient gelatin, either with or without glucose, was covered with either mineral oil or water agar containing reducing substances. Lead acetate paper suspended above peptone water containing a piece of heat coagulated egg white registered the production of H_2S within 48 hr at 27°.

Table 16 summarizes the results obtained from an investigation of organic acid utilization by two strains of this organism.

These organisms had no detectable action on egg yolk, potato slopes or glycerol (tested for acid production and the production of hydroxyacetone) and they did not grow in nutrient broth at pH 4·5. They did not produce any pigment in nutrient agar containing tyrosine. There was no characteristic appearance in the growth which developed on Dorsett's egg medium.

Bacteria of this group were considered to have some properties, particularly in respect to the changes occurring in infected eggs, similar to those of the bacteria which Florian & Trussell (1957) identified with Alcaligenes bookeri. The properties of one of their isolates are set out in Table 17. This study showed that this strain had few properties in common with either these authors' published description or the organisms isolated in this study.

The organisms isolated in this study have been identified with Pseudomonas as defined in Bergey's manual (1957). Attempts to identify at species level resulted in Pseudomonas fragi (Hussong, Long & Hammer, 1937) and Pseudomonas maltophilia (Hugh & Ryschenkow, 1960; 1961) being chosen for comparative studies, the results of which are included in Table 17. These showed that our organisms and Ps. maltophilia shared a number of properties. In particular, the reaction in litmus milk and the pattern obtained when various media were used for studies of carbohydrate utilization. In the latter case, Ps. maltophilia gave a pattern similar to that shown in Table 15. Certain differences are shown in Table 17 but it is considered that the confusion existing in the classification of non-pigmented pseudomonads prevents an assessment of their taxonomic significance. Moreover, it is considered that an unqualified acceptance of Ps. maltophilia as a new and distinct species would be premature in view of the absence of published details obtained from comparative studies with closely related pseudomonads, particularly those maltobionic acid producing strains which Kluyver, de Ley & Rijven (1951) and Bentley & Blechta (1960) examined. In the meantime, the organisms isolated in the present study will be referred to as pseudomonad E.

DISCUSSION

This study has re-emphasized the importance of Gram-negative bacteria in egg infections. From the taxonomic viewpoint, it was noted that the organisms isolated in this study were similar to those isolated by Miles & Halnan (1937) and Haines (1938) - imported South African, stored English and imported Australian eggs -, Alford, Holmes, Vickery & Scott (1950) - stored Australian eggs -, Richard &

Mohler (1950) - German eggs - and Florian & Trussell (1957) - Canadian eggs. Further evidence of this close similarity was also obtained from re-infection experiments, the results of which are discussed at a later stage. The most notable absentees from our group of isolates were Serratia marcescens, which Alford et al. (1950) found in less than 1% of the eggs which they examined, and Pseudomonas aeruginosa, an organism whose occurrence in rotten eggs has been described by Platt & Anderson (1939). It was concluded that the primary object of this study, namely the establishment of a comprehensive collection of the common contaminants of rotten eggs, had been attained.

The available evidence does suggest that these contaminants have a wide distribution in nature and that their selection, presumably by the anti-microbial defence of the white, is not appreciably influenced by geographical factors, methods of poultry husbandry and/or egg marketing procedures.

It has been stated that many of the contaminants are faecal in origin (e.g. Florian & Trussell, 1957), an assertion which is not supported by convincing evidence. The organisms isolated in this study are not considered to be typical of those normally associated with the alimentary canal of the hen. Moreover, the failure of the majority of these to grow at the hen's body temperature suggests that a source other than faeces is the principal depot of contamination. The available evidence does suggest that this is most likely to be soil and water.

It has been customary in recent studies of this type to separate the isolates into two groups depending on the changes produced on re-inoculation into eggs. In general no attempt was

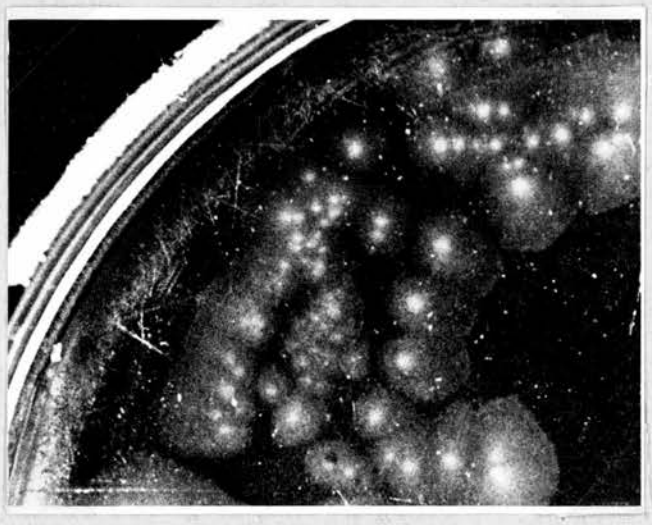
made to correlate these changes with the known metabolic attributes of the organism. Such an attempt was made in the present study but it was quickly realised that the information obtained from conventional re-infection experiments (e.g. Miles & Halnan, 1937 or Florian & Trussell, 1957) must be considered along with evidence originating from more critical examination of the infection process if a complete picture is to emerge. This aspect is considered more fully at a later stage of this thesis.

Plate 1. Morphology of colonies produced on nutrient agar after 96 hr at 27° by strains of Alcaligenes faecalis

A. Strain G36/1

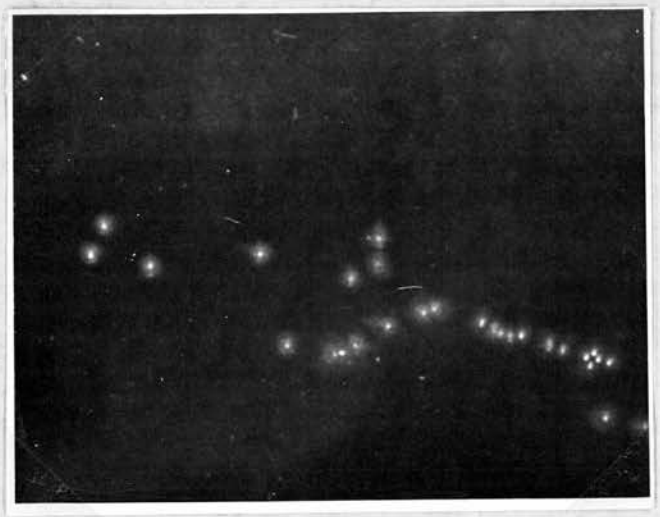
i) A shiny round raised colony with a regular border

ii) A round raised shiny plateau encircled by a flat dull area with an irregular border



B. Strain G21/3

An exaggerated form of Aii. Colonies of type Ai were rarely produced



C. Strain NCIB 8156

Produced colony type Ai and Aii

Magnification X2

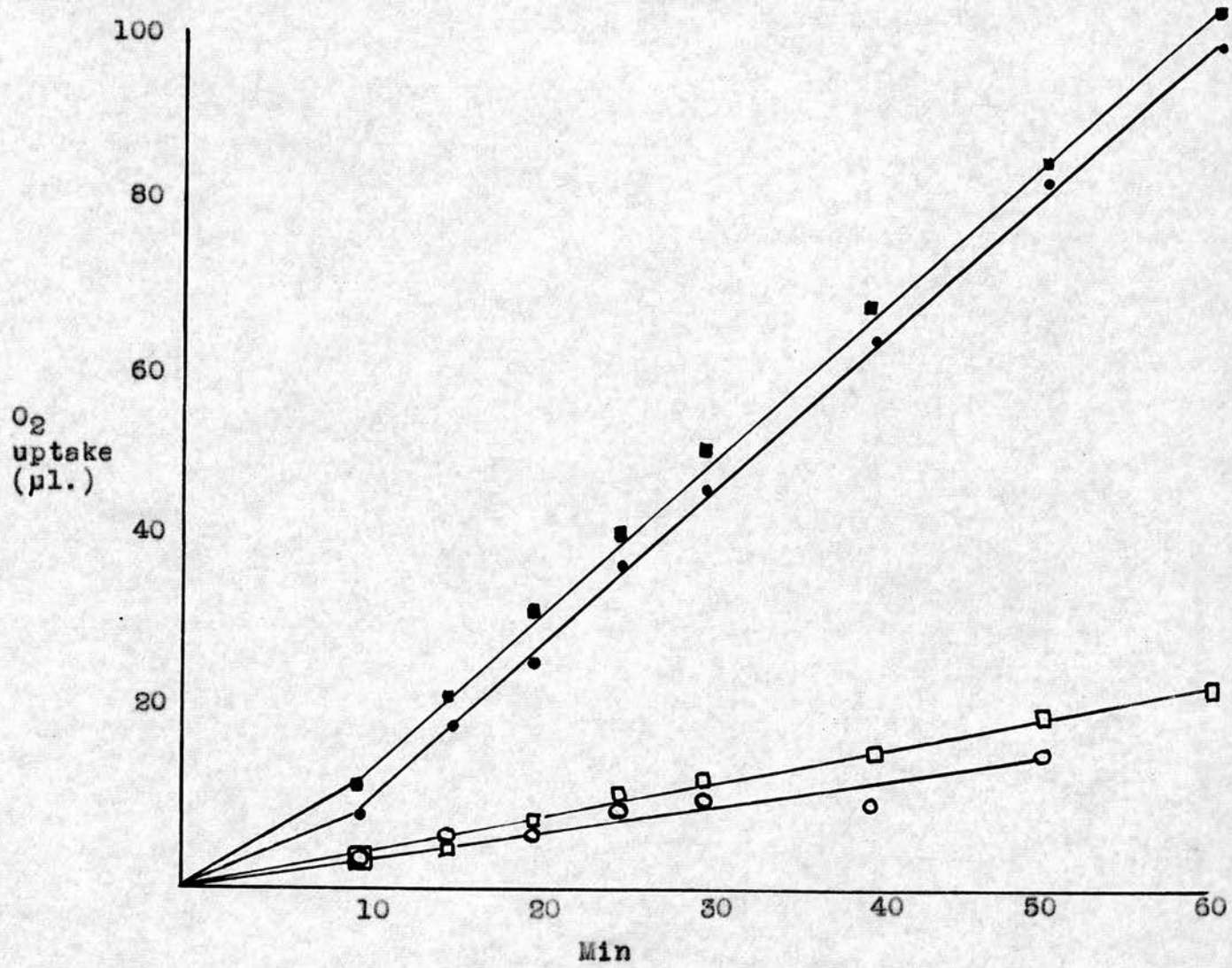


Fig. 3. Graph showing oxygen uptake at 30° by a strain of pseudomonad E (G8/1) in the presence of glucose. Flasks contained cells (equivalent to 1·25 mg dry wt./ml) 1·0 ml; M/15-phosphate buffer, pH 7·0, 1·0 ml; substrate (0·1 M-glucose or M/15-phosphate buffer for endogenous), 1·0 ml; KOH in the central well. An 18 hr nutrient agar culture was tested on glucose ■ , and a control □ ; an 18 hr glucose nutrient agar culture was also tested on glucose ● , and a control ○ .

Table 10.

The preliminary grouping of 226 strains of bacteria isolated from rotten eggs

Gram-positive	Number of isolates
Streptococci	2
Micrococci	4
Rods	2
Gram-negative	
Alcaligenes	52
Non-fluorescent pseudomonads	24
Fluorescent pseudomonads	39
Proteus	15
Coli-aerogenes*	75
Ungrouped	13

*Strains of Aeromonas are included in this group

Table 11

The occurrence of different types of Gram-negative bacteria in rotten eggs from various sources

	Laboratory induced rots			Commercial rots		
	Storage temperature			Scottish		English
	c.10°	20°	30°	A	B	A
Number of eggs	27	19	17	33	23	14
Organisms						
Alcaligenes	5	26	6	7	5	3
Non-fluorescent pseudomonads	10	8	3	9	6	3
Fluorescent pseudomonads	4	5	0	4	5	6
Proteus	0	1	8	3	3	0
Coli-aerogenes*	7	9	7	18	22	12

*Strains of Aeromonas are included in this group

Table 12.

Properties of Gram-negative bacteria isolated from rotten eggs

Obligate aerobes.

Property	Alcaligenes	Non-fluorescent pseudomonad	Fluorescent pseudomonad
Flagellation	Peritrichous	Polar	Polar
Action on glucose	-	O	O
Action on lactose	-	O	-
Hydrolysis of starch	-	-	-
Acetoin formation	-	-	-
Methyl red test	-	-	-
Gelatin hydrolysis	-	+	+6 -3
H_2S production	-	+*	-
Indole	-	-	-
Arginine test	-	-	+
Urea hydrolysis	-	-	-
Deamination of phenylalanine	-	-	-
Oxidase test	+	-	-
Aesculin	-	+	+1 -8
Action on KNO_3	-	-	d
Lecithinase	-	-	+5 -4
Growth temperature (°C)	20 - 30	4 - 30	4 - 30
Number of strains examined	9	10	9

O, acidic substances arising from an oxidative breakdown of the substrate; -, no detectable reaction; +, a positive reaction; +*, a strong positive reaction for H_2S; +', a weak positive reaction for H_2S, and d, several different reactions among the organisms examined (see Table 14)

/ continued .....

Table 12 (continued)

Properties of Gram-negative bacteria isolated from rotten eggs

	Facultative anaerobes			
	Proteus	Coli-aerogenes		Aeromonad
		A	B	
Property				
Flagellation	Peritrichous	Peritrichous		Polar
Action on glucose	F	FR	F	FR
Action on lactose	-	FR 9 / -5	F	FR 4 / -2
Hydrolysis of starch	-	-	-	+
Acetoin formation	-	+	-	+5 / -1
Methyl red test	+	-	+	-
Gelatin hydrolysis	+	+6 / -8	-	+
H_2S formation	+*	+'	+'	+*
Indole	+	-	+1 / -6	+
Arginine test	-	-	-	+
Deamination of phenylalanine	+	-	-	-
Oxidase test	-	-	-	+
Aesculin	+2 / -4	+9 / -5	+2 / -5	+3 / -3
Action on KNO_3	NO_2	NO_2	NO_2	NO_2
Lecithinase	-	+6 / -8	-	+
Growth temperature °C	20 - 40	4 - 30	4 - 30	4 - 30
Number of strains examined	6	14	7	6

F, fermentation; FR, fermentation followed by a pH reversion;

+*, strong reaction for H_2S; +', weak reaction for H_2S, and

-, no detectable reaction.

Table 13.

Utilization of organic acids by alcaligenes

	Strain			
	G21/3	CW5/2	G32/1	Alcaligenes faecalis (NCIB 8156)
Acid				
DL-aspartic	+	+	+	+
L(+)-glutamic	+	+	+	+
Glycine	+	-	NT	+
DL-asparagine	-	+	-	-
L-arginine monohydrochloride	-	+	-	-

No detectable action: DL-alanine; DL-methionine; DL-ornithine; DL-tryptophane; Valine

K acetate	+	+	+	+
Na citrate	+	+	+	+
Na succinate	+	+	+	+
Na lactate	+*	+*	+*	+*
Na formate	+	-	-	-

Slopes of the medium of Board & Holding (1960) containing the sodium salt of the organic acid (final concentration, 0·25%, w/v) were seeded with a dilute cell suspension. +, strong alkaline reaction; +*, strong alkaline reaction and amount of growth markedly greater than that on the controls; -, no difference between the medium containing the organic acid and inoculated basal medium; NT, not tested

Table 14.

Some properties of strains of fluorescent pseudomonads isolated from rotten eggs

Strain no.	Gelatin hydrolysis	Action on KNO_3	Lecithinase	Action on litmus milk
G3/1	+	Reduction gas formed	+	digested
G12/1	+	-	-	digested
CF3	+	Reduction gas formed	+	digested
CF21*	+	Reduction gas formed	+	digested
CF23/1	+	Reduction gas formed	+	digested
ED9/2	+	Reduction gas formed	+	digested
G10	-	-	-	Alkaline
G29/1	-	-	-	Alkaline
CW6/3	-	-	-	Alkaline

* This strain produced a brown pigment on initial isolation but this property was slowly lost during maintenance on laboratory media.

Table 15.

The influence of different media on the demonstration of glucose utilization by a non-fluorescent pseudomonad (G11/1)

Medium (10 ml)	Containing glucose (g)	Reaction after days at 27° 1	2	3	7
Hugh & Leifson (1953)	0	k	K	K	K
	0·05	k	-	-	-
	0·10	k	-	-	a
Hugh & Ryschenkow (1961)	0	K	K	K	K
	0·05	K	K	K	K
	0·10	K	K	K	K
Board & Holding (1960)	0	-	k	k	k
	0·05	-	-	-	A
	0·10	-	-	-	A

-, no detectable changs in the pH; k, weak alkaline reaction;
K, strong alkaline reaction; a, weak acid reaction;
A, strong acid reaction.

Table 16.

The utilization of organic acids by selected strains of a non-fluorescent pseudomonad

Acid	Strain G8/1	Strain G11/1
DL-alanine	+	+
DL-asparagine	+	+
L(+)-glutamic	+	+
DL-valine	+	+
DL-aspartic	+	±

No detectable action on: L-arginine monohydrochloride, DL-methionine, DL-ornithine, DL-tryptophane

Na citrate	+	+
Na succinate	+	+
Na lactate	+*	+*
K acetate	+'	+'
Na formate	+	+

Slopes of the medium of Board & Holding (1960) containing the sodium salt of the organic acid were seeded with a dilute cell suspension. +, strong alkaline reaction; ±, weak alkaline reaction; +*, strong alkaline reaction and the amount of growth markedly greater than that on the control basal medium; +', a strong alkaline reaction developing several days after inoculation.

Table 17.

Comparison of the non-fluorescent pseudomonad with some other bacteria

Species:	Non-fluorescent pseudomonad	Pseudomonas maltophilia	Pseudomonas fragi	Alcaligenes bookeri
Strains:	10 isolates	NCIB 9201, 9202, 9203, 9204.	NCIB 8542	29d
Obtained from:	rotten eggs	NCIB	NCIB	Dr. Garibaldi*
Action on:				
Glucose	a	a	A	-
Fructose	a	a	NT	NT
Maltose	A	A	NT	NT
Lactose	a	a	NT	NT
Arabinose	A	-	NT	NT
Zylose	a	-	NT	NT
Ethanol	a	-	NT	a
Oxidase test	-	-	+	+
Gelatin hydrolysis	+	+	+	-
Arginine test	-	-	+	NT
Litmus milk	No pH change Digestion Soft clot Almond-like odour (strong)	No pH change Digestion Soft clot Almond-like odour (weak)	- (no odour)	Reduction
Nutrient broth:	Granular turbidity	Uniform turbidity	NT	NT

-, no detectable action; a, weak acid reaction; A, strong acid reaction; NT, not tested.

* Dr. Garibaldi obtained this strain from Florian & Trussell (1957).

BACTERIAL MULTIPLICATION ON THE SHELL MEMBRANES IN VITRO

CONTENTS

INTRODUCTION

The literature dealing with the growth of micro-organisms on the shell membranes in vitro has been reviewed on pages 17-19. The report of Stuart & McNally (1943) suggested that the membranes may contain substances that are toxic to the common contaminants of rotten eggs. Subsequent investigations have indicated that this toxicity was most probably due to substances, possibly metal ions, present in the saline in which the membranes were suspended (Stokes & Osborne, 1956; Elliott & Brant, 1957; Garibaldi & Stokes, 1958). In fact, the last mentioned authors, who worked mainly with pseudomonads, found that significant bacterial multiplication occurred in suspensions of disintegrated membranes. Since Noval & Nickerson (1959) have shown that mechanical damaging of keratin can result in changes which allow the growth of organisms that are unable to utilize the native protein, the significance of this multiplication in relation to bacterial infection of the egg cannot be assessed.

Representatives of the various groups of organisms listed in Table 12 together with membranes that had not been subjected to extensive mechanical damage were used in an attempt to obtain further information on the extent of bacterial multiplication on the shell membranes in vitro. In addition, particular attention was given to the possible digestion of these structures by the organisms.

METHODS AND MATERIALS

Organisms. These were stored in nutrient broth in the laboratory. One drop from a capillary pipette of an 18 hr nutrient broth culture (incubated at 27°) in 9 ml of water was used as the inoculum in the experiments discussed hereunder.

Eggs. Naturally clean eggs obtained from a mated flock of white leghorns were used within 4 days of laying.

Colony counts. The method of Miles & Misra (1938) was used. The capillary pipettes were made in this laboratory and their accuracy was checked gravimetrically. Three drops (volume, 0·02 ml) of each dilution were placed on nutrient agar in a Petri dish and incubated at 27°.

Shell membranes. Eggs were cracked across their equators, the contents discarded and the inside of the shells washed with water. Great care was taken to minimize tearing of the membranes during their removal from the shell. The membranes were again washed in water before being held in chloroform at room temperature for 24 hr. This substance was removed in an air-stream generated by a water-pump.

Suspending medium. A medium similar to that of Garibaldi & Stokes (1958) was used. It consisted of an aqueous solution of Sørensen's phosphate buffer (final concentration, M/150) containing the following (mg/l.): $MgSO_4 \cdot 7H_2O$, 200; $ZnSO_4$, 5·0; $MnCl_2 \cdot 4h_2O$, 7·2; $FeSO_4 \cdot 7H_2O$, 10·0; $CaCl_2$, 11·0 and $CoCl_2$, 1·0. The pH, which was adjusted by varying the proportions of the two phosphates used in the buffer solution, was checked with a glass electrode after the medium had been sterilized by holding momentarily at 22 lb/in^2.

Glassware. Selected items of glassware ('Pyrex') were kept separate from the other commonly used laboratory glassware. These items were cleansed by soaking in soap solution and washed many times in water, culminating in distilled water. Cotton wool was used to plug these vessels and they were sterilized by holding momentarily at 22 lb/in^2.

Chemical tests. i) Nessler's reagent. This was used routinely to test

for protein digestion. ii) Ninhydrin. The method of Moore & Stein (1948) was used occasionally to test for protein digestion. Indanetrione hydrate (Ninhydrin; B.D.H., Laboratory reagent) was purified by treatment with charcoal and re-crystallized. The intensity of the colour reaction was determined with a Spekker absorptiometer containing the yellow filter no.606. The values were converted indirectly into absolute units by means of a standard curve prepared with leucine.

RESULTS

It was noted in some preliminary experiments that no inhibition of bacterial growth occurred when pieces of membrane were placed on the surface of nutrient agar immediately after the surface of this had been inoculated. A faint opacity was formed by bacterial growth in those regions of water agar upon whose surface pieces of membrane had been placed immediately after inoculation. This opacity was most easily seen at the edges of the membranes. The amount of growth, as judged by opacity, was not increased by previous exposure of the membranes to conditions designed to transform the proteins by either denaturation or digestion. In such tests the membranes were held in sterile solutions of either N-HCl, N-NaOH, urea, papain or trypsin for 24 hr at 27°, and they were repeatedly washed with sterile water before being placed on the agar.

Table 18 contains the results from an experiment the object of which was to check the membrane suspensions and the suspending medium for toxicity. It will be seen that neither of these had any inhibitory effect on bacterial viability. It was considered that colony counts would provide the most critical method of assessing the influence of shell membranes on bacterial viability. Shell membranes (either half an egg's membranes or 6 pieces from separate eggs which together

approximated the amount present in the former) in 15 ml of suspending medium (pH 7·2) contained in a 50 ml Erlenmeyer flask were inoculated with either 1 or 5 drops from a capillary pipette of a dilution of the organism. An uninoculated membrane was included in every experiment and a further check on the membranes' sterility was provided by inoculating pieces of membrane in nutrient broth and incubating these at 27°. The following observations were made: a) changes in the colony count, b) changes in the appearances of the membranes and suspending medium. In addition, various chemical methods were used to detect protein digestion.

Changes in colony counts. The results obtained from several experiments are summarized in Fig. 4. Bacterial multiplication occurred in the suspending medium in the majority of cases. This multiplication was less than that occurring in suspending medium containing membranes, although these differences were slight with certain organisms (cf Fig. 4e). Although the size of the inoculum did not influence the form of the growth curve, it did, in the majority of cases, determine the final size of the bacterial population. It might be inferred that this was due to nutrilites included with the inoculum. No significant differences between membranes from one egg and the aggregates from 6 eggs were noted. These results indicate that the membranes provide something which is beneficial to bacterial multiplication. This provision could be in the form of either nutrilites or in a modification of the ionic balance and/or pH of the suspending medium or some permutation of these. Information bearing upon these possibilities is discussed at a later stage. Meantime, it can be noted that non-proteolytic (cf Fig. 4a & b) as well as proteolytic bacteria multiplied in the membrane suspensions.

Effect of supplements on colony counts. Strains of pseudomonad E were used in this study. This organism resembles the bacteria which Florian & Trussell (1957) identified with Alcaligenes bookeri. The ability of strains of the latter to digest the shell membranes was noted by Garibaldi & Stokes (1958) The optimum pH of the medium for the multiplication of our strains was 7·2, a value used in all experiments. The type of result obtained is exemplified by the data given in Fig. 5. In the first part of the incubation period the rate of multiplication was increased when the suspending medium containing membranes was supplemented with trace amounts (final concentration, 0·01%, w/v) of either yeast extract, peptone, glucose, aqueous extract of deep litter material, aqueous extract of hens' faeces or pieces of shell. The ash obtained from yeast extract was also stimulatory. Calcium carbonate and calcium phosphate, two of the major components of the egg shell, did not cause stimulation. In fact, the first mentioned substance had a slight depressive effect. By the 8th day following inoculation, about the same number of viable cells were present in both supplemented and unsupplemented membrane suspensions. The supplements did not have any detectable effect on multiplication in the controls.

Changes in the appearance of the membranes and the suspending medium. The results discussed in this section were obtained with whole membranes contained in unsupplemented suspending medium. No detectable changes in the gross structure or organization of the shell membranes were produced by any of the organisms. Loss of colour of the membranes inoculated with certain organisms was noted. The shell membranes of a freshly opened egg are colourless but they become pink on exposure to the atmosphere. The pigment, the chemistry of which is in doubt

(Brooks, pers. comm.), behaves as a pH indicator. An uninoculated membrane retained its colour throughout incubation. Likewise, no loss of colour was detected when membranes were inoculated with Alc. faecalis, Ps. fluorescens or Pr. vulgaris. The colour was sometimes lost from membranes inoculated with a proteolytic strain of Cloaca and was invariably lost when either Aer. liquefaciens or pseudomonad E was used. This loss did not appear to be due to changes in pH since the pH attained in uninoculated membrane suspensions (pH 7·5) was not significantly different from that of inoculated suspensions (pH 7·6 - 8·0). The loss of pigmentation was always associated with a marked increase in the turbidity of the suspending medium. In other words, a marked increase in opacity was noted with membrane suspensions inoculated with either Aer. liquefaciens, pseudomonad E or, occasionally, Cloaca. There was no marked increase in turbidity in membrane suspensions inoculated with either Pr. vulgaris, Ps. fluorescens or Alc. faecalis. The association of these two features appears to depend on the ability of the organism to synthesize proteases.

Digestion of membranes. It was noted in the preceding section that no detectable changes in the organization of the membranes resulted from bacterial growth. Evidence of protein breakdown was therefore sought by chemical methods and Nessler's reagent was routinely used for this purpose. A positive reaction was given by the suspending medium removed from membrane suspensions in which Aer. liquefaciens, pseudomonad E and, occasionally, Cloaca had grown. Negative reactions were given by all the control suspending media, uninoculated membrane suspensions and membranes inoculated with Pr. vulgaris, Ps. fluorescens or Alc. faecalis. Fig. 6 contains typical results obtained when protein

digestion was tested by a method (Moore & Stein, 1948) in which ninhydrin is used to determine the presence of amino acids. The results obtained by this method paralleled those obtained with Nessler's reagent. It will be seen that the release of ninhydrin-reacting substances from the membranes was detectable 1-2 days following inoculation and that this release reached its peak several days later, the subsequent decrease was probably due to the breakdown of amino acids.

Hydrogen sulphide was liberated into the air-space above membrane suspensions which gave a positive reaction with Nessler's reagent.

In general, this picture was not modified when pieces of egg shell were included in the membrane suspensions. In this case, however, proteolytic strains of Cloaca tended to give more positive reactions, and the shell did appear to promote the release of ninhydrin-reacting substances by Aer. liquefaciens.

Production of proteolytic enzymes. Samples were withdrawn daily from both inoculated membrane suspensions and the control suspending medium. These were divided into 2 parts, one of which was tested with Nessler's reagent and the other, after the addition of a drop of chloroform, was placed in holes (7 mm diameter) cut from water agar (1·5%, w/v; pH 7·2) containing gelatin (Cox, Edinburgh; 0·2%, w/v) to which had been added a crystal of thymol at the time of preparation. The Petri dishes containing this medium were incubated at 37°, and after 24 hr gelatin digestion was tested by flooding the agar with acid $HgCl_2$. The results at 120 hr are given in Table 19. It will be seen that a positive reaction with Nessler's reagent was associated with the presence of proteases in the suspending medium. The amount of enzyme present was not enough to give a detectable reaction with azo-casein

(Charnley & Tomarelli, 1947) during incubation for 1 hr at 37°. At the end of the experiments, subcultures were made from the membrane suspension into nutrient gelatin. The latter were incubated at 22° and observed for liquefaction. Alc. faecalis, as was to be expected, was the only organism which failed to liquefy gelatin. All the other bacteria liquefied the gelatin in a way and at a rate which could be predicted from previous observations of this property. In the case of Pr. vulgaris and Ps. fluorescens, this was taken to be evidence that the cells developing in the membrane suspensions were not non-proteolytic variants in the sense of this property being lost at the gene level. It was concluded that these organisms were prevented from forming proteolytic enzymes, or at least those which digest the proteins of the membranes and/or gelatin, because of some inadequacy in the environment. Neither an increase in the concentration of Ca in the suspending medium nor the addition of pieces of shell rectified this inadequacy.

DISCUSSION

The explanation of the multiplication discussed in the foregoing is not clear. The organisms used in this study, which with the exception of Pr. vulgaris and pseudomonad E are of non-exacting nutritional demands, are the types which are known to multiply in nutritionally-inadequate environments such as distilled water or phosphate buffers. Although multiplication generally occurred in our suspending medium this was always less than that occurring in the suspending medium containing shell membranes. This can be accounted for by supposing that the membranes were influencing bacterial multiplication by either a) a modification of the ionic balance of the suspending medium (either by contributing ions to the suspending

medium or by altering the balance of those already present), or b) provision of nutrients. In the former case, the stimulatory effect of the ash of yeast extract does imply that the shell membranes may have made certain ions unavailable to the organisms. Although great care was taken to remove the albumen during the preparation of the membranes, the presence of trace amounts of conalbumin may have been responsible for this effect. If this implication is accepted, it can be concluded that the greater multiplication occurring in the membrane suspensions was at the expense of nutrilites present in these membranes. Although it is known (Ecker & Lockhart, 1961) that the number of viable organisms present in a medium is an unsatisfactory criterion of its initial nutritional quality, it would seem that the membranes contain only small amounts of nutrilites that are readily available to the organisms. This conclusion would appear to be supported by the observation that a marked increase in the rate of multiplication attended the supplementation of a membrane suspension with trace amounts of either yeast extract or peptone. Ecker & Lockhart (1961) found that, within certain limits, cell mass was directly proportional to the initial concentration of a nutrilite in a medium. In the experiments under discussion, there was no notable increase in the opacities of the suspending medium or the suspending medium containing membranes except in the case of membrane suspensions inoculated with Aer. liquefaciens, pseudomonad E or, occasionally, proteolytic strains of Cloaca. It was found, however, that this increase in opacity was associated with the presence of proteases and products of protein degradation in the suspending medium. Whether these organisms were able to produce proteases as a result of the nutrilites present in the membranes or whether this production was influenced by the

composition of the suspending medium cannot be decided. It was concluded, therefore, that the shell membranes *in vitro* can support the growth of the commonly occurring contaminants of rotten eggs. This conclusion is in concord with those of Stokes & Osborne (1956), Elliott & Brant (1957) and Garibaldi & Stokes (1958).

It would be dangerous to make an uncritical acceptance of this conclusion and use it when discussing the roles that the shell membranes may or may not play in the egg's defence. Brooks (1960b) found that the rate of multiplication of pseudomonads in albumen *in vitro* was significantly slower than that in egg white containing shell membranes, the greatest increase occurred in albumen containing membranes from aged eggs. Brooks considered the conclusions of the authors cited above but concluded that this increase in the rate of multiplication was not due to nutrilites present in the membranes. He considered that this conclusion was supported by his observation that the rate of multiplication was not increased by the addition of glycine or ammonium salts to albumen. Instead, he favoured the conclusion that the important contribution by the membranes was iron and, possibly, other metal ions, a conclusion which is not in concord with his views of the changes occurring in the shell membranes in eggs on the 12th day of storage *viz*. "a spontaneous change occurs in the membrane which makes it more susceptible to bacterial attack". It would seem that the conclusion arrived at by Brooks and that arrived at in this study can be reconciled in the following manner. Although Brooks (1960b) does not give information concerning the form of the membranes and the method of their incorporation in the albumen, it would seem reasonable to assume that egg white containing shell membranes would not be a homogeneous medium. It is conceivable that in such a medium,

bacterial multiplication would be confined to the shell membranes and that the white would be contaminated from these loci - this concept of the manner of contamination of the white is in agreement with the sequence of events which Brooks (1960b) found in eggs whose air cells had been inoculated with pseudomonads. The acceptance of this concept, support for which would appear to be given by the analogous situation occurring with the growth of obligate anaerobes in liquid media containing particulate matter - the 'nidus effect' - Knight (1941), allows one to suggest that bacterial multiplication was being aided by iron in the membranes, as suggested by Brooks (1960b), and the presence in the membranes of the nutrilites whose occurrence was implied by the results given above. It would seem, however, that an assessment of the contribution, if any, which these make to bacterial multiplication during the infection of eggs would be best postponed until more information is available concerning the changes that occur in the shell membranes of stored eggs. This aspect is considered in the next part of this thesis.

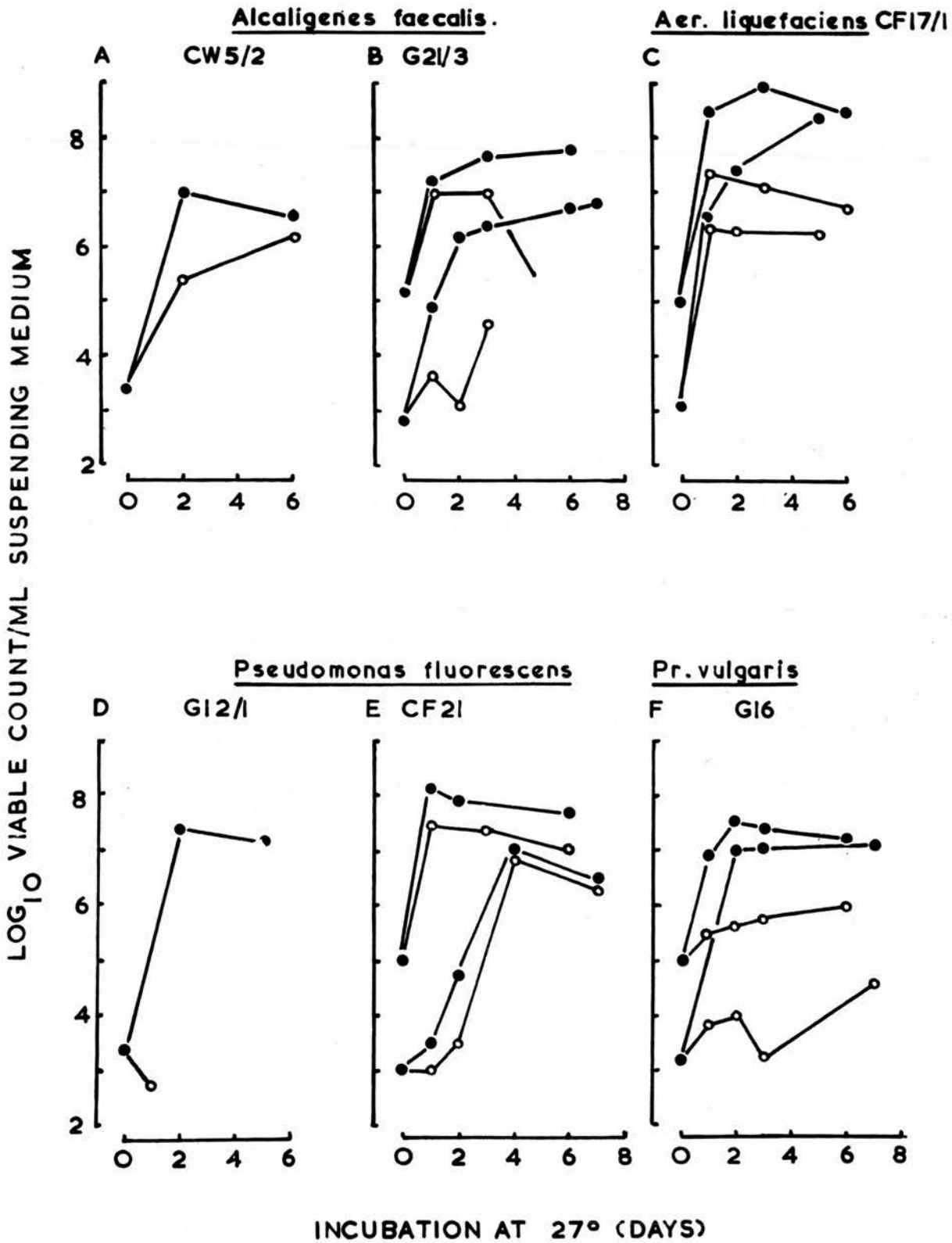


Fig.4. (continued)

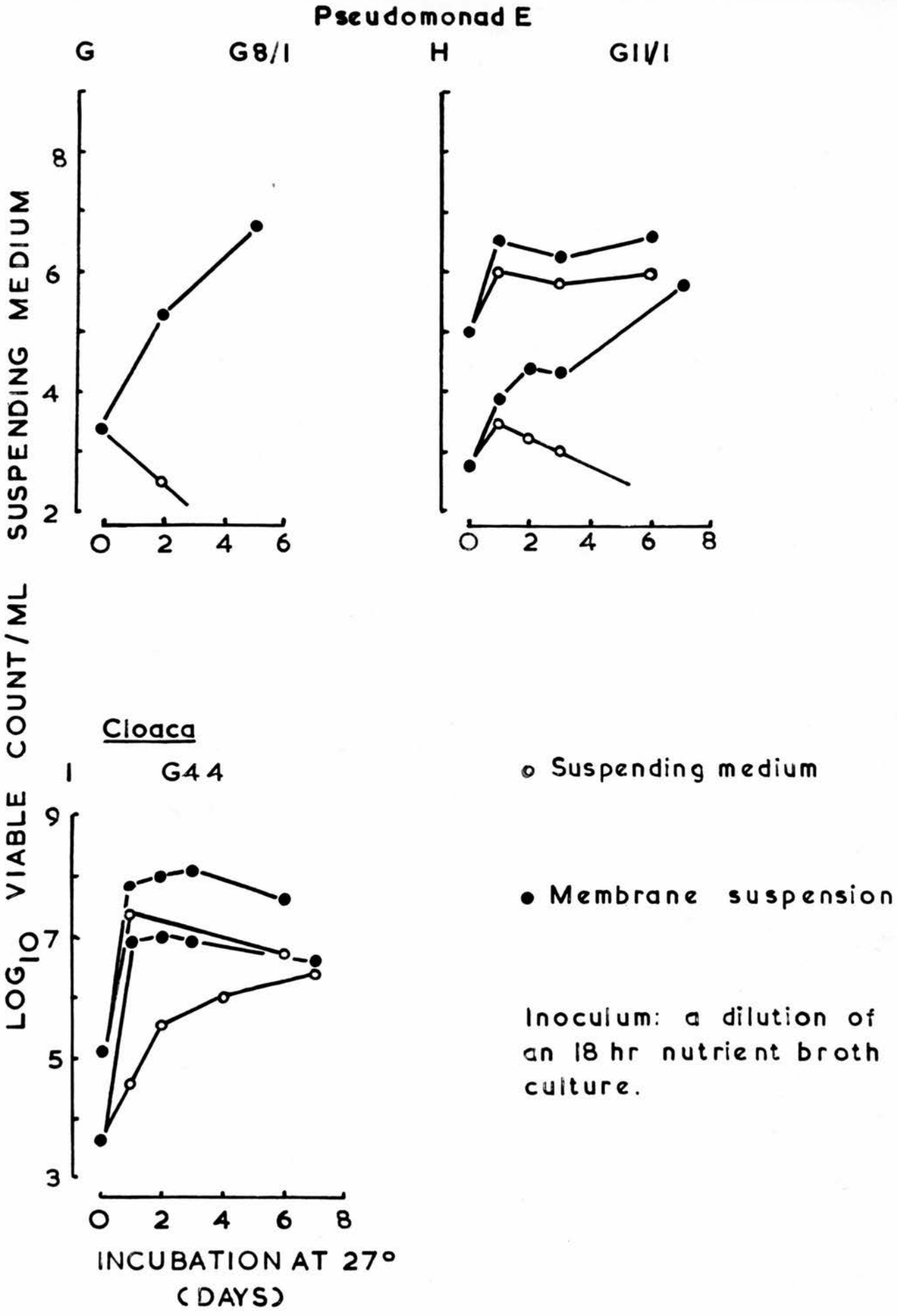


Fig.4. GROWTH OF BACTERIA IN SUSPENSIONS OF SHELL MEMBRANES

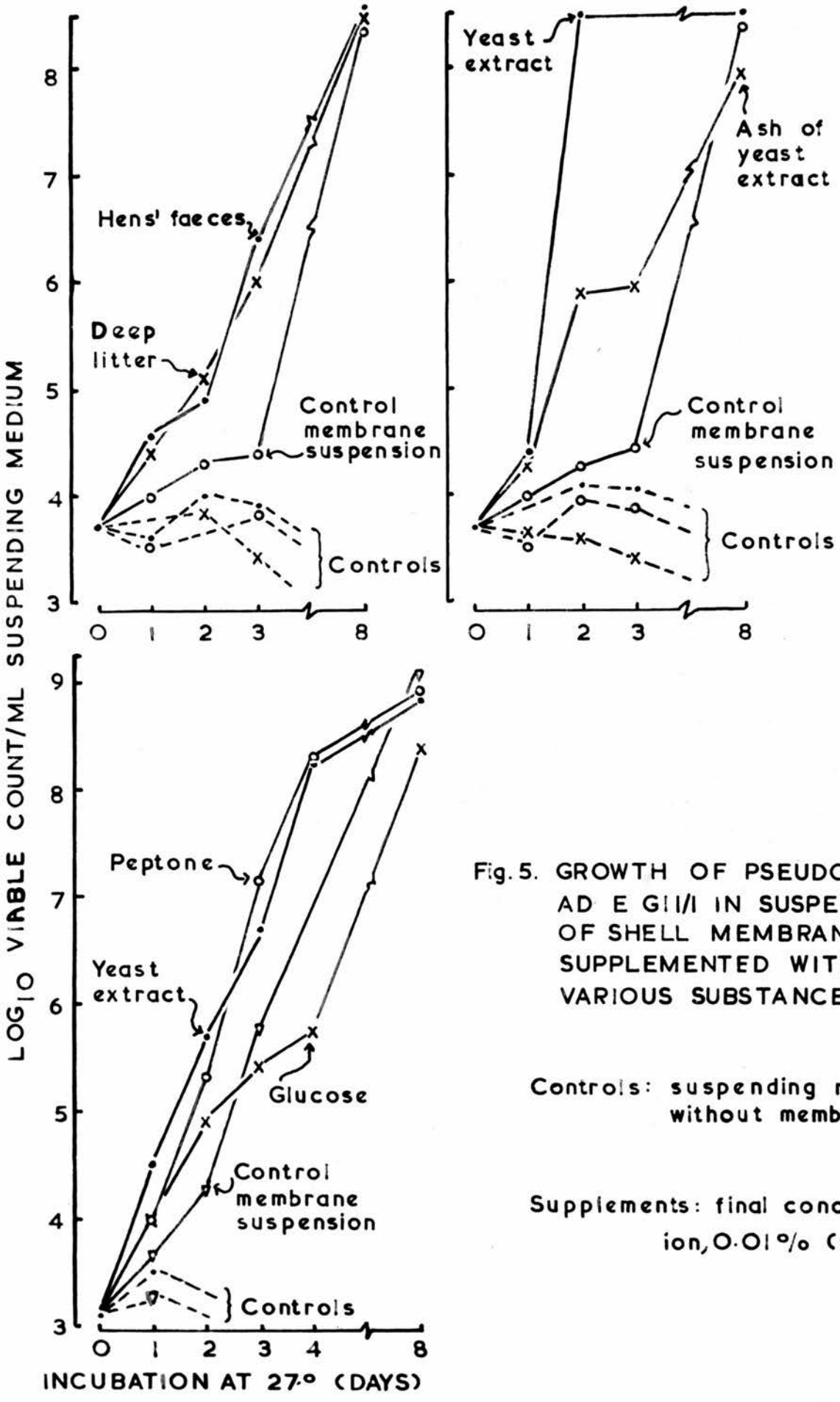


Fig. 5. GROWTH OF PSEUDOMONAD E GII/I IN SUSPENSIONS OF SHELL MEMBRANES SUPPLEMENTED WITH VARIOUS SUBSTANCES

Controls: suspending media without membranes

Supplements: final concentration, 0·01 % (w/v)

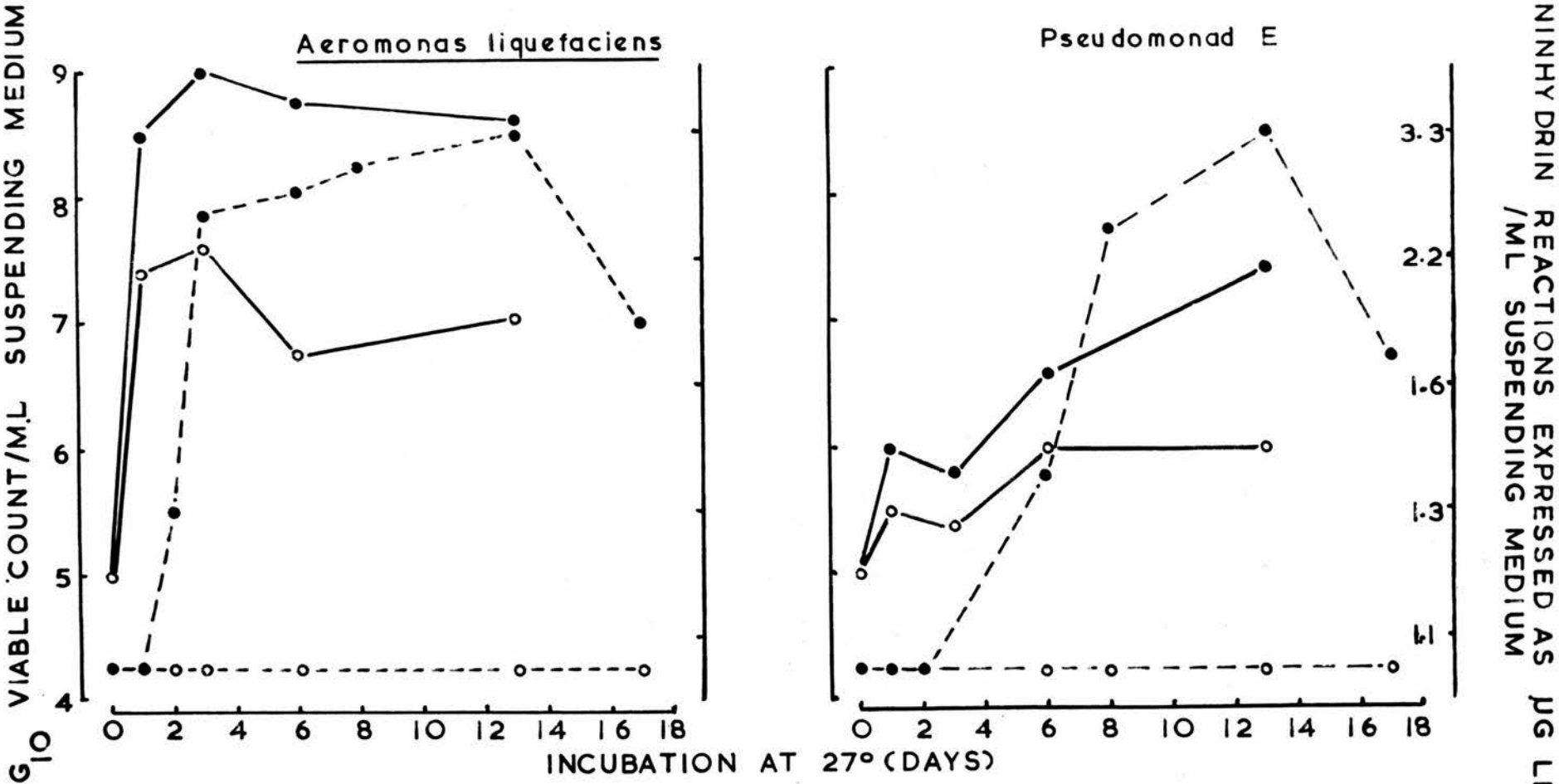


Fig. 6. RELEASE OF NINHYDRIN-REACTING SUBSTANCES FROM SHELL MEMBRANES DURING THE GROWTH OF BACTERIA

●: Membrane suspension (6 pieces from separate eggs which together approximated that of half an egg's shell membranes). o: Suspending medium. ——: Viable count. – – –: Ninhydrin-reacting substances. Uninoculated membrane suspensions did not give a measurable reaction with ninhydrin.

Table 18.

Survival of bacteria in suspensions of shell membranes

Organism	No. of strains	Suspending medium plus membrane			Suspending medium		
		Days incubation at 27°			Days incubation at 27°		
		3	14	33	3	14	33
Alcaligenes faecalis	2	+	+	-	+	+	-
pseudomonad E	2	±	±	+	±	-	-
Pseudomonas fluorescens	2	+	+	+	+	+	+
Proteus vulgaris	2	+	+	+	+	+	+
Cloaca							
non-proteolytic	1	+	+	+	+	+	+
proteolytic	4	+	+	+	+	+	+

Viability tested by streaking a loopful of the suspending medium on nutrient agar. +, confluent growth along the streak; ±, isolated colonies along the streak; -, no growth.

Table 19.

Production of proteolytic enzymes by bacteria growing in suspensions of shell membranes

Organism	Suspending medium				Suspending medium plus membranes			
	Reaction with Nessler's reagent		Proteolytic enzymes		Reaction with Nessler's reagent		Proteolytic enzyme	
	Incubation (hr)		Incubation (hr)		Incubation (hr)		Incubation (hr)	
	0	120	0	120	0	120	0	120
Alcaligenes faecalis	-	-	-	-	-	-	-	-
Pseudomonas fluorescens	-	-	-	-	-	-	-	-
Proteus vulgaris	-	-	-	-	-	-	-	-
Cloaca	-	-	-	-	-	±	-	7·5
Aeromonas liquefaciens	-	-	-	2·0	-	+	-	7·5
pseudomonad E	-	-	-	-	-	+	-	10·0
Uninoculated controls	-	-	-	-	-	-	-	-

-, no detectable reaction; ±, weak reaction with Nessler's reagent; +, strong reaction with Nessler's reagent; Numerals, width (mm) of clearing of gelatin around holes (7 mm diameter) cut from water agar (1·5%, w/v) containing gelatin to which had been added thymol and chloroform.

THE COURSE OF BACTERIAL INFECTION OF THE HEN'S EGG

CONTENTS

INTRODUCTION

The literature dealing with the course of bacterial infection of the hen's egg has been considered on pages 43 - 47 . A number of investigators (Gillespie & Scott, 1950; Elliott, 1954; Stokes et al. 1956; Fromm & Monroe, 1960) have presented evidence, derived mainly from empirical observations, which suggests that the anti-microbial defence becomes exhausted 10 - 15 days following the infection of a newly laid egg. Brooks (1960b), who examined the course of infection in eggs whose air cells had been inoculated with pseudomonads, obtained evidence which indicated that bacterial multiplication was confined to the shell membranes during the initial phase of infection and that the white and yolk were contaminated with migrants from this source. The rate of multiplication during the initial phase was slow when compared with that occurring in a second phase whose beginning was determined by the age of an egg at the time of inoculation. In the case of newly laid eggs, this second phase began on about the 12th day following inoculation but this period was markedly reduced if the eggs were stored for 1-7 days prior to inoculation. This second phase of multiplication was associated with a heavy contamination of an egg's contents and the first macroscopic signs of infection of the yolk and white. Brooks considered that this change was due to a spontaneous change in the properties of the shell membranes. The nature of this change awaits elucidation.

The object of the work about to be described was to gain a clearer understanding of the changes which result in the apparent exhaustion of the anti-microbial defence of an infected egg. Bacteriological and chemical methods were used to trace the course of infection in

eggs whose air cells had been inoculated with a dilute cell suspension.

METHODS AND MATERIALS

Eggs. These were produced by a mated-flock of White leghorns. The hens were fed a layers' ration and they were kept on deep litter. Eggs produced during one day were used in any one experiment and they were stored at room temperature prior to inoculation. All the eggs were candled and those of poor internal quality were discarded.

Organisms. The properties of the organisms used in this study are listed in Table 12. The organisms were stored in nutrient broth at room temperature. An 18 hr nutrient broth culture was used in all experiments. The cells were harvested by centrifugation and twice washed with Sørensen's phosphate buffer (pH 7·2; M/15) and resuspended in distilled water.

Bacterial penetration of the shell and shell membranes. The methods used were based on those described by Garibaldi & Stokes (1958). The apparatus is depicted in Fig. 7. There were differences, however, in the preparation of the shells. In the present study, the surface of the shell was wiped with 70% ethanol, the pointed end of the shell removed and the contents discarded. The shell, held in a pair of sterile forceps, was again wiped with ethanol before being placed in a crystallizing dish containing 15 ml of an 18 hr nutrient broth culture. Nutrient broth (15 ml) was introduced into the inside of the shell and incubation at 27° followed. Penetration of the shell and shell membranes and purity of the culture were tested at 24 hr intervals by streaking a loopful of the broth from within the shell on nutrient agar and incubating at 27°. Each bacterium was tested with a minimum of three eggs.

Inoculation and sampling of eggs. Method 1

The technique is illustrated in Fig.8. The shell above the air cell was swabbed with 70% ethanol. A carborundum-disk was used to drill 3 channels in the shell above the air cell (the 3 joined to form a triangle) but the outer shell membrane was not perforated. A glass ring (internal diameter, 15 mm; height, 7 mm) was cemented (paraffin wax) onto the shell surrounding the triangle. The inside of the ring and the surface of the shell were wiped with a burning pledget of cotton wool containing ethanol. The triangular shaped piece of shell was removed and, with the egg resting on its side, the outer membrane was removed from the area freed from shell. A vaseline-coated coverslip was sealed on the top of the glass ring. If the contents of the egg were removed, the shell was rinsed with sterile distilled water and filled with water agar (pH 7·2; agar previously washed with water). This proved to be the most satisfactory method of preventing the desiccation of the shell membranes. Pieces of filter paper (Whatman No.1; diameter, 6 mm) soaked in an appropriate dilution of an organism were placed on the inner surface of the air cell. Four of these disks would fit into the air cell of a 3 or 4-day old egg. A disk was removed after a suitable period of incubation and comminuted by shaking in a screw-capped glass vial containing 6 glass beads and 2 ml of water. One millilitre of this was used in subsequent manipulations.

Inoculation and sampling of eggs. Method 2.

Inoculation. The outline of the air cell was marked (with a lead pencil) on the shell at the time of candling. A carborundum-disk was used to drill a hole in the shell, previously swabbed with 70% ethanol. The needle of a Record-type hypodermic syringe was pushed

through the shell membrane and 0·1 ml of a bacterial suspension or, in the case of controls, 0·1 ml of the suspending medium was expelled from the syringe. The needle was positioned roughly tangentially to the shell during its introduction into the air cell. The direct evidence which was used to ascertain the inner membrane's freedom from perforation will be discussed at a later stage. The hole in the shell was sealed with sterile vaseline (c. 80° at the time of application) and the eggs were held in Keyes trays during incubation at 27°.

Sampling. The eggs were candled and particular attention was paid to: a) the freedom of movement of the yolk, and b) the presence of a "running air cell"; this phenomenon occurred when the inner membrane of an air cell had been punctured with the hypodermic needle and eggs showing this condition were discarded. The shells were swabbed with 70% ethanol, the pointed end of the shell was fractured with a sterile scalpel and the contents of the egg were poured into a Petri dish. The contents of the egg and the inside of the shell were inspected and, when appropiate, examined under ultra-violet irradiation. The whole of the white was transferred to a 50 ml Erlenmeyer flask containing 10 glass beads and it was shaken for 5 min with a "Microid" flask shaker (Griffin & Tatlock Ltd., London). Serial decimal dilutions (diluent, 1/4-strength Ringer's solution) were prepared from 1 ml of homogenized albumen and samples from these were either included in nutrient agar containing 0·05% (w/v) iron citrate (1 ml diluent: 10 ml medium) or inoculated onto nutrient agar by the method of Miles & Misra (1938). The Petri dishes were incubated at 27° for 1 week.

The shell membranes were freed from albumen by repeated flushings with sterile water; in the case of eggs in advanced stages of rotting, the removal of material from the inner membrane of the air cell called for extensive flushing. This operation also gave information on: a) the efficiency of the sealing of the injection-port; if this was improperly sealed, the air cell collapsed under the weight of the water, and b) changes in the water-retaining property of the inner membrane of an air cell. The diameter of the air cell was measured with a pair of screw-adjusted callipers. The inner membrane was excised along its boundary of contact with the outer shell membrane. This disk of membrane was ground to a paste with a pestle in a mortar containing sand (the pestle and mortar were sterilized with formaldehyde and flushed with sterile water). The paste was suspended in 9 ml of 1/4-strength Ringer's solution (this suspension was regarded as a 10^{-1} dilution in all subsequent manipulations) and serial decimal dilutions were prepared in the same medium. Standard volumes (0·02 ml) of these were inoculated on nutrient agar by the method of Miles & Misra (1938). Incubation was at 27° until the size of the colonies was optimal for enumeration.

Chemical methods. The pH of an albumen was determined with a glass electrode. The amount of free glucose in the albumen was determined in the following manner. Ten millilitres of albumen were slowly added to 25 ml of absolute ethanol. The coagulum was disintegrated with a glass rod and stored at room temperature for 24 hr. The coagulum was removed by filtration and the filtrate was made up to 50 ml in a volumetric flask. Two 5 ml amounts of this were used to determine the free glucose by the method of Somogyi (1945).

RESULTS

Preliminary experiments

Bacterial penetration of the shell and shell membrane. The effect of albumen remaining in the egg shell on bacterial growth and the efficacy of the method of sterilizing the shell were investigated in preliminary experiments. No differences in the rate or amount of growth (visual estimation) were detected when growth in nutrient broth was compared with that in nutrient broth (5 ml) containing albumen (0·2 ml). Spore forming bacilli grew when ethanol-treated shell were incubated in nutrient broth. In consequence, all growth arising in tests made with the common contaminants of rotten eggs was scrutinized and, when necessary, examined by other methods.

Fig. 18 contains a summary of the results obtained from an investigation of bacterial penetration of the shell and shell membranes. It was found that the time taken for different shells to be penetrated by the same organism varied. This is illustrated by the results set out in Table 21. Moreover, the rate of penetration of a number of shells by various strains of the same bacterium varied. This variation will be seen in Fig. 18. It would seem that the rate of penetration is governed by several factors. The differences in the rate of penetration of different shells by the same bacterium may have been due to variations in the thickness of the membranes. Such variation was frequently noted in subsequent work but its importance in the subject under discussion was not tested. No success attended attempts to correlate the different penetration rates with any known properties of the organisms. All the organisms were motile but the speed of penetration did not appear to be influenced by the type of flagellar arrangement. In addition, proteolytic activity did not

appear to be important. This is clearly seen in the results set out in Fig. 18. For example, the non-proteolytic Alc. faecalis behaved in a manner similar to that of the pseudomonad E, an organism which digests the membranes.

The technique used in the experiments described above gives a very inadequate simulation of the conditions which could be expected to operate in practice. This criticism was stressed by Richard & Mohler (1950) in the course of their review of the many investigations in which the above type of experiment had been used. Consequently, attention was turned to investigations using whole eggs. Meantime, however, it can be noted that the shell and shell membranes impose a temporary barrier to bacterial penetration and that this does not appear to be destroyed by bacterial proteases.

Bacterial growth in nutrient agar containing albumen. The results set out in Table 22 show that the addition of albumen to nutrient agar can inhibit bacterial growth. This was overcome by the addition of iron citrate (final concentration, 0·05%, w/v) to the medium. It was concluded that this inhibition was due to the organic ligand, conalbumin; the influence of this substance on the growth of other common contaminants of rotten eggs is discussed at a later stage. As a result of this observation, nutrient agar supplemented with iron citrate (0·05%, w/v) was used when testing albumen for bacterial contamination.

Resistance to the lytic action of lysozyme. Fig. 9 illustrates the change in opacity which followed the addition of lysozyme (final concentration, 5 μg/ml) to an 18 hr nutrient broth culture of Micrococcus lysodeikticus (NCTC 2665). The opacity of an 18 hr nutrient broth culture of any of the organisms discussed in the first

section was not detectably changed by the addition of lysozyme. Moreover, a small inoculum (a 4 mm loopful of an 18 hr nutrient broth culture) of any of these organisms initiated growth in nutrient broth containing lysozyme (final concentration, 5 μg/ml). It was concluded that none of the organisms used in this study was sensitive to the lytic action of lysozyme.

Bacterial growth in filter paper resting on the inner shell membrane.

The inner membrane at the air cell end was inoculated with an appropriate dilution of bacteria contained in disks of filter paper. The eggs were incubated at 27°. Four disks were placed in each air cell and at daily intervals one or more disks were used to determine changes in the number of viable bacteria. This method was developed with the object of obtaining information concerning the behaviour of organisms placed on the inner shell membrane of whole eggs.

In preliminary experiments the behaviour of bacteria was examined when filter paper disks were incubated on inert materials at 27°. Inoculated disks were placed on either water agar (1·5%, w/v; pH 7·2; the agar previously washed with water) or collodion membranes supported on filter paper (Whatman, No. 17) moistened with Sørensen's phosphate buffer (pH 7·2; M/15). The number of viable cells was determined at 24 hr intervals. The results presented in Fig. 10 include those obtained with a nutritionally non-exacting organism, Ps. fluorescens, and others obtained with Pr. vulgaris, an organism which does not grow on a glucose ammonium salts medium. It will be noted that significant multiplication occurred and that this was not prevented by previous washing of the cells. In addition, there were differences in counts given by different disks removed from the medium at the same time.

This variation has a bearing on the results of subsequent work on shell membranes. The experiments also demonstrated that the colony counts were not adversely affected by the cellulose fibres produced by comminution of the filter paper. For instance, no marked association of bacterial colonies with cellulose fibres was apparent when drops from the 2 ml of water in which the filter paper was comminuted were placed on nutrient agar. In fact, deformation of colony form was rarely noted even when bacterial growth occurred on or near to heavy deposits of cellulose fibres. The satisfactory nature of colony counts obtained with successive decimal dilution provided conclusive evidence that the cellulose fibres did not interfere with the counting technique.

Response of small inocula placed on the inner shell membrane. The results obtained with Ps. fluorescens G12/1, Cloaca G75/2 and Pr. vulgaris G16 are presented in Fig. 11. The results obtained with the following organisms were essentially similar to those shown in Fig. 11 (bracketed figures refer to the number of organisms tested): Ps. fluorescens (1), Cloaca (2), Aer. liquefaciens (1), pseudomonad E (2), Pr. vulgaris (2) and Alc. faecalis (1). It will be seen from Fig.11 that significant bacterial multiplications occurred in disks of filter paper resting on the inner membrane in situ. Moreover, it will be seen that bacterial multiplication was not increased by storing the eggs for 14 days prior to inoculation. A noteworthy feature of these experiments was the fact that the populations occurring in filter paper disks resting on the shell membranes of intact eggs were normally greater than those developing in eggs in which the contents had been replaced with water agar. This is illustrated by the results set out in Fig. 11A and 11B. This observation suggested that the albumen was assisting bacterial multiplication in the filter paper disks. Further

evidence of this assistance was obtained when Cloaca G75/2 was used. This organism forms a viscous substance during growth in a number of commonly used media. This substance was formed when the organisms grew on the inner membrane of intact eggs but not on the membrane of eggs whose contents had been replaced with water agar. The presence of this substance caused the filter paper to adhere to the membranes and the removal of the disks without rupturing the membranes called for the greatest care. Further evidence of the influence of the albumen was obtained from experiments in which strains of Ps. fluorescens were used. The contents and the membranes of eggs inoculated with these organisms were examined under ultra-violet irradiation. Marked fluorescence was generally associated with the membrane and albumen of intact eggs but it was never seen in the membranes of eggs whose contents had been replaced with water agar. This information suggested that the conditions obtaining in the filter paper disks were unlike those which Brooks (1960b) considered to exist in the membranes themselves. Moreover, the observation that the storage of eggs did not benefit multiplication was in marked contrast to that of Brooks (1960b). These differences, together with the fact that the behaviour of an organism could not be studied for more than 96 hr (i.e. a filter paper disk sampled every 24 hr), were responsible for the adoption of the technique of Elliott (1954) for further investigations of the course of bacterial infection in eggs. Meantime, the results discussed above indicate that a number of commonly occurring contaminants can grow in filter paper disks resting on the inner shell membrane in situ.

The pattern of bacterial multiplication in eggs

A dilution (1/100) of washed cells obtained from an 18 hr nutrient

broth culture was used to inoculate the air cells (0·1 ml/air cell) of 3 or 4-day old eggs and the course of infection was followed by bacteriological and chemical methods.

A proteolytic (CF21) and a non-proteolytic strain (G10) of Ps. fluorescens were used in attempts to repeat the observations of Brooks (1960b). The inoculated eggs were incubated with their air cells uppermost at 27°. At daily intervals 10 eggs were randomly selected for bacteriological examination.

Changes in the viable counts in the inner membrane of the air cell. The sequence of events observed in this investigation is exemplified by the data presented in Fig. 12. For comparative purposes the viable counts were calculated as the number of organisms/10 mm (diameter) disk of membrane. It will be seen that there was a significant increase in the size of the populations in the 48 hr following inoculation. The size of the populations became more widely scattered during the next 3 days, but a consideration of the mean count for each day does suggest that the populations declined in size after a short period during which the size appeared to be static. This situation was disrupted on the 7th day (i.e. when the eggs were eleven days old) at which time the eggs fell into 2 types: a) those in which there appeared to be a slight reduction in the size of the populations and in which there was no visible evidence of infection of the eggs' contents, and b) those in which there was a marked increase in the size of the populations in the air cell membrane and in which there was fluorescent green pigment in the membrane and, normally, in the contents of the eggs. It will be seen from the data presented in Table 23 that this pigment was normally present on the surface of

the yolk. It is noteworthy that this was always either on or in close proximity to the blastoderm. The occurrence of large bacterial populations in the inner membrane of the air cell was generally associated with gross contamination of the albumen (Table 23). A discussion of the loss of the water retaining-property of the inner membrane (see Table 23) will be reserved for a later stage.

Changes in the viable counts in the albumen. The data set out in Table 24 show that viable organisms were recovered from only a few of the albumen in the 5 days following inoculation. Since only 1 ml of albumen was tested, these results only indicate freedom from gross contamination. Some of the albumen contained large numbers of organisms on the 8th and subsequent days of incubation. It will be seen from Table 23 that, with the exception of egg no. 10 on the 7th day following inoculation, this was always associated with the presence of fluorescent green pigment in the membrane and, generally, on the surface of the yolk. It will be seen, however, that the presence of this pigment in the membrane was not always associated with either detectable or gross contamination of the albumen.

Changes in the internal properties of the eggs. The following changes were observed when the eggs were candled: a) a progressive enlargement of the air cell, and b) a progressive increase in the freedom of movement and buoyancy of the yolk. Useful indices of the latter were provided by the speed and the extent of the gyration of the yolk on turning the egg. Both these changes reached their maxima on the 7 - 10th days of incubation at which time, also, the yolk finally came to rest in the uppermost part of an egg. This meant that on the 7th and subsequent days of incubation, the yolks were in close proximity to the inoculated membranes. These changes in the movement of the yolk were also associated with a

progressive decrease in the quality and definition of the thick albumen, properties that were observed in the broken-out egg.

The changes in the internal properties of the egg suggested that the sudden increase in the size of the bacterial populations in the inner membrane of an air cell resulted from a contact between the yolk and the inoculated membrane. This supposition was given support by the observation that the first signs of infection of an egg's contents were normally associated with the blastoderm. It is known that the yolk, as a result of its gravimetrical asymmetry, tends to a resting position in which the blastoderm is uppermost. Differences in the rate of ascent of the yolks, different rates of increase in the size of the air cells and the fact (as shown by injection of a dye) that the inoculum was normally eccentrically positioned in the air cell were reasons which could account for the sudden increase, in the size of the populations in the inner membrane of the air cell being scattered in the way that is illustrated in Fig. 12.

Influence of the yolk on bacterial multiplication. An attempt was made to correlate the sudden increase in the size of the populations in the inner membranes with the deterioration of some property of the albumen. Experiments similar to that described above were carried out and the deterioration of the quality of the albumen was assayed by several methods. These were chosen from those discussed by Wesley & Stadelman (1959). No success attended these experiments and it was considered that this was due to the lack of precision of the methods used to assay the deterioration of the quality of the albumen. Consequently, the following method was used. The air cells of 10 dozen 3-day old eggs were inoculated with 0·1 ml of a 1/100 dilution of a washed cell suspension. Half the eggs were incubated with their air cells uppermost whereas the

remaining eggs were incubated in the reverse position. At frequent intervals 5 eggs were randomly selected from each group and, after candling, colony counts were made on the inner membrane of the air cells and the albumen.

The results obtained with Ps. fluorescens CF 21 are set out in Fig. 13 and Table 25. It will be seen from Fig. 13 that in those eggs in which the air cells were uppermost the sequence of events was essentially similar to that discussed above. It is noteworthy that the inner membrane of the air cells and the albumen of all the eggs contained large numbers of organisms on the 12th and subsequent days of incubation. A different pattern was given by the eggs that were incubated in the reverse position i.e. eggs in which the yolk moved away from the inoculated membrane. It will be seen from Fig. 13 that the initial increase in the size of the populations was followed by a period (9 - 19 days) during which there was a decline in the size of the populations. Moreover, on the 9 - 16th days of incubation the albumen was not detectably contaminated. On the 19th, 23rd and 30th days following inoculation, there was an increase in both the incidence and magnitude of contamination of the albumen and an increase in the size of the populations in the inner membrane of some of the air cells. The results obtained during this period are summarized in Table 26 from which it will be seen that the contamination of an albumen was not always associated with visible evidence of infection in the contents of an egg. It was noteworthy, however, that when fluorescent green pigment did occur, it was always associated with the yolk, particularly that part which was in contact with the shell membranes.

The above type of experiment was repeated with organisms other than

pseudomonads. The results obtained with Aer. liquefaciens CF10/1 are given in Fig. 14 and Table 27 and those obtained with other organisms are given in Table 28 (Cloaca G75/2), Table 29 (pseudomonad E), Table 30 (Alc. faecalis) and Table 31 (Aer. liquefaciens CF17/1). It will be seen that all these organisms gave a sequence of events which, with minor exceptions, was essentially similar to that described above. The exceptions are discussed in the following section. As a result of these experiments it was concluded that there were two phases of multiplication during the course of bacterial infection of an egg.

Primary phase of bacterial multiplication. This occurred during the 48 hr following the inoculation of the membrane. The low incidence of contamination of the albumen did imply that the multiplication was confined to the shell membranes and that migrants from this locus were unable to multiply in the albumen. Evidence in support of this assumption was obtained in the following experiments. The air cells of eggs were inoculated with 0·1 ml of an 18 hr nutrient broth culture. Eggs were randomly selected at frequent intervals and colony counts made on the inner shell membrane of the air cells and the albumen; the H-ion concentration of the albumen was measured with a glass electrode and the concentration of free glucose was determined by the method of Somogyi (1945). Some of the results obtained with Aer. liquefaciens are set out in Table 32. It will be seen that the large inoculum used in this experiment resulted in large bacterial populations in the shell membranes and gross contamination of the albumen from an early stage in the infection. Nevertheless, changes in either the H-ion or glucose concentration were found only in albumen of eggs in which there were definite signs of infection on the surface of the yolk. This took

the form of a patch of custard-like material on or near to the blastoderm. Results similar to these were obtained with a proteolytic (CF21) and a non-proteolytic strain (G10) of Ps. fluorescens. These results indicated that the migrants which accumulated in the albumen during the primary phase of multiplication were unable to utilize the glucose present in the albumen. It could be inferred that this failure to utilize the most freely available and plentiful energy source of the albumen was responsible for the non-multiplication of the primary invaders of the albumen.

The results set out in Fig. 15A were obtained from an experiment in which the multiplication in the shell membrane of pseudomonad E G11/1 suspended in distilled water was compared with that of the same organism suspended in a deep litter infusion (0·5 g deep litter: 100 ml of distilled water). It will be seen that the inoculum suspended in a deep litter infusion multiplied to a greater extent than did the inoculum suspended in water. The experiment was repeated but in this case the organism was suspended in either a deep litter infusion which had been extracted with 8-hydroxyquinoline (0·5 g) dissolved in chloroform (100 ml) or this medium containing $FeSO_4$ (100 µg/ml). It will be seen from Fig. 15C that the presence of $FeSO_4$ increased the extent of bacterial multiplication in the inner membrane of the air cell. Moreover, it was found (Fig. 15B) that the addition of $FeSO_4$ (100 µg/ml) to a 0·5% (w/v) solution of yeast extract (Difco), in which the concentration of metal ions had been reduced by extraction with 8-hydroxyquinoline, also increased the extent of the primary phase of multiplication. Results (Fig. 15D, E and F) similar to those discussed above were obtained with Ps. fluorescens CF21. The amount

of $FeSO_4$ (10 µg) introduced into the air cell was about 1/2,500th of the amount which would be required to saturate the binding-potential of the conalbumin present in the white. It was noted, however, that the $FeSO_4$ produced a salmon-pink discolouration in the inner shell membrane and that the intensity of this discolouration did not change in the course of incubation. This observation indicated that the iron was not being removed by the conalbumin present in the albumen. It was concluded that, in the case of the washed cell suspensions used in the experiments discussed above, the extent of the primary phase of multiplication was controlled by the availability of iron in the shell membranes.

On one occasion (Table 23, 7th day of incubation, egg no. 10) a bacterial population of above-average size was present in the inner membrane of the air cell of an egg in which there were no visible signs of infection other than the loss of the water-retaining property of the membrane. This feature, which is discussed at a later stage, is considered to be evidence of bacterial action on some structural component(s) of the shell membrane. This observation implies that bacterial utilization of these can, under certain unknown conditions, allow the extent of growth of the primary phase of multiplication to exceed that which normally occurs.

The primary phase of multiplication was followed by a phase during which there was a reduction in the size of the bacterial populations in the inner membrane of the air cells. This was most clearly seen in eggs in which the yolk moved away from the site of the inoculum. Moreover, the extent of this reduction appeared to be determined by some inherent property of an organism. For example,

Cloaca G75/2 (Table 28) showed a most pronounced decline whereas Aer. liquefaciens CF 17/1 (Table 31) did not. Although insufficient work has been done on this aspect, it would seem reasonable to assume that this decline resulted from: a) a slowing down of bacterial multiplication, b) migration of the organisms from the membrane to the albumen, and c) death of some of the organisms present in the membranes. The latter possibility was suggested by the results obtained with pseudomonad E. It was found that on the 14 - 23rd day of incubation the formation of colonies by the organisms in the membranes of eggs in which the yolk moved away from the site of the inoculum required a longer period of incubation than did the organisms present at the beginning of the experiment (48 hr compared with 24 hr for colony formation). It was inferred that the organisms had been rendered moribund through starvation.

Secondary phase of multiplication. The results discussed above indicated that this phase may have been induced in several ways.

In the first case the induction resulted from a contact between the inoculated membrane and the yolk. Information concerning the possible nature of this induction will be considered at a later stage. It will be recalled (Table 24) that the occurrence of an above-average number of pseudomonads in a membrane tinted with the organism's pigment was not always associated with either detectable or significant contamination of the albumen. It would seem, therefore, that the initial stage of the secondary phase of multiplication was confined to the shell membranes. The data set out in Table 32 suggested that the contaminants in the white were able to utilize the nutrients present in the latter soon after the yolk had come into contact with an inoculated membrane. It

was concluded, therefore, that the secondary phase of bacterial multiplication began in the shell membrane when this was touched by the yolk and that it then continued in the albumen.

The available evidence did not allow generalizations to be made concerning the other methods of induction. Nevertheless the results discussed above did indicate two possibilities. Firstly, the association of gross contamination of albumen with pseudomonads and the occurrence of the organism's pigment on the yolk (Table 23) inferred that the secondary phase of multiplication may have been initiated by migrants that had reached the surface of the yolk. Secondly, it will be seen from Table 23 that an appreciable contamination of the white did occur in the absence of any detectable change in the appearance of an egg's contents. This was also a notable feature in eggs inoculated with Cloaca G75/2 (Table 28) or Aer. liquefaciens CF17/1 (Table 31). Whether this multiplication was induced by organisms that had reached the surface of the yolk and multiplied before the development of macroscopic changes in the appearance of the yolk, or whether this multiplication was induced by some change in the properties of the albumen (e.g. diffusion of iron and/or amino acids from the yolk) cannot be decided.

The effect of iron on bacterial multiplication in eggs. The investigations of Brook (1960b) and Garibaldi (1960) have shown that, in the case of the commonly occurring contaminants of rotten eggs, bacterial growth in albumen is inhibited because iron is rendered unavailable through chelation with conalbumin. The experiments described hereunder were done with the object of demonstrating the inhibitory action of this ligand in intact eggs.

In preliminary experiments, the object was to obtain information concerning the effect of iron on the growth on albumen *in vitro* of organisms that had been isolated from rotten eggs. Sterile albumen (10 parts) - for preparation, see appendix - was mixed with a 100 parts (by volume) of liquefied (45°) water agar ("Ionagar" No.2; Oxoid, London) and 10 ml aliquots of this were placed in Petri dishes. The surface of this medium was covered with 5 ml of the water agar containing a 4 mm loopful of an 18 hr nutrient broth culture. A disk of Whatman no.1 filter paper (6 mm diameter) saturated with an aqueous solution of $FeCl_3$ (0·001 g/ml) was placed on the inoculated agar. Filter paper soaked with distilled water or aqueous solutions of $CaCl_2$ or $MgSO_4$ (the concentration of metal in these solutions was equivalent to that of iron in the $FeCl_3$) were used as controls. The plates were incubated at 27°. It was found that iron alone influenced bacterial multiplication. This occurred in two forms. A. The type of response described hereunder was given by strains of *Ps. fluorescens* and *Aer. liquefaciens*. Colonies were present around the filter paper containing $FeCl_3$ after 24 hr incubation; macroscopic colonies were not present elsewhere in the inoculated agar. Colonies were easily visible throughout the agar after a further 24 hr incubation. B. The type of response described hereunder was given by strains of *Pr. vulgaris*, pseudomonad E, *Cloaca* and *Alc. faecalis*. A band of growth visible to the naked eye was present around the filter paper containing the $FeCl_3$ on the 3rd day of incubation. Microscopic examination revealed that the size of the lenticular-shaped colonies in the agar became progressively less as their distance from the edge of the filter paper increased. Moreover this decrease was associated with a loss of symmetry of the colonies. In fact, at a distance of *c.* 15 mm from the

edge of the filter paper microscopic colonies of very irregular outline were present and these occurred throughout the remainder of the agar.

The effect of iron on bacterial multiplication was tested in the following manner. The air cells of 3-day old eggs were inoculated with 0·1 ml of a 1/100 dilution of a washed cell suspension. The eggs were incubated for 5 days at 27°, during which time their air cells were downward so that the yolk would not influence the organisms present in the inner membrane of an air cell. It was considered that this period of incubation would exhaust the cells of endogenous materials present at the time of inoculation, thereby providing a critical test of the ability of iron to influence bacterial growth in the intact egg. On the 5th day following inoculation, 0·1 ml of an aqueous solution of $FeSO_4$ (2·5 g/100 ml distilled water) or, in the case of controls, 0·1 ml of distilled water was injected into the albumen immediately below the inner membrane of the air cell. The eggs, with their air cells downward, were incubated at 27° for a further 2 days.

The $FeSO_4$ did not have any detectable effect on the pH of the albumen. Moreover, it spread slowly through the thin white in the 24 hr following injection and through the thick white in the 24-48 hr period following injection. This diffusion resulted in the albumen becoming a salmon-pink colour. The results set out in Fig. 16 were obtained with a strain of pseudomonad E, an organism that will not grow in a glucose ammonium medium. It will be seen that the addition of iron to the albumen resulted in an increase in the extent of growth of the organisms in the shell membranes. Moreover it was found that the albumen containing iron was more heavily contaminated than that containing distilled water. The results obtained with other organisms are summarized in Table 33. It will be seen that the sizes of the populations in the membranes of eggs containing iron were always greater than those in the membranes of

eggs injected with distilled water. It was considered that the yolk's induction of the secondary phase of multiplication may have been due to the provision of iron to the organisms present in the shell membranes. Time did not permit an investigation of the effect of the ash of yolk material on the growth of organisms in an inoculated membrane. As far as can be ascertained, the results discussed above have provided the first direct evidence in support of the widely accepted belief that the chelation of iron is the main component of the anti-microbial defence of the egg.

The loss of the water-retaining property of the inner membrane of the air cell.

When water was placed on the inner membrane *in situ* of an uninoculated egg of any age there was no obvious signs that the water passed through the membrane. It was noted that when certain inoculated membranes were treated in this manner, water passed through and formed drops - reminiscent of dew drops - on the outer surface of the membrane. Moreover, such membranes were more easily ground to a paste than were membranes which did not show this phenomenon. This feature was not made a subject of investigation but the information obtained from the experiments discussed above will be considered in this section.

This condition was never observed in eggs in which the yolk moved away from the inoculated membrane. Moreover, the phenomenon was generally associated with definite evidence of a union between the membrane and the yolk. An exception to this generalization has been discussed on page97 . On another occasion, drops were observed on the membrane of air cells inoculated with an 18 hr nutrient broth culture of *Aer. liquefaciens* before the yolk had made contact with the membranes.

It was concluded, therefore, that this loss of the water-retaining property was due to bacterial action and that, in the case of the washed cell suspensions used in the above experiments, it was induced by substances present in the yolk. This effect was observed in eggs inoculated with proteolytic organisms (Ps. fluorescens, Cloaca, pseudomonad E, Aer. liquefaciens) as well as in eggs inoculated with non-proteolytic organisms (Alc. faecalis). This implies that this phenomenon is not entirely due to bacterial proteases but that it may be due to the breakdown of the mucin fibres in the shell membranes. The organisms were not tested for mucolytic activity.

Changes in eggs produced by different species of bacteria

Information obtained from the experiments discussed above together with that obtained from re-infection experiments will be discussed in this section. The results obtained from the re-infection experiments are summarized in Table 34. The method of Florian & Trussell (1957) was used to infect the air cell of 3-day old eggs and these were incubated at 27°. Each organism was inoculated into 4 eggs. On the 42nd day following inoculation, the eggs were opened and the rots were identified with the descriptions given by Haines (1939) or Florian & Trussell (1957). In addition, a 4 mm loopful of the contents of an egg was subcultured on nutrient agar and incubated at 27°.

Changes in the appearance of inoculated eggs. It will be recalled that the pattern of multiplication discussed above was common to all the organisms tested and that the primary phase of multiplication was rarely associated with signs of infection in the contents of an egg. Changes in the appearance of an egg were associated with the secondary phase

of multiplication and then only in the case of eggs infected with certain organisms. It will be seen from Table 30 that Alc. faecalis multiplied extensively when the yolk made contact with an inoculated membrane. Although this resulted in the albumen being contaminated with c. 1 x 10^9 organisms/ml, this gross contamination was not associated with detectable changes in either a candled or broken-out egg. In fact, inoculated eggs could not be distinguished from uninoculated eggs that had been incubated for a similar period of time. It was found that eggs inoculated with Citrobacter or non-proteolytic strains of Cloaca contained large numbers of organisms on the 42nd day of incubation and yet the eggs did not exhibit any signs of infection. In the case of eggs inoculated with non-proteolytic strains of Ps. fluorescens, the organisms' green pigment was formed in the albumen and this was the only feature that distinguished an inoculated egg from an uninoculated egg which had been incubated for a similar period of time. The most profound changes occurred in eggs inoculated with proteolytic organisms. In all cases the onset of the secondary phase of multiplication was associated with the occurrence of a patch of custard-like material on or in close proximity to the blastoderm. These patches increased in size and/or in appearance during subsequent incubation. Although there were variations in the 5 eggs opened at the same time, it was possible to arrange the changes in what is believed to be a true sequence.

The sequences observed with various organisms were as follows (the bracketed figures refer to the number of days following the union of the yolk and the inoculated membrane). Proteolytic strain (Cf81) of Pseudomonas fluorescens: patches of green fluorescent pigment were present in the inner membrane of the air cell and, normally, on the

surface of the yolk and, occasionally, in the albumen (1): the pigment was present throughout the albumen (2-5): custard-like material was present on the surface of the yolk and the contents of the yolk were of an oily appearance and the albumen was turbid and fluoresced under ultra-violet irradiation (6-10). Pseudomonad E: a patch of custard-like material was present on the yolk (1-8): the yolks were gelatinous, of an amber-like appearance and their surfaces were striped with an olive-green pigment; a strong almond-like odour was present (9-20); similar to the foregoing but the white was salmon-pink (21). Aer. liquefaciens: a patch of custard-like material was present on the yolk (1-9): this patch was olive-green in colour; the yolk was gelatinous and amber-like in appearance (10): the custard-like material had been reduced to a few patches; the yolk was completely gelatinous and its centre was amber-like in appearance whereas its surface was olive-green or black; the white was slightly turbid and salmon-pink (14). Cloaca: a patch of custard-like material was present on the surface of the yolk, the centre of the yolk was oily in appearance and the white was very viscous (1-4): the custard-like material was olive-green and the white, which was very viscous, was salmon-pink (5-7): the whole surface of the yolk was covered with custard-like material and that part of it which was in contact with the inoculated membrane was olive green; the centre of the yolk was of an oily appearance; the albumens were very viscous and salmon-pink (10).

On the 42nd day following inoculation, the appearance of an infected egg was characteristic of a particular organism. This allowed the various rots to be identified with the descriptions given by Haines (1939) or Florian & Trussell (1957). The results of this identification together with the salient features of each rot are summarized in Table 34.

An attempt was made to correlate the salient features of the various types of rot with the metabolic properties of the causative organisms. The results are given in Table 35. It was noted that, with the exception of non-proteolytic strains of Ps. fluorescens, the main difference between organisms that produced profound changes in eggs and those that did not was that the former produced proteolytic enzymes. Furthermore, there appeared to be a correlation between the rate of decomposition of the contents of an egg and the rate at which an organism liquefied gelatin. The most active liquefiers, Aer. liquefaciens and Pr. vulgaris, produced the most extensive decomposition; pseudomonad E, Cloaca and Ps. fluorescens, all of which liquefied gelatin slowly, produced relatively less decomposition. Miles & Halnan (1937) presented evidence which indicated that the blackening of an egg was due to the production of H_2S. Similarly, in this study it was found that a strong odour of H_2S was present in eggs that contained either olive-green pigment or complete blackening and that these discolourations could be removed with H_2O_2. Moreover, there was a correlation between the extent of blackening of an egg's contents and the rate of blackening of a piece of lead acetate paper held in the atmosphere above the egg, the fastest blackening occurred in eggs whose yolks were completely blackened. It was noted, moreover, that there was a correlation between the extent of blackening of an egg's contents and the rate at which an organism liquefied gelatin and produced H_2S from heat-coagulated egg white. The most extensive blackening was seen in eggs infected with Aer. liquefaciens or Pr. vulgaris, two active liquefiers of gelatin and producers of large amounts of H_2S. pseudomonad E and Cloaca, both of which produced large amounts of H_2S from heat-coagulated egg white but were relatively slow liquefiers of gelatin, produced only patches of

blackening on the yolk of infected eggs. Finally, the proteolytic strains of Ps. fluorescens did not produce H_2S from coagulated egg white and the blackening of the eggs' contents was never observed with these organisms.

Certain other features of the rots could be presumptively attributed to other metabolic properties of the organisms. For example, pseudomonad E produced an almond-like odour during growth in media containing proteins; this odour was a notable feature in eggs infected with these organisms. Similarly, Cloaca strains G44 and G75/2 produced very viscous substances during growth in a number of commonly used laboratory media. It would seem reasonable to assume that this property was responsible for the marked increase in the viscosity of the albumen of infected eggs. In addition, the 6 strains of Cloaca examined in this study all produced a thick layer of custard-like material around the yolk of an infected egg. It is noteworthy that a very heavy scum was a notable feature in nutrient broth containing egg yolk in which these organisms had grown. It was concluded, therefore, that this feature of the rot was due to lecithinase produced by these organisms. It will be seen from the information given above that a salmon-pink discolouration of the albumen was present in eggs inoculated with certain organisms. The marked similarity between this discolouration and that obtained when $FeSO_4$ was added to albumen suggested that the pigment was most probably the conalbumin-iron-complex. This implies that mechanisms (the semipermeable vitelline membrane and the diffusion gradient in the yolk) which hinder the physico-chemical systems of the yolk from reaching equilibria with those in the white are disrupted during the course of bacterial infection of an egg. In view of this observation, it was concluded that the available evidence did not warrant further presumptive correlations of the appearance of a rot with the metabolic attributes of the organism.

Resistance of the albumen to bacterial digestion. The resistance of the albumen to microbial decomposition was a notable feature of infected eggs. It was only towards the 42nd day following inoculation that decomposition of the albumen was observed and, even then, only in eggs inoculated with Aer. liquefaciens or Pr. vulgaris. Moreover, it was found that the watery grey albumen present in such eggs was coagulated with ethanol. The strength of such coagula, as judged by their resistance to shaking, was much less than that of coagula produced from uninoculated eggs. The strength of the coagula of albumen infected with proteolytic strains of Cloaca or Ps. fluorescens was intermediate between these two extremes.

The possibility that this resistance may have been due to ovomucoid was tested in the following manner. An organism was grown in nutrient broth for 6 days at 27° at which time the cells were removed by centrifugation and the supernatant was sterilized by the addition of a drop of chloroform. Two millilitres of the supernatant were added to 10 ml azo-casein (Charnley & Tomarelli, 1947) containing either 1 ml distilled water or 1 ml of a 2% (w/v) aqueous solution of ovomucoid. This substance was prepared by the method of Lineweaver and Murray (1947). These mixtures, contained in 18 x 150 mm test tubes, were incubated in a water bath maintained at 37°. At frequent intervals 1 ml aliquots were transferred to 5 ml of a 5% (w/v) aqueous solution of trichloracetic acid and the amount of liberated dye was measured in an absorptiometer. A 0·05% (w/v) aqueous solution of trypsin (B.D.H., England) was used as the control. The inhibition of trypsin with ovomucoid is shown in Fig. 17. Ovomucoid had no detectable effect on the digestion of azo-casein by any of the following organisms (bracketed figures refer to the number of strains tested): Ps. fluorescens (2), Pr. vulgaris (2), pseudomonad E (2),

Aer. liquefaciens (2) and *Cloacs* (2). It was concluded, therefore, that ovomucoid was not responsible for the resistance of the white to bacterial digestion.

DISCUSSION

A more comprehensive view of the course of bacterial infection of an egg appears to have become available. Gillespie & Scott (1950), using information derived mainly from empirical observations, considered the following to be possible stages in the infection of an egg: a) contamination of the shell, b) penetration of the shell, c) colonization and penetration of the shell membranes, and d) infection of the contents of an egg.

The present investigation, though not concerned with contamination or penetration of the shell, provided information which indicated that, in the case of commercial eggs, these are the two critical stages in the infection of an egg.

It is known that the incidence of soiling of egg shells can be reduced by paying strict attention to the hygiene of the nesting materials and the egg collecting methods. Nevertheless, some eggs will always be soiled and it would appear from the available evidence (Haines, 1939; Winter *et al*, 1952; Brooks & Taylor, 1955) that this dirt should be removed by dry-cleaning methods if bacterial penetration of the shell is to be minimized.

A number of investigators (Gillespie & Scott, 1950; Stokes *et al*. 1956; Fromm & Monroe, 1960; Garibaldi & Bayne, 1960) have presented evidence which suggested that effective bacterial infection is confined to the shell membranes for 10-15 days following bacterial penetration of the shells of newly laid eggs. Brooks (1960b) found that, in the case of pseudomonads inoculated into the air cell, this period was

characterized by a relatively slow rate of multiplication of the organisms in the inner membranes and a slow accumulation of contaminants in the albumen. The duration of this period was determined by the age of an egg at the time of inoculation; it lasted for about 12 days in the case of newly laid eggs but the period was considerably reduced if the eggs were stored for 1-7 days prior to inoculation. At the end of this period the rate of multiplication of the organism in the inner shell membranes increased and the contents of the eggs became more heavily contaminated. Brooks suggested that the increase in the rate of multiplication resulted from a spontaneous change in the property of the shell membranes which makes them more susceptible to bacterial attack.

The results obtained in the present investigations have shown that this sequence of events is common to organisms other than pseudomonads. Moreover, the use of an inoculum ($1{\cdot}0 \times 10^3$) smaller than that used by Brooks ($5{\cdot}0 \times 10^6$) has given greater emphasis to the stages in the sequence. The observation that the primary phase of multiplication was confined to the shell membranes and that migrants from this source remained inactive in the albumen accords with the findings of Brooks (1960b). The evidence obtained in this investigation suggests that 2 factors are responsible for this confinement. The first, and apparently the least important, is the mechanical barrier which the shell membranes impose to bacterial penetration. It will be recalled that the shell membranes can act as bacterial filters (Haines & Moran, 1940; Garibaldi & Stokes, 1958) but that this property is lost during contact of the membranes with bacteria (Walden et al. 1956; Garibaldi & Stokes, 1958). It was found in the present investigation (Fig. 18) that the duration of effective restraint by the shell membranes in vitro was apparently determined by some property of the membranes and not by any attribute

(e.g. proteolytic activity) of the organism. A similar situation was found in the case of whole eggs. Although the method whereby bacteria penetrate the shell membranes awaits elucidation, it would seem reasonable to assume that the method may be analogous to that which occurs in the penetration of a bacterial filter, an explanation offered by Gillespie & Scott (1950). The low incidence of contamination of the albumen during the primary phase of the infection together with the apparent inability of the contaminants to multiply in the albumen suggested that the properties of the albumen were responsible for the confinement of the organisms to the shell membranes. Since the secondary phase of multiplication could be induced by the addition of iron to the albumen, it would appear that, in the case of the contaminants of rotten eggs, conalbumin by ensuring the absence of available iron was primarily responsible for the confinement of the early stages of the infection to the shell membranes.

Indirect evidence (Gillespie & Scott, 1950; Schmidt & Stadelman, 1957) has indicated that neither disinfectants nor antibiotics are able to control bacterial multiplication in the shell membranes. In fact, the only commercially significant control of rotting in eggs has been obtained with a method of heat treatment - the eggs were held at 145°F/3 min - Funk (1943) Knowles (1956). The practical application of this technique has been hindered by various technological and economic difficulties. In the meantime, therefore, the results of Stokes *et al.* (1956) and Orel (1959b) indicate that cold storage would be the most practicable method of controlling infection. This method doubtless works simply by retarding bacterial multiplication and decreasing the rate of deterioration of egg quality.

It will be recalled that the secondary phase of multiplication was induced in various ways. In the first case, induction occurred when the yolk made contact with an inoculated membrane. It is noteworthy that this occurred 12-20 days following the laying of an egg. Not only does this suggest a possible explanation of the information given at the beginning of this discussion, but it also permits another interpretation of the results presented by Brooks (1960b). In fact, it was concluded that the secondary phase of multiplication was induced by substances, possibly iron, present in the yolk. Further evidence against a spontaneous change in the properties of the membranes (cf. Brooks, 1960b) was obtained from eggs in which the yolk moved away from the inoculated membrane. It will be recalled that in this case the primary phase of multiplication was followed by a period during which there was a decline in the number of organisms in the inner membrane of an air cell. In some eggs, particularly those inoculated with Aer. liquefaciens, Cloaca or Ps. fluorescens, there was a notable increase in the level of contamination of the albumen and, occasionally, the inner membrane of an air cell on the 20-30th day following inoculation. The reasons for this are not clear but 2 possibilities suggested themselves: a) the multiplication of migrants which had reached the surface of the yolk, or b) the multiplication of contaminants in the albumen as a result of some change in the properties of the latter.

These results suggest that the site of infection may determine the rate and, possibly, the incidence of rotting during the storage of dry-cleaned eggs. This supposition is supported by the observations of Lorenz et al. (1952). They found the highest incidence (28·6 - 29·2%) of rotting in eggs whose blunt poles had been contaminated with a pseudomonad and the lowest incidence (17·4 - 18·2%) in eggs whose

pointed ends had been contaminated. Since washing is the most commonly used method of cleaning dirty eggs, it is possible that potential contaminants would be lodged over the whole of the shell thus predisposing such eggs to a secondary phase of multiplication induced by a union of the yolk and contaminated membranes. In fact, this would appear to be the most probable interpretation of the results presented by Fromm & Monroe (1960). They dipped eggs in cultures of pseudomonads both of which were at the same temperature; this would ensure that the membranes would be contaminated with water drawn through the pores of the shell by capillary attraction. The course of infection was followed by candling the eggs in a lamp fitted with a source of ultra-violet irradiation. It is noteworthy that they found a sudden increase in the incidence of rotting on the 10-15th day of storage, i.e. at a time when the yolks could be expected to have made contact with the shell membranes. Similarly, the induction of the secondary phase of multiplication by the union of the yolk and infected membrane would appear to be a possible interpretation of the results presented by Stokes et al. (1956). These authors, who infected eggs by allowing them to contract in cold suspensions of salmonellas, found a very marked increase in the level of contamination of the albumen on the 14-20th day of incubation. This evidence suggests that in commercial eggs the secondary phase of multiplication would be induced principally by contact of the yolk and infected membrane. It is noteworthy that a sequence of events different from those of Stokes et al. (1956) and Fromm & Monroe (1960) was reported by Garibaldi & Bayne (1960) for eggs which had been dipped in bacterial suspensions containing $FeSO_4$. In this case, a high incidence of infection was present on the 4th day following inoculation. This observation implies that the contamination of water with iron may

play an important part in bacterial rotting of commercial eggs. It would seem that this aspect is worthy of further exploration.

The results obtained in the present study raise questions concerning the terms that have been used to denote the role that a organism plays in the infection of an egg. It will be recalled (page 59) that although 70% of the eggs examined at the beginning of this investigation contained a mixed bacterial infection, the majority of rots could be identified with the descriptions given by Haines (1939) or Florian & Trussell (1957). It was found, however, that only certain of the isolates reproduced rots when inoculated into fresh eggs. It is noteworthy that, with the exception of non-proteolytic Ps. fluorescens, the rot-producing bacteria were proteolytic whereas the others were not. This observation is in agreement with the views of Garibaldi & Stokes (1958). It was found, however, that both types of organism gave an essentially similar pattern of multiplication in the course of an egg's infection. It is noteworthy that Brooks (1960b) found a similar pattern in eggs inoculated with Ps. putida or Ps. schuykilliensis, two organisms that may have originated from eggs suffering from off-odours. (Brooks & Hale (1954) stated that they had obtained both species from Richard & Mohler (1950) who had isolated them from eggs suffering from off-odours). This information implies that the use of the term 'secondary invaders' (e.g. Florian & Trussell, 1957) is unwarranted. It would appear that a utilitarian classification would be possible if stress was given to the type of change occurring in an infected egg and appending to this the name of the organism that was responsible for the salient features of these changes. The organism might be recognized from these changes or by laboratory tests. In any case, if the terms of ecology are applied, this organism would be regarded as the

'dominant' species and the use of this term does not imply numerical preponderance. The envisaged scheme is set out in Table 36.

The re-infection experiments also revealed a fact whose practical implications are worthy of comment. This concerns a possible source of contamination of frozen egg and egg powder; these products can contain many viable organisms (50,000 - 500,000 organisms /g) but no one has accounted for the source of contamination (Brooks & Taylor, 1955). It is conceivable that many of these contaminants may be introduced by eggs infected with non-proteolytic bacteria. It will be recalled that such eggs did not exhibit signs of infection although their contents harboured many millions of viable organisms. Consequently, they would not be eliminated by the "look and smell" test of the egg breakers. Similarly, eggs can be infected with salmonellas and yet show no signs of infection (Stokes et al. 1956). Such eggs could make a greater contribution to the contamination of egg products with these organisms than the various sources reviewed by Heller, Knowles & Bryce Jones (1958). It would appear, therefore, that the bacteriological quality of egg products might be improved by the use of newly laid eggs.

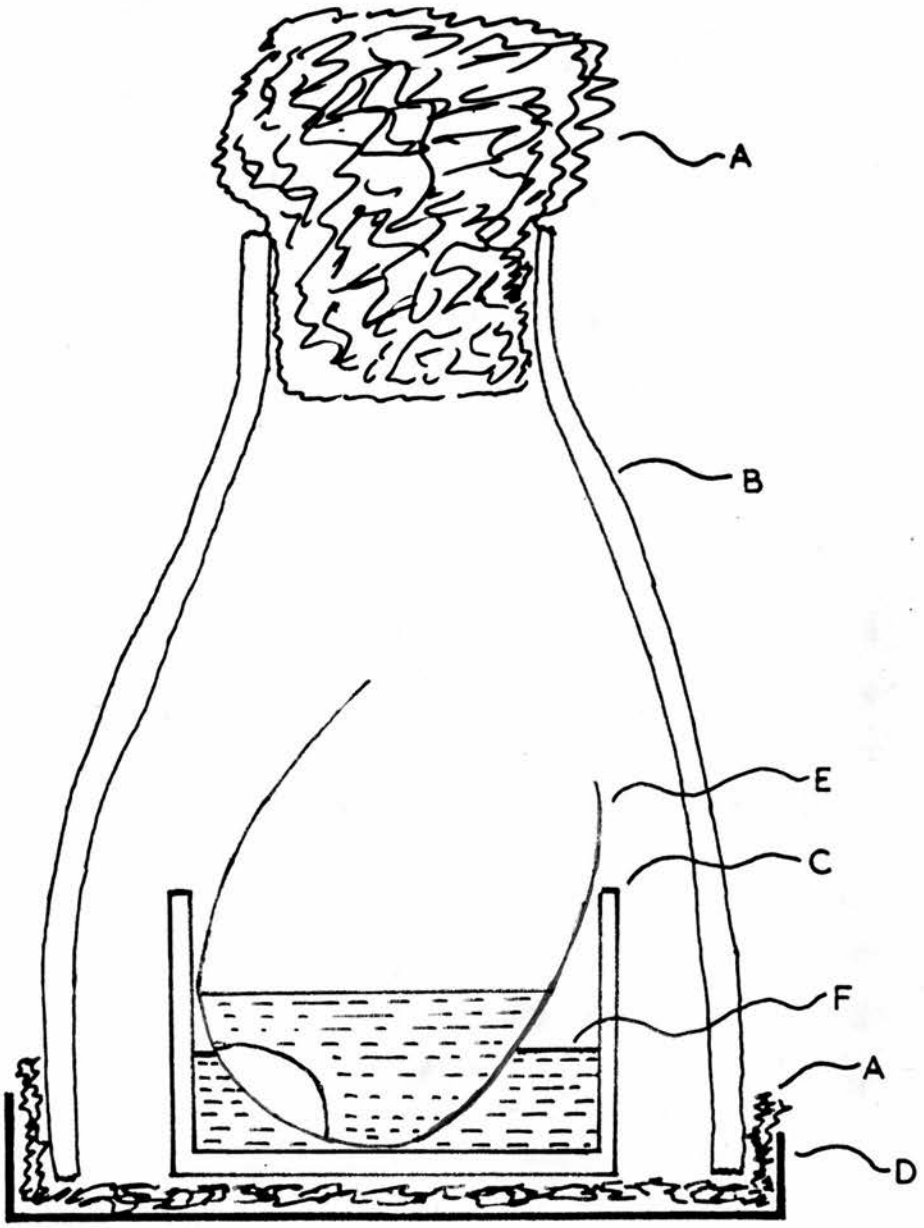
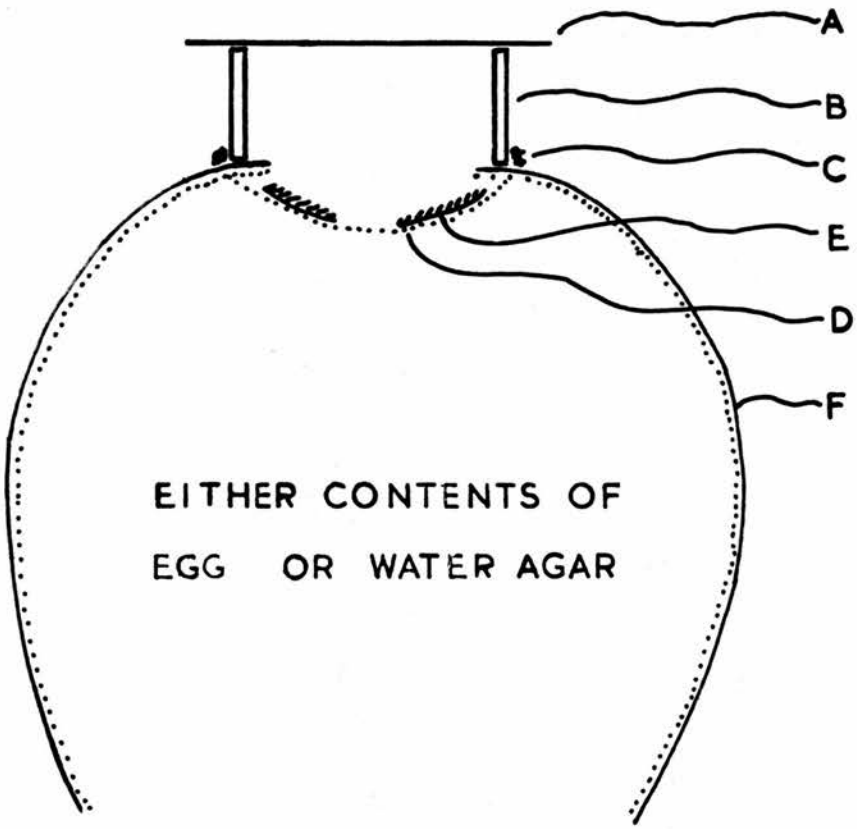


Fig. 7. THE APPARATUS USED FOR AN INVESTIGATION OF BACTERIAL PENETRATION OF THE SHELL AND SHELL MEMBRANES. THE ASSEMBLED APPARATUS WAS WRAPPED IN PAPER AND STERILIZED BY HOLDING MOMENTARILY AT 22 lb/in^2.

A: Cotton wool. B: Top of a milk bottle. C: Crystallizing dish. D: Top of a Petri dish. E: Egg shell. F: Nutrient broth.

Drawn to scale.



Fiq.8. METHOD USED TO STUDY THE GROWTH OF BACTERIA IN FILTER PAPER DISKS RESTING ON THE INNER MEMBRANE OF THE AIR CELL.

A: Vasiline-coated cover slip. B: Glass ring. C: Paraffin wax. D: Inner shell membrane. E: Filter paper disk. F: Shell and outer shell membrane.

Scale: X 2.

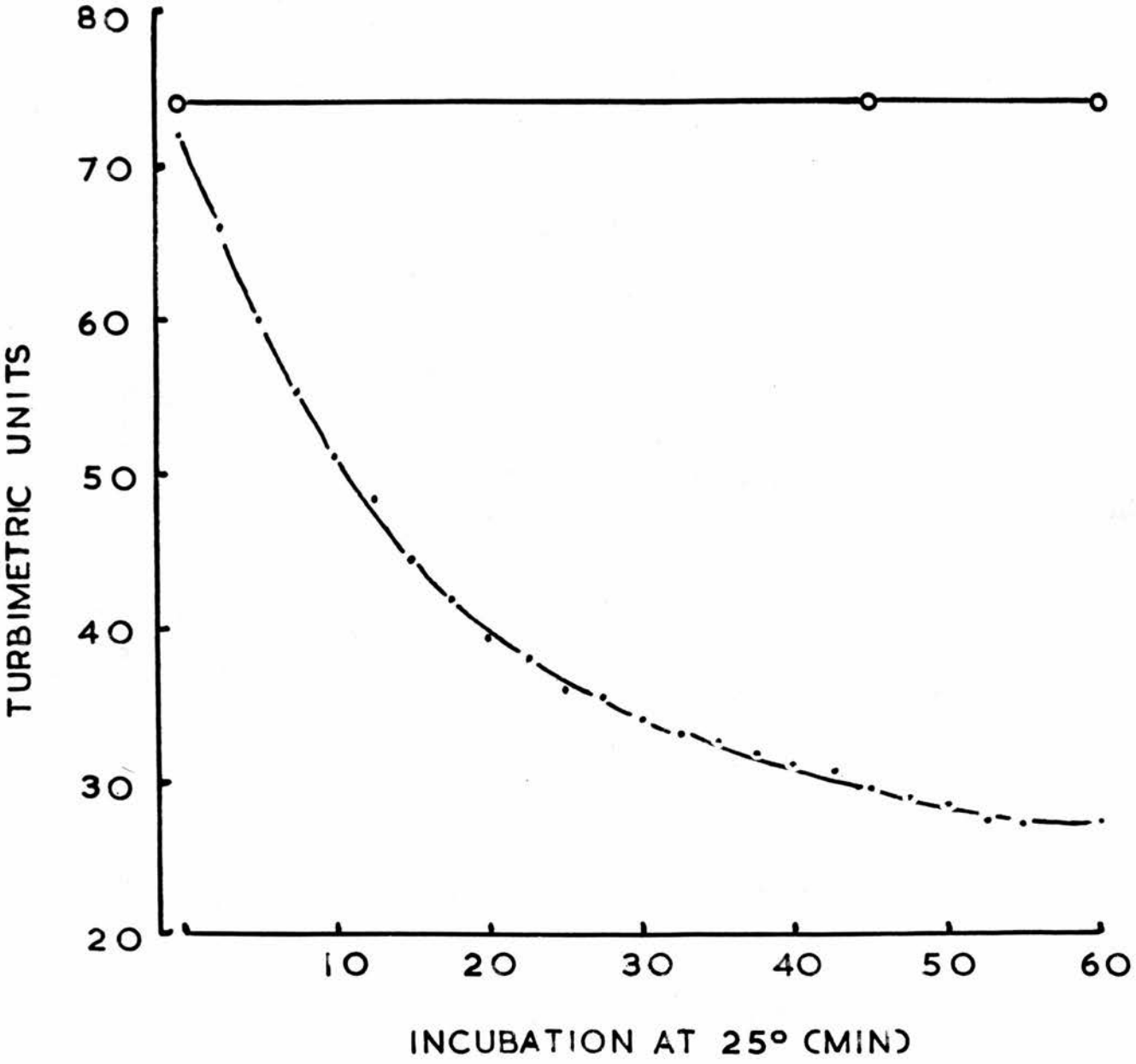
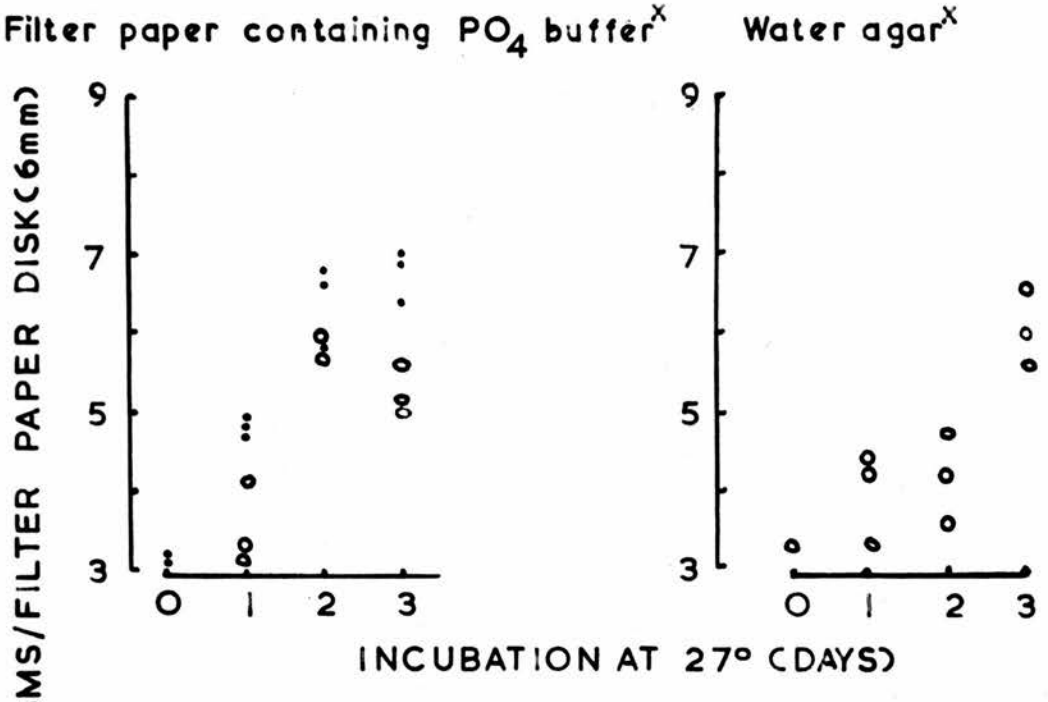


Fig.9. CLEARING OF AN 18 HR BROTH CULTURE OF MICROCOCCUS LYSODEIKTICUS. BY LYSOZYME.

•: 9.5 ml of a nutrient broth culture plus 0.5 ml of a solution (100 µg/ml) lysozyme.

°: 9.5 ml of a nutrient broth culture plus 0.5 ml of water.

Proteus vulgaris



Pseudomonas fluorescens

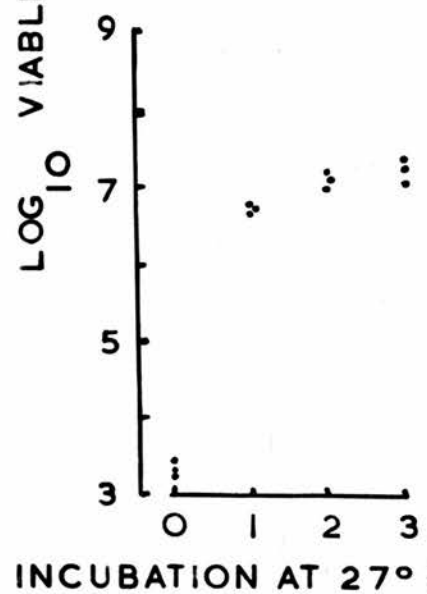


Fig.10. BACTERIAL MULTIPLICATION IN FILTER PAPER RESTING ON NUTRITIONALLY IN-ADEQUATE MEDIA.

•: Dilution of an 18hr nutrient broth culture used to inoculate the filter paper

o: Washed cell suspension prepared from an 18 hr nutrient broth culture used to inoculate the filter paper.

x: Medium on which the filter paper was placed.

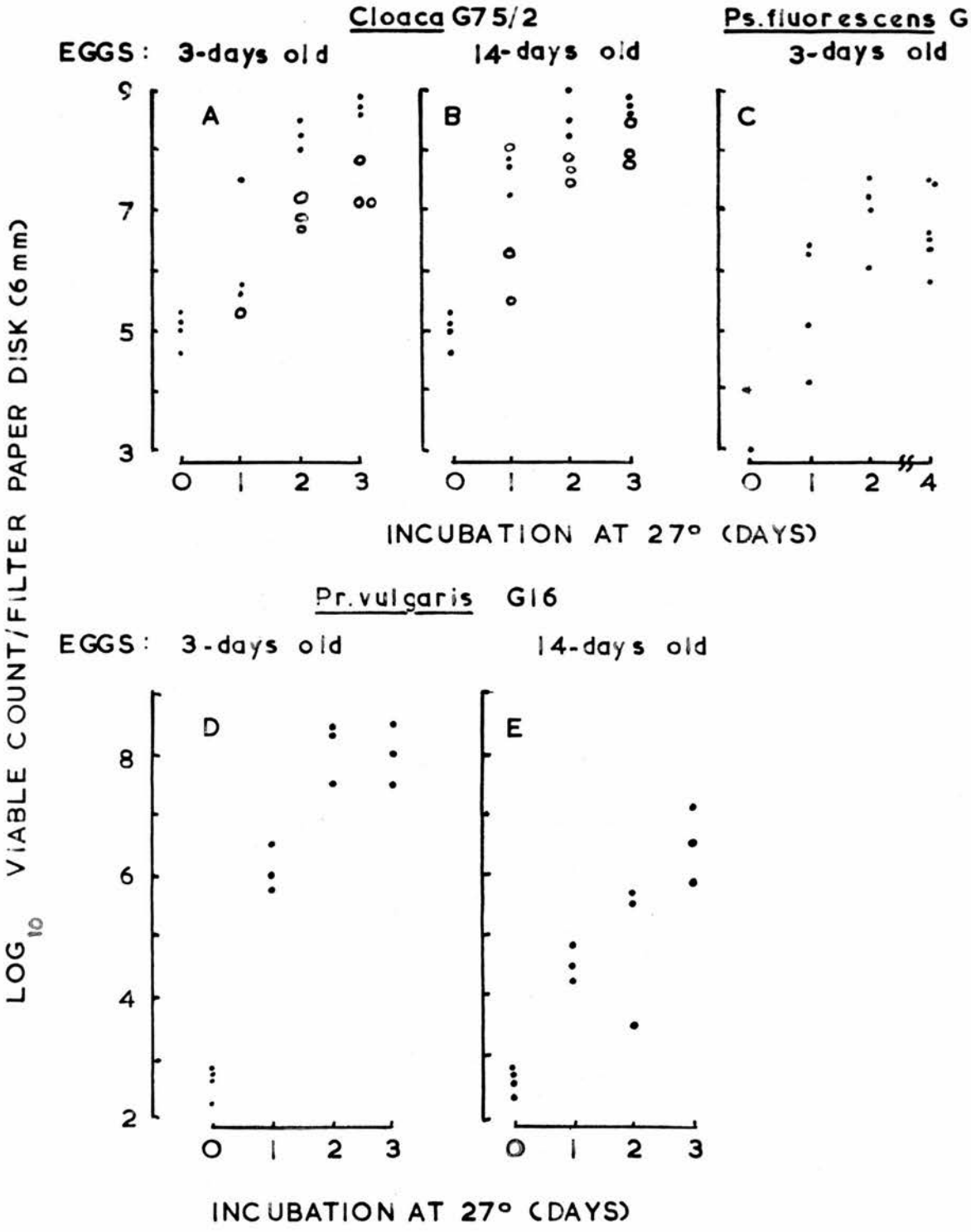


Fig.11. BACTERIAL MULTIPLICATION IN FILTER PAPER RESTING ON THE INNER MEMBRANE OF THE AIR CELL.

Inoculum: a dilution of an 18 hr nutrient broth culture

•: Intact eggs

°: Eggs in which the contents had been replaced with water agar

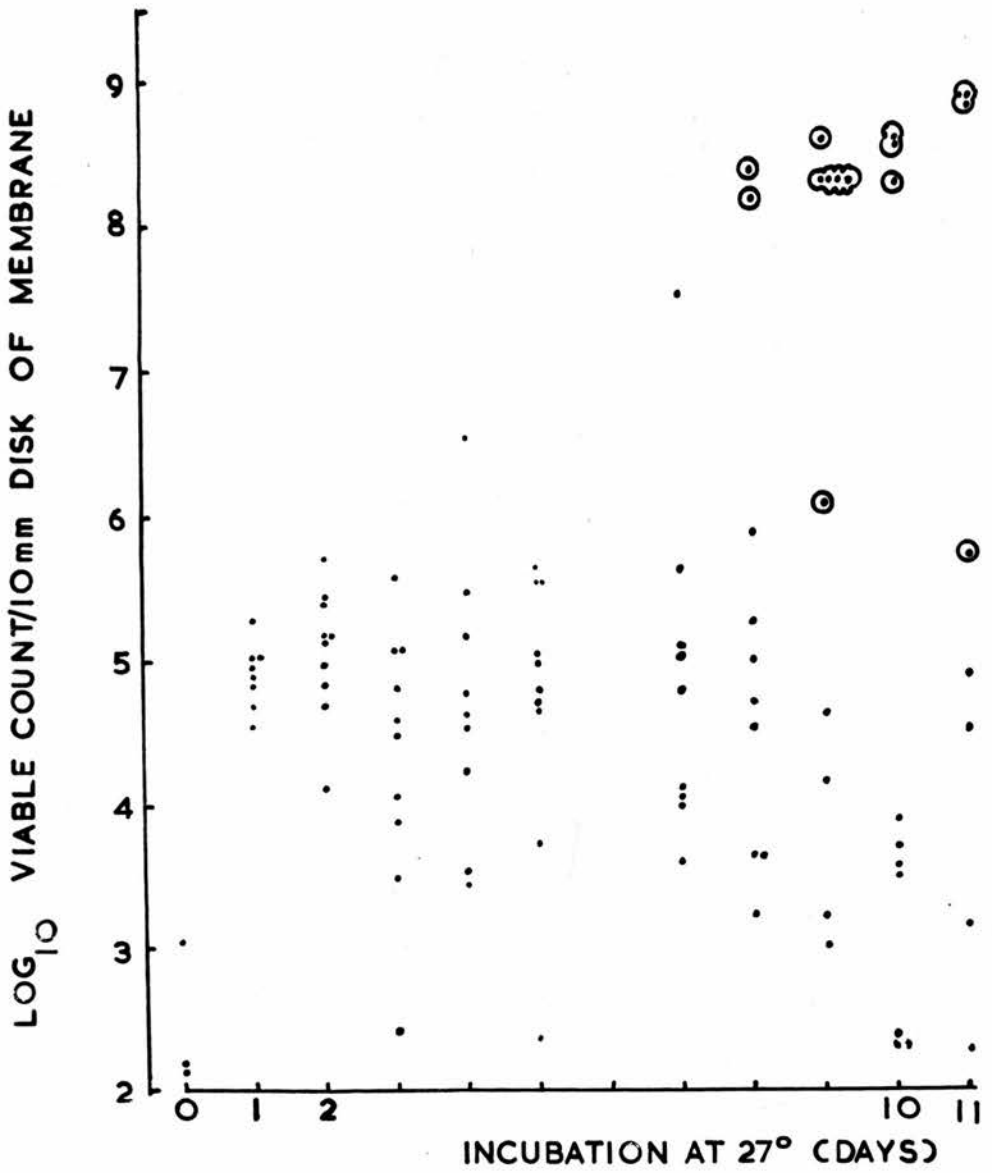


Fig.12. THE GROWTH OF PSEUDOMONAS FLUORESCENS CF21 IN THE INNER MEMBRANE OF THE AIR CELLS OF EGGS THAT WERE 3-DAYS OLD AT THE TIME OF INOCULATION. THE AIR CELLS WERE HELD UPPEPMOST.

• = ONE EGG. O = FLUORESCENT GREEN PIGMENT IN MEMBRANE

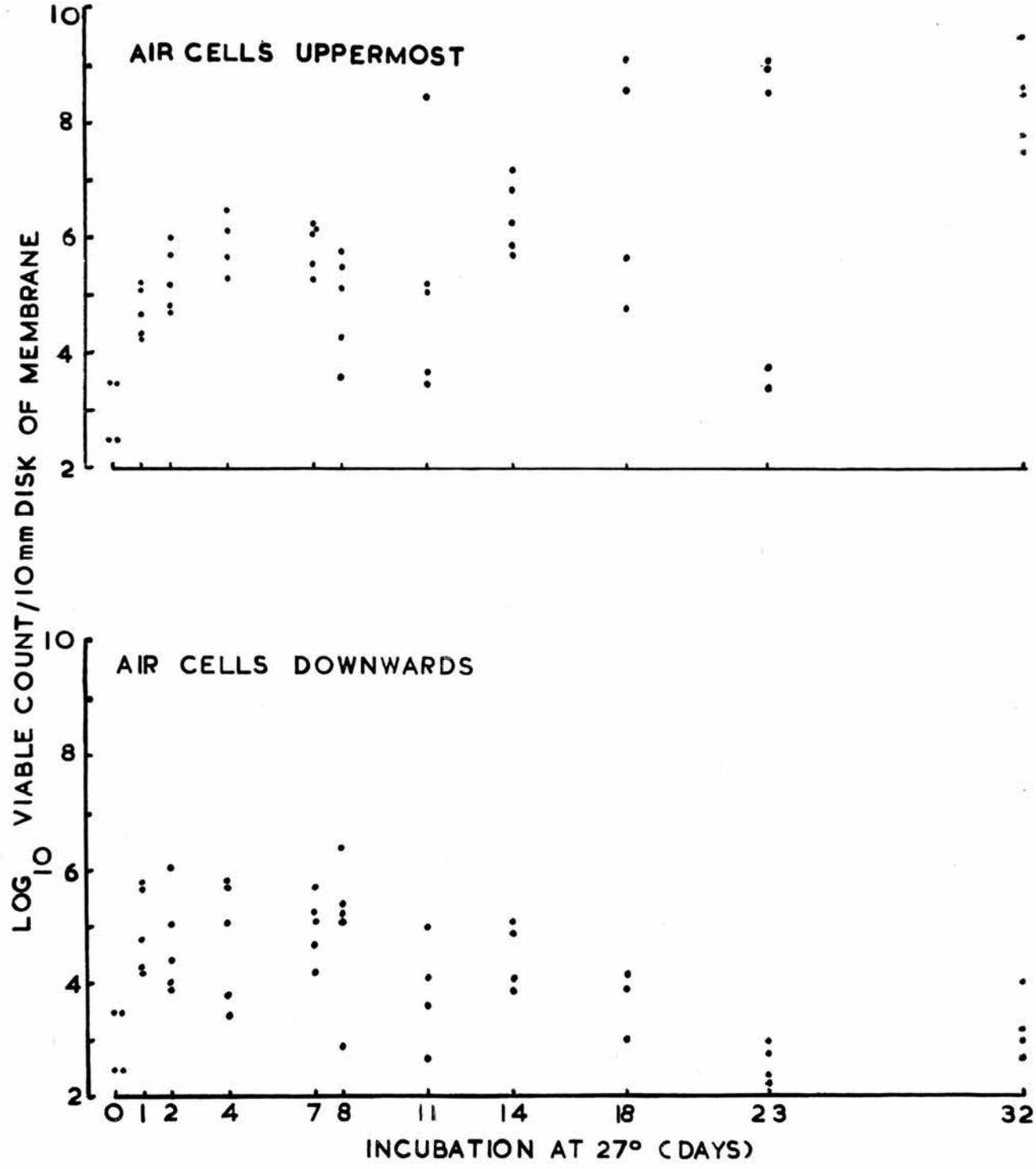


Fig. 14. THE GROWTH OF AEROMONAS LIQUEFACIENS CF10/1 IN THE INNER MEMBRANE OF THE AIR CELLS OF EGGS THAT WERE 1-DAY OLD AT TIME OF INOCULATION.

• = ONE EGG

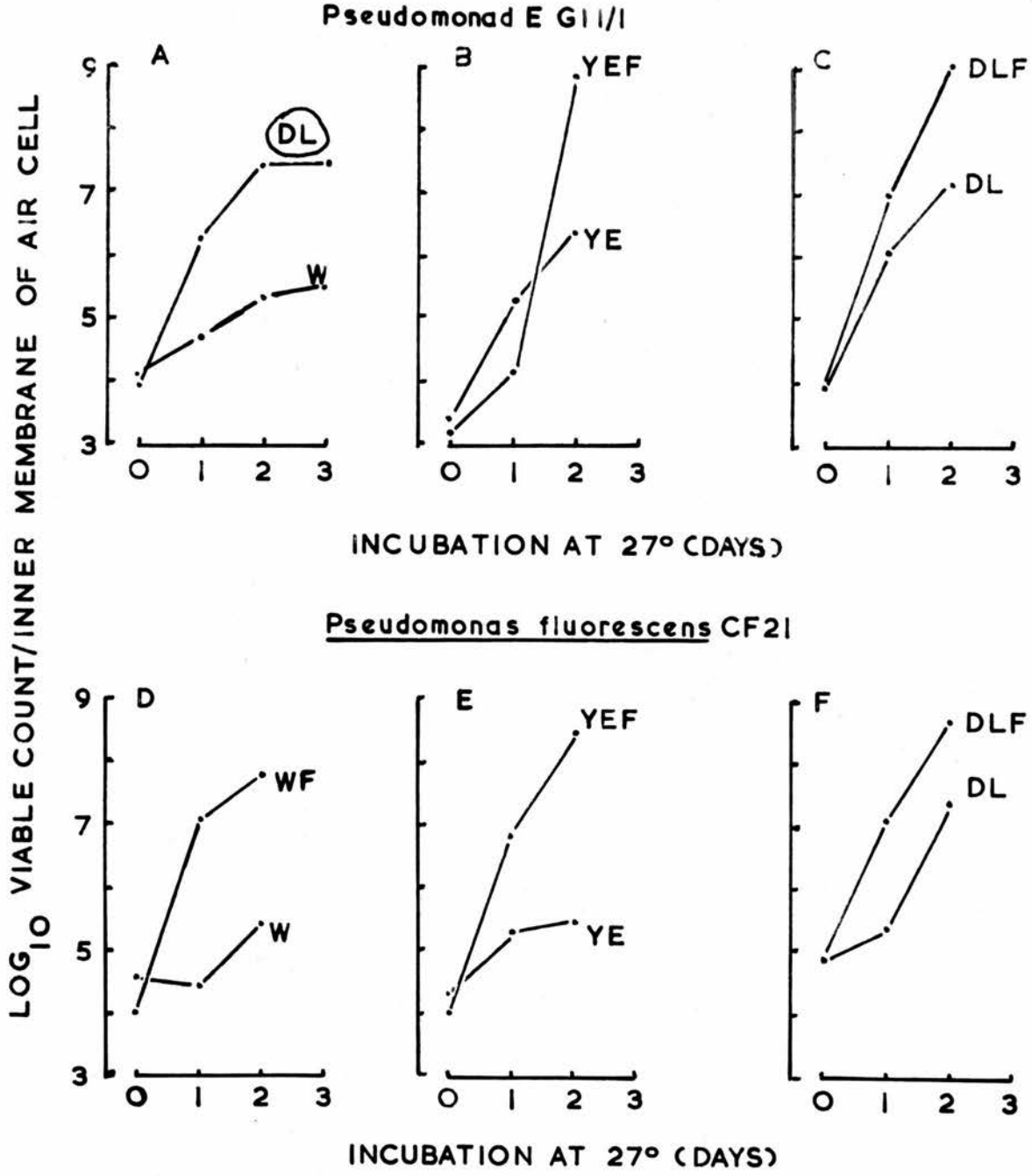


Fig.15. THE INFLUENCE OF VARIOUS SUSPENDING MEDIA ON BACTERIAL MULTIPLICATION IN THE INNER MEMBRANE OF THE AIR CELL.

(DL): deep litter infusion (0.5g deep litter:100 ml water). DL: deep litter infusion extracted with 8-hydroxyquinoline. W: distilled water. YE: 0.5% (w/v) yeast extract (Difco) extracted with 8-hydroxyquinoline. The suffix F indicates that the medium had been contaminated with $FeSO_4$ (final concentration 100 µg/ml). •: an average obtained from 5 eggs. The eggs were 3-days old at the time of inoculation. Eggs incubated with their air cells downwards.

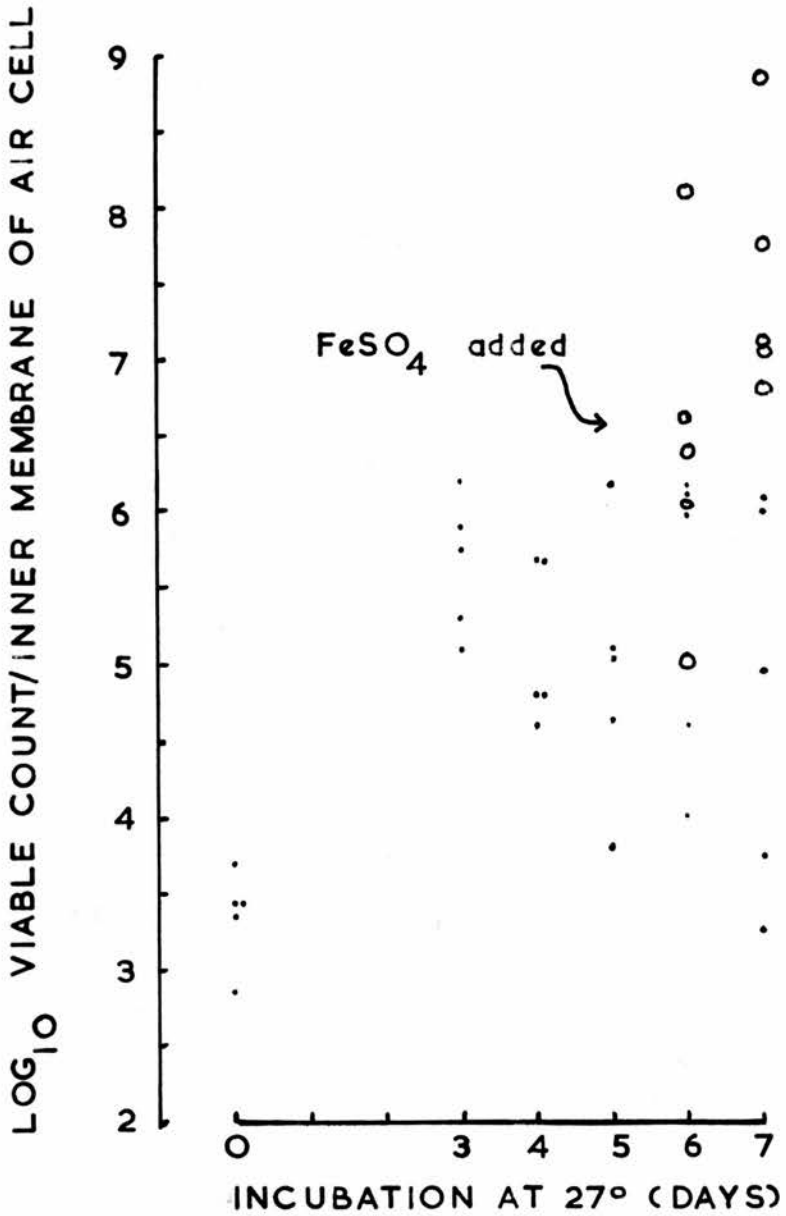


Fig.16. THE EFFECT OF THE ADDITION OF $FeSO_4$ TO THE ALBUMEN ON THE MULTIPLICATION OF PSEUDOMONAD E IN THE INNER MEMBRANE OF THE AIR CELL

•: Normal eggs or, after the 5th day of incubation, eggs whose albumen had been injected with 0.1 ml of water. °: Eggs whose albumen had been injected with 0.1 ml of a 2.5% (w/v) solution of $FeSO_4 \cdot 7H_2O$.

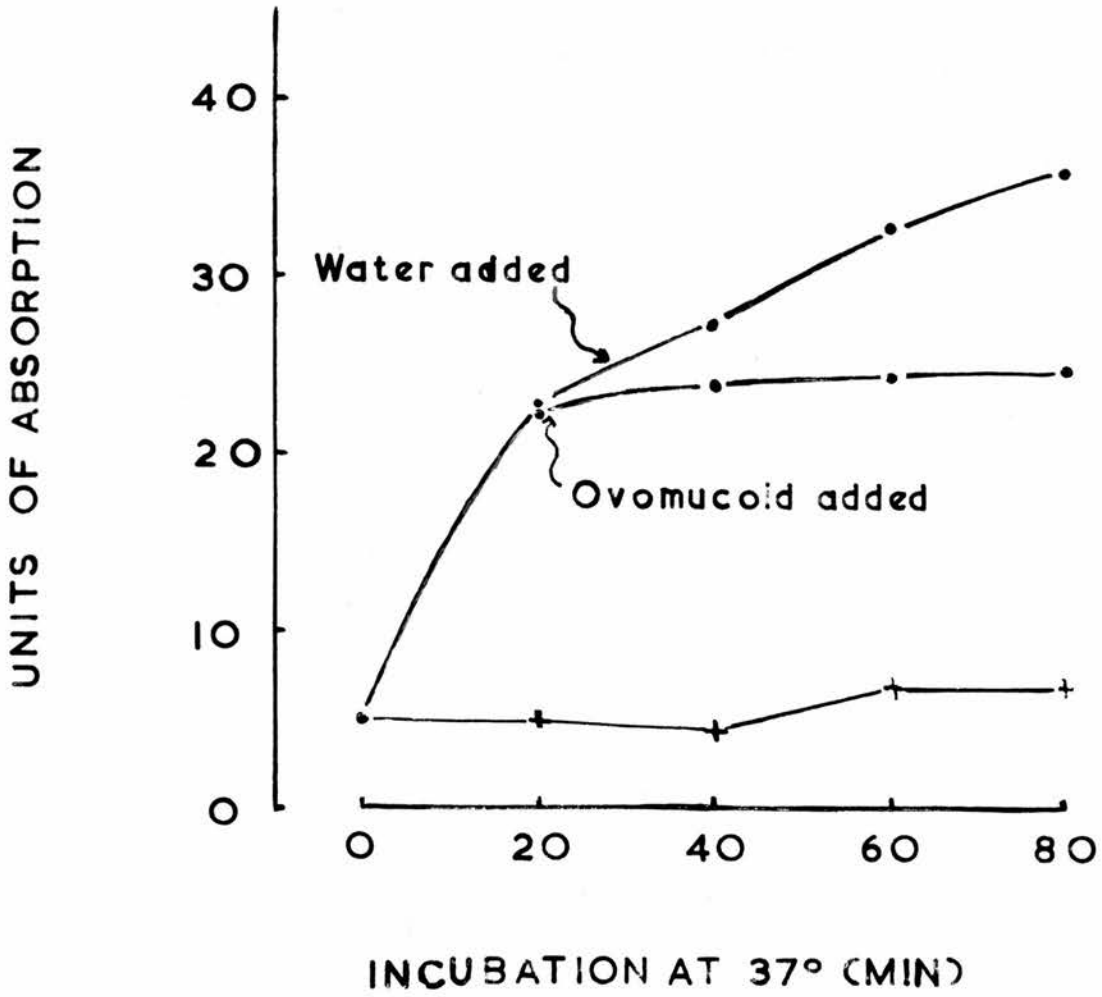


Fig.17. INHIBITION OF TRYPSIN BY OVOMUCOID

•: 10 ml azo-casein plus 2 ml of 0·05% (w/v) trypsin plus either 1 ml of a 2% (w/v) solution of ovomucoid or 1 ml of distilled water.

+ As above except that the trypsin had been held at 100° for 10 min.

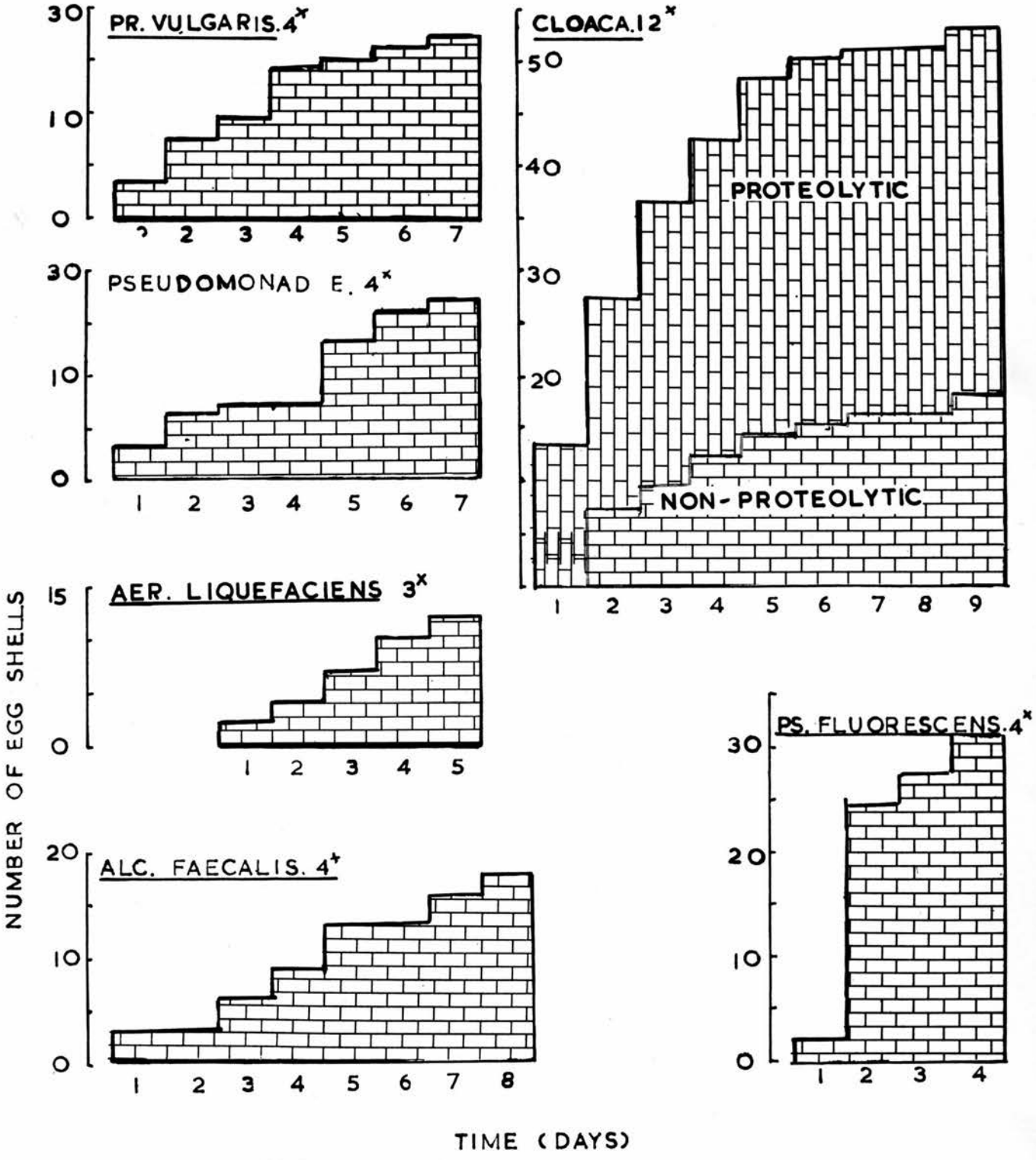


Fig. 18. BACTERIAL PENETRATION OF EGG SHELLS AT 27°

x NO. STRAINS EACH ORGANISM TESTED IN 3 EGG SHELLS

Table 21

Penetration of the shell and shell membranes by Proteus vulgaris G16

Number of eggs	Number of shells penetrated	Incubation at 27° (hr)
8	2	24
	2	48
	-	72
	1	96
	1	120
	1	144
	1	168

Table 22.

The inhibitory effect of albumen on the growth of pseudomonad E in nutrient agar

Medium	Number of colonies at dilutions: 10^{-5}	10^{-6}
Inoculated nutrient agar* (11 ml) containing:		
1 ml distilled water	496	54
1 ml distilled water and 0·05% (w/v) iron citrate	380	36
1 ml albumen	No growth"	
1 ml albumen and 0·05% (w/v) iron citrate	364	42

*, Nutrient agar (10 ml) was inoculated with 1 ml of a dilution of pseudomonad E (G11/1) and poured into a Petri dish containing the supplement.

", Final recording made after 14 days at 27°.

Table 23.

Changes in the appearance of eggs on the 7-11th days following inoculation with Pseudomonas fluorescens CF21*

Incubation at 27° (days)	Egg no.	Log_{10} viable bacteria/ inner membrane of air cell (10 mm disk)	Log_{10} viable bacteria/ 1 ml albumen	Membrane's water-retaining property lost	Fluorescent green pigment: Inner membrane air cell	Fluorescent green pigment: yolk	Fluorescent green pigment: albumen
7	10	7·5327	2·3979	+	-	-	-
8	5	8·1692	4·3010	-	F	F	-
8	9	8·3736	> 6·4771	-	F	F	-
9	1	8·2966	NR	-	F	F	-
9	2	8·2966	> 7·0000	-	F	F	F
9	4	6·9031	NR	-	F	-	-
9	5	8·2966	2·0000	-	F	F	-
9	6	8·2966	> 6·0000	-	F	F	F
9	7	8·5471	> 7·0000	+	F	F	F
10	4	8·5780	6·6021	-	F	-	F
10	8	8·2808	6·7782	-	F	F	E
10	9	8·5441	> 3·0000	-	F	F	F
11	5	5·7337	NR	+	F	-	F
11	6	8·8502	6·6990	+	F	F	E
11	7	8·7959	NT	+	F	-	-

*, Eggs stored with their air cells uppermost. NR, Viable bacteria not recovered. NT, not tested. +, water-retaining property lost. -, water-retaining property not lost, or no reaction with ultra-violet light. F, Fluorescent green pigment occurring in patch(es). E, fluorescent green pigment disseminated throughout albumen.

Table 24.

Recovery of viable organisms from the albumen of eggs whose air cells had been inoculated with Pseudomonas fluorescens CP21

Days at 27°C	Egg no: 1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	(+)
2	-	-	-	1·3010	1·3010	2·3979	1·3979	1·3010		2·0645
3	-	-	-	-	-	-	-	-	-	-
4	-	-	-	2·6532	-	-	-	-	1·6721	1·4314
5	-	-	-	-	-	-	-	-	-	-
7	-	-	2·1913	-	-	-	-	1·6232	-	2·3979
8	-	-	-	-	4·3010	-	>2·6990	1·4914	>6·4771	-
9	(-)	>7·0000	-	(-)	2·0000	>6·0000	>7·0000	1·3010	-	-
10	-	-	-	6·6021	-	-	-	-	6·7782	>3·0000
11	-	-	-	-	(-)	6·6990	NT	NT	NT	NT

Numerals, Log_{10} viable organisms/1 ml albumen, numerals (underlined), fluorescent green pigment in the inner membrane of air cell.

-, viable organisms not recovered from 1 ml albumen. (-), viable organisms not recovered from 1 ml of the albumen of an egg whose air cell membrane was tinted with a fluorescent green pigment. NT, not tested.

Table 25.

Recovery of viable organisms from the albumen of eggs whose air cells had been inoculated with Pseudomonas fluorescens CF 21

Days at 27°	Egg no: air cell uppermost					air cell downward				
	1	2	3	4	5	6	7	8	9	10
1	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	2·6990	-	-	-	-
4	-	-	-	NT	2·1761	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	2·5211
9	(-)	7·1931	3·0000	6·0000	3·0000	-	-	-	-	-
12	-	5·6532	6·5502	7·9294	7·6335	-	-	-	-	-
14	9·0969	8·6990	6·3010	1·3802	8·7540	-	-	-	-	-
16	8·5441	8·6990	9·0914	9·1761	9·0212	-	-	-	-	-
19	9·4314	8·5798	8·5563	8·3979	8·6021	2·2380	8·0000	2·9031	3·0000	2·6021
23	6·6990	8·3979	8·3802	9·0000	7·6990	1·3424	2·8451	2·4771	2·9031	6·1139
30	8·4698	8·6990	8·6990	8·0000	9·0969	(+)	-	(+)	-	3·0000

Numerals, Log_{10} viable organisms/ ml albumen. Numerals (underlined), fluorescent green pigment in the inner membrane of the air cell.
-, viable organisms not recovered from 1 ml of albumen. (-), viable organisms not recovered from the albumen of an egg in whose membrane there was fluorescent green pigment. +, more than 500 viable organisms/ml of albumen. (+), more than 500 viable organisms/ml albumen of an egg in which there was fluorescent green pigment.
NT, not tested.

Table 26.

Changes in the appearance of eggs on the 19th, 23rd and 30th days following inoculation with Pseudomonas fluorescens CF21

Days at 27°	Egg no.	Log_{10} viable organisms/ inner membrane of air cell (10 mm disk)	Log_{10} viable organisms/ 1 ml albumen	Water-retaining property of air cell membrane lost	Fluorescent green pigment: Inner membrane of air cell	Fluorescent green pigment: albumen	Fluorescent green pigment: surface of yolk
19	6	4·0090	2·2380	-	-	F	F
	7	4·0000	8·0000	-	-	-	-
	8	4·7782	2·9031	-	-	-	-
	9	2·6021	3·0000	-	-	-	-
	10	3·6876	2·6021	-	-	-	-
23	6	3·8870	1·3424	-	-	-	-
	7	3·6797	2·8451	-	-	-	-
	8	3·9082	2·4771	-	-	-	-
	9	3·9603	2·9031	-	-	-	-
	10	6·5850	6·1139	-	-	F	F
30	6	6·3011	+	-	F	F	F
	7	4·4150	NR	-	-	-	-
	8	7·0253	+	-	-	-	F
	9	6·3803	NR	-	-	-	-
	10	5·9031	3·0000	+	-	-	-

-, viable organisms not recovered from 1 ml of albumen, or no reaction under ultra-violet irradiation. +, more than 500 viable organisms/ml albumen. F, fluorescent green pigment present. NR, viable organisms not recovered.

Table 27.

Recovery of viable organisms from the albumen of eggs whose air cells had been inoculated with Aeromonas liquefaciens CF10/1

Days at 27°	Egg no: air cells uppermost 1	2	3	4	5	air cells downward 6	7	8	9	10
0	-	-	-	-	-					
1	-	3·3240	-	-	2·6021	-	2·3979	-	-	-
2	-	-	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-	-	-
7	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	3·0569	-	+	-	-
11	-	-	-	-	-	-	-	-	-	-
14	-	-	-	-	-	-	-	-	-	-
18	-	5·6990	6·0000	2·1461	M	-	-	-	-	2·6682
23	8·1139	8·0969	4·3617	8·7482	4·4857	-	-	-	-	-
32	9·0969	9·0607	8·3979	8·6021	9·6435	1·0943	-	-	-	-

Numerals, $\log_{10}$ viable organisms/1 ml albumen. Numerals (underlined), a patch of custard-like material on the surface of the yolk.

+, more than 500 viable organism/1 ml of albumen. -, viable organisms not recovered from 1 ml albumen. M, egg contaminated with a mould.

Age of egg at the time of inoculation: 1 day.

Table 28.

The size of bacterial populations in the inner membrane of the air cells and albumen of eggs inoculated with Cloaca G75/2

Eggs incubated with their air cells uppermost

Incubation at 27° (days)	Log_{10} viable organisms/	Egg no: 1	2	3	4	5
0	Membrane*	-	-	4·4040	4·2218	
	Albumen"	-	-	-	-	
2	Membrane	5·4686	4·3717	6·2024	4·8373	-
	Albumen	-	1·9494	2·5315	2·8621	2·4216
6	Membrane	2·6576	-	-	4·2139	3·9420
	Albumen	-	-	-	-	-
10	Membrane	3·9393	3·0362	8·6118	C	-
	Albumen	-	-	8·3010	C	-
15	Membrane	(9·5000 9·7076)	(9·5000 7·9542)	8·5627	6·9301	6·5850
	Albumen			5·1761	-	4·0000
20	Membrane	5·1871	(9·5850 9·3685)	(9·5000 9·9240)	(9·5000 9·2553)	4·5144
	Albumen	-				3·3979
24	Membrane	(9·4437 10.1303)	(9·4407 9·8751)	(8·0074 9·3522)	(8·8494 9·0212)	(9·5000 9·5119)
	Albumen					

/continued.

Table 38 (continued).

Incubation at 27° (days)	Log_{10} viable organisms/	Eggs incubated with their air cells downward — Egg no: 6	7	8	9	10
2	Membrane	4·5478	-	3·5363	4·1120	3·8303
	Albumen	-	-	1·3010	2·5185	2·9638
6	Membrane	3·9420	4·3011	4·0555	3·3980	4·2258
	Albumen	-	-	-	-	-
10	Membrane	3·4358	4·4513	-	3·4472	3·2597
	Albumen	1·2788	1·2788	-	-	-
15	Membrane	4·4881	-	4·7522	-	2·9829
	Albumen	-	1·2041	-	-	-
20	Membrane	-	2·9829	5·6021	-	-
	Albumen	-	-	-	-	1·3424
24	Membrane	3·3400	2·9655	-	3·0670	-
	Albumen	-	-	-	1·4314	1·9085
29	Membrane	4·8553	3·6990	2·9065	3·6457	NT
	Albumen	3·6128	1·7404	4·6990	3·3729	NT
34	Membrane	-	5·2366	2·9355	3·2158	C
	Albumen	2·0334	3·1303	1·9445	2·8573	C

*, log_{10} viable organisms/ disk (10 mm diameter) of inner membrane of air cell. " log_{10} viable organism/ 1 ml albumen. (numerals), a patch of custard-like material on the surface of the yolk. C, egg contaminated with a mould.

Age of the eggs at the time of inoculation: 3 days

Table 29.

The size of bacterial populations in the inner membrane of the air cells and albumen of eggs inoculated with pseudomonad G11/1

Eggs incubated with their air cells uppermost

Incubation at 27° (days)	Log_{10} viable organisms/	Egg no. 1	2	3	4	5
0	Membrane*	-	-	-	-	NT
	Albumen"	-	-	-	-	NT
1	Membrane	3·8928	3·8373	3·4686	5·2431	4·4560
	Albumen	2·1139	2·6435	-	-	-
2	Membrane	4·3337	4·8416	5·0019	6·0000	6·0000
	Albumen	-	-	-	-	-
7	Membrane	6·0607	7·2431	6·3768	6·2906	5·1250
	Albumen	-	4·7292	-	-	-
9	Membrane	-	4·2511	5·2672	8·5327	5·6990
	Albumen	-	-	-	-	-
14	Membrane	(5·8693	(5·0448	(6·8687	(9·3400	7·2378
	Albumen	4·9031)	-)	3·0000)	7·6946)	-
16	Membrane	(8·2071	(8·9913	(5·2833	(8·8472	(9·3980
	Albumen	6·4624)	6·0000)	-)	5·7404)	5·4698)
23	Membrane	(9·6021	(9·6021	(9·6383	(9·5686	(9·2919
	Albumen	8·6180)	8·5051)	9·6812)	8·9542)	9·4594)
30	Membrane	(7·4172	(9·0000	M	(9·5000	(9·8993
	Albumen	8·2041)	7·1761)	M	8·8751)	7·5441)

/continued

Table 29 (continued).

Eggs incubated with their air cells downward

Incubation at 27° (days)	Log_{10} viable organisms/	Egg no: 6	7	8	9	10
1	Membrane	4·3980	3·9262	5·0512	4·4491	5·1761
	Albumen	-	-	3·3345	-	-
2	Membrane	5·7959	4·9457	5·1928	3·1426	3·9737
	Albumen	-	-	-	1·6232	-
7	Membrane	-	5·2038	2·6775	5·0928	2·3373
	Albumen	-	-	-	-	-
9	Membrane	4·8190	4·8880	5·8037	2·3566 (*)	4·1226 (*)
	Albumen	-	-	-	-	-
14	Membrane	r	M	4·1462 (*)	6·9243 (*)	4·2703 (*)
	Albumen	-	M	-	-	-
16	Membrane	4·6990 (*)	5·5850 (*)	M	5·0335 (*)	3·6990 (*)
	Albumen	-	3·6990	M	1·0414	2·6902
23	Membrane	3·2633 (*)	2·7137 (*)	7·1866	-	2·5051 (*)
	Albumen	0·8451	3·1761	2·6812	3·0253	-
30	Membrane	2·5229	7·5528	2·6847	-	-
	Albumen	-	-	-	-	-

*, log_{10} viable organisms/disk (10 mm diameter) of inner membrane of air cell. ", log_{10} viable organisms/ 1 ml albumen. Bracketed numerals, macroscopic changes in the contents of an egg.

NT, not tested. M, egg contaminated with a mould. -, viable organisms not recovered. (*), 48 hr at 27° required for colony formation.

Age of eggs at the time of inoculation: 1 day.

Table 30.

The size of bacterial populations in the inner membrane of the air cells and the albumen of eggs inoculated with Alcaligenes faecalis G21/3

Incubation at 27° (days)	Log_{10} viable organisms/	Eggs incubated with their air cells uppermost Egg no: 1	2	3	4	5
1	Membrane*	5·7553	6·0000	6·0706	5·6147	5·8110
	Albumen"	-	-	-	-	-
6	Membrane	6·0000	5·5027	7·2542	5·0193	6·0000
	Albumen	-	1·0000	-	-	5·0000
7	Membrane	4·8936	5·5414	2·3373	4·9905	4·8144
	Albumen	-	-	-	-	-
14	Membrane	4·8804	3·5975	3·3980	9·1112	8·9174
	Albumen	2·6990	3·9031	-	8·1761	2·6990
20	Membrane	9·0082	-	7·2932	8·6989	4·6989
	Albumen	8·8129	-	3·0000	9·0531	3·6021
23	Membrane	8·5463	9·5686	9·5686	9·2676	8·9303
	Albumen	9·0000	8·4472	4·0414	7·9777	9·0000

		Eggs incubated with their air cells downward Egg no: 6	7	8	9	10
7	Membrane	5·2001	4·9807	5·1434	4·1081	4·7879
	Albumen	-	-	-	-	-
14	Membrane	3·6100	4·6990	5·6805	4·5563	3·7727
	Albumen	-	-	-	-	-
20	Membrane	3·7324	2·6990	3·8025	5·1932	4·0734
	Albumen	-	-	-	-	-
23	Membrane	2·8696	3·0969	3·5676	5·0147	NT
	Albumen	-	-	-	-	NT

*, log_{10} viable organisms/disk (10 mm diameter) of the inner membrane of an air cell. ", log_{10} viable organism/ 1 ml albumen.

NT, not tested. -, viable organisms not recovered.

Age of the eggs at the time of inoculation: 3 days.

Table 31.

The size of bacterial populations in the inner membrane of the air cells and albumen of eggs inoculated with Aeromonas liquefaciens CF17/1

Incubation at 27° (days)	Log_{10} viable organisms/	Eggs incubated with their air cells uppermost				
		Egg no:				
		1	2	3	4	5
0	Membrane*	3·9378	3·8229	3·2218	NT	NT
	Albumen"	-	-	-	NT	NT
3	Membrane	5·8451	5·8129	5·4394	5·9890	5·9031
	Albumen	-	-	-	-	-
7	Membrane	5·6435	5·0621	5·7715	7·0890	5·9393
	Albumen	-	-	-	-	-
15	Membrane	(9·5134, 10·0969)	(9·2615, 10·0000)	8·8465	7·3980	(9·1700, 9·5502)
	Albumen			5·5502	4·9294	

Eggs not sampled on the 20th and 30th days of incubation because all of them exhibited Black rot type 1 (Haines, 1939).

		Eggs incubated with their air cells downward				
		Egg no:				
		6	7	8	9	10
3	Membrane	5·6532	6·1973	6·0000	6·2566	6·3129
	Albumen	-	-	-	-	-
7	Membrane	5·2065	3·9051	5·0489	4·6118	5·1633
	Albumen	-	-	-	-	-
15	Membrane	5·6264	3·9174	2·6383	3·9543	5·8386
	Albumen	1·0000	5·0000	3·6284	2·1139	3·0792
20	Membrane	5·6831	5·3815	4·5070	2·9355	4·4980
	Albumen	2·5798	3·3802	3·2430	2·7924	3·1303
26	Membrane	-	6·5741	3·6515	5·4771	4·8846
	Albumen	2·4771	1·0000	3·7482	3·6335	3·4771

*, log_{10} viable organisms/ disk (10 mm diameter) inner membrane of air cell

", log_{10} viable organisms/ 1 ml albumen. -, viable organisms not recovered. NT, not tested. Bracketed numbers, Black rot type 1 (Haines, 1939)

Age of eggs at the time of inoculation: 3 days.

Table 32.

Changes in the H-ion and glucose concentrations in the albumen of eggs inoculated with Aeromonas liquefaciens CF10/1

Incubation at 27° (days)	Egg no.	Viable count in membrane*	Viable count in albumen"	Custard-like material on yolk	Water-retaining property of membrane lost	Albumen pH	Albumen glucose (mg/ml)
3	1	7·4393	5.5119	-	-	9·3	2·43
	2	4·3424	6·1461	-	-	9·3	3·40
	3	7·4393	5·9294	-	-	9·3	3·07
	4	7·3010	5·5563	-	-	9·35	3·86
	5C	-	-	-	-	9·4	2·05
	6C	-	-	-	-	9·4	3·11
10	1	6·7782	-	-	-	9·3	1·91
	2	5·2041	-	-	+	9·3	1·63
	3	9·4624	8·1761	+	+	8·6	0·31
	5C	-	-	-	-	9·2	2·00
	6C	-	-	-	-	9·2	1·80
16	1	NT	8·7782	+	+	8·1	NT
	2	9·0128	-	-	-	9·3	1·83
	3	NT	7·0000	+	+	8·7	0·22
	4	NT	8·5441	+	+	8·6	0·14
	5C	-	-	-	-	9·6	1·98
	6C	-	-	-	-	9·6	1·74

*, $\log_{10}$ viable organisms/ disk (10 mm diameter) of inner membrane of air cell. ", $\log_{10}$ viable organisms/ 1 ml albumen.

Eggs no. 1-4 inoculated with 0·1 ml of 18 hr nutrient broth culture.

Eggs 5C and 6C inoculated with 0·1 ml of sterile nutrient broth.

-, viable organisms not recovered, or water retaining property not lost.

+, custard-like material present on the surface of yolk, or the water-retaining property of the membrane lost.

NT, not tested.

Table 33.

The effect of the addition of $FeSO_4$ to albumen on the extent of bacterial multiplication in the inner membrane of the air cell

Organism	Strain	Log_{10} viable organisms/ 20 mm (diameter) disk of inner membrane of air cells of eggs injected with:					
		Distilled water*			$FeSO_4$"		
		Egg no:			Egg no.		
		1	2	3	1	2	3
Alcaligenes faecalis	G21/3	5·8451	5·8751	6·1461	7·8751	7·6021	8·3324
	G32/1	-	4·5441	4·9294	8·0212	8·6532	8·3324
pseudomonad E	G8/1	6·7782	4·1761	-	9·4771	8·8451	9·3979
	G11/1	5·8451	5·6607	5·2430	6·2068	9·0414	8·3541
Pseudomonas fluorescens	CF21	-	4·8751	4·3010	8·4393	8·9294	8·0212
	G10	-	3·9031	3·7782	5·2041	8·4393	5·0969
Proteus vulgaris	G14	3·9542	4·0969	4·6990	8·0969	7·8451	7·9542
	G16	6·4624	7·0414	B	8·2788	8·3424	8·1761
Cloaca	G75/2	3·1761	5·3010	-	7·9294	6·6021	7·8129
Aeromonas liquefaciens	CF17/1	6·9294	6·2900	6·9777	8·6021	9·0414	B

The eggs were incubated for 5 days at 27° before the addition of $FeSO_4$ to the albumen and colony count was made 2 days after the addition of the $FeSO_4$. *, 0·1 ml of distilled water injected into the albumen. ", 0·1 ml of a 2·5% (w/v) aqueous solution of $FeSO_4$ injected into the albumen. -, viable organisms not recovered. B, egg broken.

Table 34.

Changes in eggs produced in 42 days at 27° by different bacterial species

Organism	No. of strains	Time at which rot was first detected by candling (days)	Type of rot'	Salient features of rot.
Aeromonas liquefaciens	8"	8-12	Black rot type 1 (a)	Gelatinous yolk blackened throughout. Grey albumen. Strong odour of H_2S.
Proteus vulgaris	4	18-24	Black rot type 2 (a)	Dark brown mealy yolk, Dark brown white.
Pseudomonas fluorescens				
proteolytic	3	12-24	Pink rot (a)	Mealy yolk, turbid, orange albumen.
non-proteolytic	2	42	Fluorescent green rot (b)	Fluorescent green albumen.
pseudomonad E	4	24-42	Green rot (b)	Gelatinous amber-like yolk with olive-green peripheral stripes. Strong almond-like odour.
Cloaca				
proteolytic	6	12-36	Custard rot (b)	Yolk encrusted with custard-like material.
non-proteolytic	8	-	Colourless rot	None
Citrobacter	7	-	Colourless rot	None
Alcaligenes faecalis	4	-	Colourless rot	None

The eggs were inoculated by the method of Florian & Trussell (1957).

', The rots were identified with the descriptions of Haines (1939) - marked, (a) - or Florian & Trussell (1957) - marked, (b).

", Pseudomonas fermentans L417 (von Wolzogen Kuhr, 1932) and Aeromonas hydrophila included.

Table 35.

Presumptive correlation of the salient features of rotten eggs with metabolic attributes of the causative organisms

	Metabolic attribute				Salient feature of rot
	Proteolytic	H_2S" Produced	Lecithinase produced	Pigment produced	
Aeromonas liquefaciens	+++	+++	(+)	-	Gelatinous yolk blackened throughout. Grey watery albumen.
Proteus vulgaris	+++	+++	-	-	Dark brown mealy yolk Dark brown albumen.
Pseudomonas fluorescens	+	-	+	+	Mealy yolk, fluorescent green albumen.
	-	-	-	+	Fluorescent green albumen.
pseudomonad E	+	+	-	-	Gelatinous amber-like yolk striped with olive-green pigment.
Cloaca	(+)	(+)	+	-	Yolk encrusted with custard-like material.
	-	-	-	-	No change in infected egg.
Citrobacter	-	-	-	-	No change in infected egg.
Alcaligenes faecalis	-	-	-	-	No change in infected egg.

+, +++ , positive reaction. -, negative reaction. (+), although positive, the property did not appear to be concerned with the rotting of an egg. ", peptone water (1%, w/v) containing a piece of heat-coagulated albumen was used to test for H_2S production.

Table 36.

A proposed scheme for the classification of eggs infected with bacteria

Type of change produced in egg		'Dominant' organism	
A. ROTS	Black rot type 1	*Aeromonas liquefaciens*	
	Black rot type 2	*Proteus vulgaris*	
	Fluorescent green rot	*Pseudomonas fluorescens*	
	Pink rot	*Pseudomonas fluorescens*	
	Green rot	pseudomonad E	
	Custard rot	*Cloaca*	
	Red rot	*Serratia marcescens*	
	Colourless rot	*Alcaligenes faecalis*, non-proteolytic *Cloaca*, *Citrobacter*	
B. OFF-ODOURS		*Achromobacter perolens*	'
		Pseudomonas putida	
		Pseudomonas ovalis	
		Pseudomonas shuyllkilliensis.	
		Aerobacter cloacae	
		Pseudomonas spp.	
		Alcaligenes spp.	
C. OFF-FLAVOURS		*Escherichia coli*	"
		Pseudomonas spp.	

' and ", the investigators who isolated these organisms are listed in Table 7.

SUMMARY

A collection of bacteria was assembled from eggs which had rotted on the premises of the producer and others which had been induced to rot in the laboratory at 10°, 20° or 30°. The majority of isolates were Gram-negative rods. Representatives of the numerically predominant types were examined in detail. The following species were recognized: Alcaligenes faecalis, Aeromonas liquefaciens, Proteus vulgaris, Cloaca sp. Citrobacter sp. and Pseudomonas fluorescens. A non-fluorescent pseudomonad (pseudomonad E) could not be identified at species level.

Representatives of these organisms multiplied in whole shell membranes suspended in a solution of mineral salts. The organisms divided into 2 groups: a) Ps. fluorescens, Pr. vulgaris and Alc. faecalis which produced no definite turbidity, and b) Aer. liquefaciens, pseudomonad E, and, occasionally, a proteolytic strain of Cloaca which produced a definite turbidity. The organisms of the latter group differed from those of the former in showing the ability to form a protease during growth in the membrane suspensions. This synthesis was associated with the loss of pigment from the membrane and the appearance in the suspending medium of products of protein degradation.

Bacteriological and chemical methods were used to follow the course of infection in eggs, incubated at 27°, whose air cells had been inoculated with a washed bacterial suspension. The pattern of multiplication was essentially similar for all the organisms tested. In the 48 hr following inoculation a primary phase of multiplication occurred in the inner membrane of the air cell and a small number of migrants appeared in the albumen. The extent of this multiplication appeared to be limited by the availability of iron in the shell

membrane. This phase of multiplication was followed by a period during which there was a decline in the number of viable organisms in the membrane. The extent of this decline appeared to be determined by inherent properties of the organisms and its duration was determined by the position of the egg during incubation. In the case of eggs whose air cells were uppermost, a renewed multiplication occurred when the yolk made contact with the inner membrane of the air cell. This phase was iniated on the 12-20th day following laying. The secondary phase of multiplication was associated with changes in pH and glucose concentration of the albumen and, in the case of pigment-producing and/or proteolytic bacteria, with the first macroscopic effects on the contents of the egg. In the case of eggs with their air cells downward, the number of viable organisms in the inner membrane of the air cell continued to decline for a longer period. In this case the albumen remained free from contamination for a period that varied with the organism. One strain of Aer. liquefaciens produced heavy contamination of the albumen on the 15th day following inoculation whereas with another strain of the same species the albumen was not grossly contaminated on the 32nd day following inoculation. What is responsible for this contamination when the yolk does not make contact with the inoculated membrane has not been explained, but two possibilities suggest themselves: a) the induction of multiplication by changes in the properties of the albumen, and b) the capacity of organisms to travel to the surface of the yolk.

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APPENDIX

Nutrient broth: (g/l.), peptone (Evans), 10; meat extract (Lemco), 10; dissolved in tap water and sterilized by holding momentarily at 22lb/in^2. pH, 7·0.

Nutrient agar: the above solidified with 1·5% (w/v) agar (Davis, New Zealand).

Sterile albumen. The shells of newly laid eggs were scrubbed with a proprietary egg-cleaning compound ("Ovosan", British Extract Co. (Sales) Ltd., England). They were soaked for 3 min in a 0·5% (w/v) aqueous solution of "Cetavlon" (I.C.I., Manchester, England). An egg was held in a pair of beaker tongs - Fisher pattern - (Griffin & George Ltd., England) and then dipped into 95% ethanol. The ethanol on the shell was ignited. The shell at the equator of the egg was punctured with a sterile scalpel and the opening was enlarged by applying gentle pressure with the tongs. The egg was then placed (long axis horizontal) on the crystallizing dish in the apparatus depicted in Fig. 7. The albumen slowly drained from the shell but the yolk did not escape. The albumen from several eggs was removed to a 200 ml Erlenmeyer flask containing several glass beads and mixed.

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