OBSERVATIONS ON THE BACTERIAL FLORA OF THE HEN'S EGG, WITH A DESCRIPTION OF NEW SPECIES OF *PROTEUS* AND *PSEUDOMONAS* CAUSING ROTS IN EGGS

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Introduction

During storage trials with eggs, an account of which has been given elsewhere (Moran, 1937), a certain proportion of the eggs developed rots. In order to follow the mechanism of the production of rots, and as part of the assessment of the value of the different methods of storage, examination was made of the bacterial flora of these eggs at the beginning and the end of storage. In addition, a number of rots in imported Australian and New Zealand eggs have been examined bacteriologically, and the organisms isolated compared with those obtained from rots in English eggs, and with *Proteus melanovogenes*[1] previously isolated by Miles & Halnan (1937) from black rot in South African eggs.

Bacterial flora of the new-laid egg

The majority of the eggs were sampled immediately on receipt from the farms, but in a few cases samples were not taken until after candling and grading. No significant difference in the bacterial flora of the eggs was detected between those examined immediately and comparable batches allowed to stand for 4 days at room temperature while candling and grading were carried out.

Bacteriological examination of the shell was carried out as follows. The egg was cracked, the contents discarded, and the shell together with the shell membrane dropped into a wide-necked, sterile bottle. Previously a stout glass rod and glass beads had been put in the bottle. The shell was pounded up with a rod, then shaken vigorously for 5 min. with glass beads and added saline (generally 20 c.c.). Preliminary tests showed that no further organisms were removed from the shell by continuing the shaking for a longer period. Suitable volumes of the saline suspension were then plated, using nutrient agar *p*H 7·4 and malt agar *p*H 5·5–6·0. Plates were incubated, at least in duplicate, at 20 and 37° C. No anaerobic tests were made on the shells, nor were any enrichment methods for particular organisms used. 130 shells were examined at the commencement of storage.

For bacteriological examination of white and yolk, the egg was first soaked for 5 min. in 3% Lysol. It was then well rinsed, soaked in alcohol, the alcohol burned off, and the shell thoroughly flamed on a glass triangle. Triangle and egg were transferred, without handling the egg, to a closed glass inoculating chamber fitted with rubber sleeves. The egg was dropped into a sterile dish and cut open. The shell was discarded. With specially made wide-ended

[1] Kindly supplied by Dr Miles.

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pipettes, 2 c.c. quantities of white and yolk were separately removed to Petri dishes. Before pipetting the white, thick and thin white were well mixed by beating with the pipette. As a routine procedure plates were made in which the white or yolk was emulsified with the agar and also in which the white or yolk was poured on to previously solidified agar. The latter method was found preferable with the yolk, since after emulsification of yolk with agar, the numerous globules were very difficult to distinguish from bacterial colonies, apart from the laborious method of picking off and examining microscopically. Nutrient agar *p*H 7·4 and malt agar *p*H 5·5–6·0 were used, plates being incubated at 20 and 37° C. In addition, deep stab inoculations in Veillon agar were made to test for the presence of anaerobes. These were later abandoned, since on no occasion was anaerobic growth obtained from white or yolk, in samples from about sixty eggs.

About 300 samples each of white and yolk were taken from 112 eggs at the beginning of storage, and similar numbers were examined after 6 and 9 months, except that the yolks of the latter were not examined. The results are summarized in Table I. It was found that, in general, about 75 % of the plates poured were free from growths of any kind. Approximately a further 17 % contained a few colonies ($\leqslant 3$), chiefly mould fungi or common saprophytes such as *B. vulgatus*, and these were assumed to be contaminants. Finally, a small number of the plates showed growths such as micrococci, sporing rods and mould fungi, thought to be too numerous or too characteristic to be contaminants. These may have represented actual infections in the egg. It is possible, of course, that an egg from which no growths were obtained was not absolutely sterile, since the total volume of the samples, neglecting the anaerobic tubes, was 6 c.c. of white and yolk respectively, the average volume of white and yolk in the egg being about 28 and 16 c.c. respectively. In view of the number of eggs sampled, however, this possibility is neglected. It is also possible that the white contained organisms unable to develop owing to the inhibitory action egg-white has been shown to exert (Rettger & Sperry, 1912). Experiments in which a number of organisms were seeded on to agar containing egg-white in the proportion used in the routine examinations negatived this possibility, since good growth was obtained in all cases. Growth was, however, slow in some instances, so that as a routine procedure all plates were incubated for at least a week. In Table I eggs from which the plates showed no growths, or less than four growths, are classed as "probably sterile", the remainder as "possibly infected".

The results suggest that a high proportion, 98 %, of the whites of new-laid eggs are sterile. The number of infected yolks is possibly slightly greater, 93 % being adjudged to be sterile. Apparently little change took place in the number of infected yolks during storage, but there is probably some increase in the number of infected whites. The magnitude of the infection in a given egg may, of course, be much greater at the end of storage, some of the plates showing several thousand colonies. The organisms isolated from "possibly infected" eggs at the beginning of storage included Gram-positive sporing rods, mould fungi, and certain Gram-positive cocci. Organisms of the *Proteus*, *B. coli* and *Pseudomonas* groups have not been found in the white or yolk of

the fresh egg. Their entire absence cannot be taken as shown, however, without the use of enrichment methods, since they might be overgrown by the more luxuriant types. About 4 % of the eggs examined after 9 months' storage were heavily infected with *Pseudomonas*.

Table I. *Bacterial content of white and yolk of eggs, new-laid and after storage*

Time	No. of samples		Percentage of eggs: Probably sterile	Percentage of eggs: Possibly infected
Beginning of storage	316 samples white and 301 samples yolk from 112 eggs	White	98	2
		Yolk	93	7
6 months' storage at 0° C.	399 samples each of white and yolk from 133 eggs	White	92	8
		Yolk	90	10
9 months' storage at 0° C.	268 samples of white from 268 eggs	White	92	8

Counts were made on the shells at the beginning of storage and after 6 months. The results are summarized in Table II. There was a big variation in the number of organisms per shell, sometimes of the order of a hundredfold, but no significant difference was found in the mean count of eggs from five different farms. The count on the shells of eggs from battery-bred hens was not appreciably lower than on those from clean eggs of hens on grass on the same farm. After 6 months' storage at 0° C. there was no significant difference in the counts in the different gas mixtures at a given humidity,[1] and hence the mean of all the shells examined is given in the table. There did appear, however, to be a difference between those shells stored in a saturated atmosphere and those stored at 80–85 % relative humidity. The mean count on all the shells examined from eggs stored in a saturated atmosphere did not differ appreciably from the initial count, but a few individual shells showed high counts, due mainly to *Pseudomonas* organisms. On the shells of eggs stored at 85 % relative humidity the counts tended to be smaller, indicating death of some of the bacteria during storage.

Table II. *Bacterial counts on egg-shells, new-laid and stored*

Age of egg	No. of shells	Conditions of storage	Mean count per shell: 37° C.	Mean count per shell: 20° C.	Mean count per shell: 20° C. *p*H 5·8	Maximum count: 37° C.	Maximum count: 20° C.	Maximum count: 20° C. *p*H 5·8
New laid	130	—	35,000	130,000	13,000	$1{\cdot}6 \times 10^6$	8×10^6	200,000
6 months' storage	100	0° C. saturated atmosphere	24,000	545,000	71,000	400,000	40×10^6	4×10^6
	60	0° C. 80–85 % relative humidity	9,000	17,000	8,000	40,000	200,000	40,000

In order to gain some idea of the flora present on the shells, about 100 organisms were picked off the plates at random, subcultured, and their morphological and biochemical characteristics studied in sufficient detail to

[1] The gas mixtures were 100 % CO_2, 60 % CO_2, 85 % CO_2 + 15 % N_2, at 100 % relative humidity, and air, 2·5 % CO_2, 5 % CO_2 and 10 % CO_2 at 80–85 % relative humidity.

assign them tentatively to their respective genera. No attempt was made to establish the identity of the organisms absolutely. The composition of the flora thus obtained from the shells (capable of growth on nutrient agar *p*H 7·4 at 20° C.) is shown in Table III. It may be seen that the flora is very heterogeneous. The coliform and *Proteus* organisms were atypical, most of them growing poorly if at all at 37° C.

Table III. *Approximate composition of the flora obtained in washings from egg-shells on nutrient agar* pH 7·4 *at* 20° C.

Non-sporing rods 38%	*Achromobacter*	19%
	Pseudomonas	6%
	Alkaligenes	4%
	Coliform	4%
	Flavobacteria	3%
	Proteus	1%
	Aerogenes	1%
Sporing rods 30%	*B. vulgatus*	12%
	B. subtilis	10%
	Unidentified	6%
	B. mycoides	2%
Cocci 25%	Micrococci	18%
	Staphylococci	5%
	Sarcinae	2%
Yeasts 4%		
Actinomyces 3%		

Disregarding genital infection, which has been shown to occur (Fromme, 1934), it is obvious that the hen's egg is exposed to external infection from a wide variety of sources, the three chief being presumably faeces, manure, and the soil. Infection from the last two is likely to be particularly undesirable in the case of eggs that are to be cold-stored, since species from these sources frequently grow comparatively rapidly at 0° C. and are the chief cause of spoilage of other animal tissues in cold store (Haines, 1933*a*). It is clear that the egg carries with it on its shell a load of potential rot-producing organisms, so that its successful storage will depend on the maintenance intact of its natural defence mechanisms, reinforced possibly with artificial ones.

EXAMINATION OF ROTS

Several hundred rots in imported eggs from New Zealand and Australia and in English eggs, have been examined. The eggs were first candled, then broken open into sterile 15 cm. Petri dishes, and, where desired, cultures made into broth or agar. From the point of view of appearance, the rots may conveniently be grouped into the following classes.

Black rot type 1

On candling, the egg is dark and opaque, in the worst cases transmitting scarcely any light at all. If the shell is cracked without breaking the shell membrane, the latter can sometimes be seen to be distended with gas, the pressure occasionally being sufficient to burst the membrane and scatter the

contents. On breaking, the yolk is seen to be hard, black and solid. The white is entirely liquefied, sometimes turbid, granular and occasionally light brown or greenish brown. The odour is faecal. In the later stages of the *Proteus melanovogenes* type of black rot, the yolk is always black, hard and compact, and blackening sets in very rapidly. The black rot obtained with other strains of *Proteus* is often softer, the yolk consisting of an outer "custard-like" layer with a black core, especially in the early stages.

Black rot type 2

Over the lamp, the yolk appears a liquid opaque mass, floating freely in the white. When opened, the white appears liquefied, fluorescent green or greenish brown, sometimes granular and viscous, and the yolk a soft greenish black mass. All stages of blackening, beginning at the periphery of the yolk, may be found. The odour varies, being sometimes putrid: occasionally there is a penetrating "saccharine" smell, sometimes a strong "cabbage-water" smell.

This type of rot may be due to infection of the egg with certain species of *Pseudomonas* bacteria. Not all the members of this genus are equally active in this respect: many of them produce a green rot without any tendency to blackening, however long the egg is incubated. Such rots are among the most difficult to classify at sight: inoculation into suitable enrichment media from eggs with green whites and slightly blackened yolks will often show the presence of *Proteus* bacteria, in which case it is assumed that they are responsible for the blackening. On the other hand, certain strains of *Pseudomonas* by themselves injected into fresh eggs produce some blackening.

Red or pink rot

After candling, eggs of this type may be rejected as early black rots, or a pronounced reddish tinge may be seen, especially in the yolk. Subsequent examination discloses coloured patches in the white, varying from golden brown through rusty red to deep blood red. The yolk membrane is opaque, in places dense white, or often bright pink, and brittle. The white may be entirely liquefied, or viscous and greyish. The smell varies from "crayfish" to "cabbage water".

Green rot

Over the lamp, the yolk is seen to be floating more freely than in a fresh egg, but may often not be appreciably opaque, and the air space can still be distinguished. If the egg is swung over about its short axis a greenish tinge may sometimes be observed as the air space comes round. Green rot in its early stages often escapes detection entirely, however, by the candler, and hence is particularly insidious from a commercial point of view. At first in a green rot, the white is bright yellowish green and fluorescent. The yolk may not be appreciably affected. In later stages the yolk membrane becomes thick, white and opaque, and subsequently some blackening may or may not be

obtained. Green rot is due to infection of the white with species of *Pseudomonas*. Comparatively few species of *Pseudomonas* give blackening, however, so that many of the green rots never become black.

Miscellaneous rots

Spots of varying degrees of opacity are often seen over the lamp. Apart from "blood spots" of physiological origin, these are generally due to infection either with bacteria or mould fungi, and subsequently develop into rots. A type of red rot in its early stages consists of *Sporotrichum* spreading along the chalazae to the yolk. Occasionally opaque patches on the yolk, beneath the membrane, appear to be due to the growth of bacteria or yeasts within the yolk.

Of the above rots, green rot is by far the most common.

BACTERIAL ISOLATIONS FROM ROTS

In general, the egg was opened into a sterile 15 cm. Petri dish, a few drops of the white or yolk removed with a pipette, emulsified, and suitable dilutions plated on nutrient agar. (In many of the more advanced rots it was impossible to separate white from yolk and a little of the mixture was emulsified.) Plates were always incubated both at 20 and 37° C. Broth cultures were sometimes made before plating.

The bacterial content of rotten eggs was usually of the order of 10^6 organisms per c.c. on agar at 20° C., considerably less and sometimes nil on agar at 37° C. After incubation, a selected plate was worked over, and a representative of each type of organism discernible by macroscopic and microscopic examination picked off and subcultured. The subcultures were then examined in detail by morphological and biochemical tests. When any doubt as to the purity of the subculture arose, it was replated.

Briefly, almost all the rots examined contained organisms of the *Pseudomonas* group. The black rots appeared to contain the most complex flora, in addition to *Pseudomonas*, organisms placed in the genera *Proteus*, *Coli*, *Alkaligenes*, and *Aerogenes* having been found. Green rots in most cases seemed to contain mainly *Pseudomonas* bacteria, *Achromobacter* and certain sarcinae and cocci being sometimes associated with them. Occasionally sporing rods and yeasts have been isolated.

In these cases of advanced rotting, containing a heterogeneous flora, it was clearly impossible to decide *a priori* which were the significant organisms, and which merely adventitious. A series of inoculation experiments with fresh eggs was therefore made. The technique was as follows. An area of about one square centimetre on the side of a clean, fresh egg, just below the air space, was painted with an alcoholic solution of iodine and allowed to dry. The shell was then bored in the centre of this area with a sharp steel needle, and about 1 c.c. of a 24 hr. broth culture forced into the egg from a Pasteur pipette, into the

white but not breaking the yolk membrane. The site of inoculation was then swabbed and sealed with wax, and the egg incubated. Controls inoculated with sterile broth were put up at the same time. The progress of rotting could then be followed by periodical candlings. In general, marked rotting developed at 20° C. in about 8–14 days, and at 0° C. in about 5 weeks, the controls remaining unaffected. Experiments of this type showed that the organisms responsible for definite rotting could be limited to two groups, *Proteus* and *Pseudomonas*. Turbidity and slightly unpleasant "fishy" odours were sometimes produced by coliform and other organisms, but attention has been confined to the definite rots produced by *Proteus* and *Pseudomonas* bacteria. The biochemical characteristics of these and the coliform organisms have been studied in some detail and are described subsequently. Table IV contains a selection of the results obtained by inoculation experiments. In these the candling was done independently by an expert candler. The classification of rots in the trade is done

Table IV. *Examples of type of rot produced by artificial inoculation with pure cultures previously isolated from rotten eggs*

Organism	Time of incubation	Temperature of incubation ° C.	Candling	Examination of opened egg
Proteus melanovogenes	9 days	20	Black rot	White liquefied, yolk soft and blackened. Putrid smell
	14 days	20	Advanced black rot. Opaque	White entirely liquefied, turbid, yolk hard, solid, shrunken, dense black. Faecal odour
SE 60 *Proteus*	9 days	20	Black rot	White liquefied and yolk blackened. Putrid smell
	14 days	20	Advanced black rot	White entirely liquefied, turbid, yolk soft, black, with thick "custard" envelope round blackened core
SE 66 *Proteus*	9 days	20	Red rot	White liquefied, yolk soft with opaque spots on membrane. No blackening.
	14 days	20	Black rot	White entirely liquefied, turbid, yolk liquid, partly black
SE 47 *Pseudomonas*	9 days	20	Early black rot	White liquefied, opaque patches on yolk, no blackening. White fluorescent
	14 days	20	Early black rot	White entirely liquefied, bright green and fluorescent. No blackening
SE 49 *Pseudomonas*	9 days	20	Early black rot	White partially liquefied, turbid
	14 days	20	Early black rot	White liquefied, pink colour, yolk slightly black round periphery
SE 82 *Pseudomonas*	9 days	20	Early black rot	White liquefied, turbid
	14 days	20	Black rot	White entirely liquefied, bright bluish green, turbid, yolk liquid, partly blackened. Putrid smell
SE 49 *Pseudomonas*	3 months	0	Red rot	Bright pink to red colour in yolk and golden red in white. Some blackening
SE 47 *Pseudomonas*	3 months	0	Black rot	White turbid, reddish, thick opaque yolk membrane, some blackening. "Cabbage-water" smell

practically entirely by candling: obviously there cannot be complete correlation between a classification arrived at by candling and a classification on the basis of the opened egg: in the former case any type of decomposition rendering the yolk opaque will appear over the lamp as a black rot.

Taking into consideration the isolations from typical cases, together with the effects obtained by inoculation with pure cultures, the organisms responsible for the types of rot listed previously have been found to be as follows.

Black rot type 1

Caused by *Proteus melanovogenes*,[1] and *Proteus* strains SE 45, 60, 66, and 68.

Black rot type 2

Caused by *Pseudomonas* strains SE 103, 104, 87, 8, 49.

Red rot

Generally caused by *Pseudomonas* strains, but some commercial red rots are due to infection with mould fungi (especially *Sporotrichum*), red rots by inoculation obtained from *Pseudomonas* strains SE 82, 63, 18, 4, D, 28, 67.

Green rots

Always caused by a strain of *Pseudomonas*. Strains SE 64, 16, 49, 75, 47, 48 etc., have been isolated from typical cases. *Pseudomonas aeruginosa* (*B. pyocyaneus*) also produces a green rot on inoculation.

THE MECHANISM OF THE PRODUCTION OF ROTS

The foregoing experiments suggest that most eggs carry on their shells bacteria capable of producing rot. The number of rots in commercially stored eggs, although it may be large in the aggregate, in any given batch is not generally more than 5%, and is often less. It follows that the egg must possess good defence mechanisms against bacterial invasion, which are absent or break down in certain cases. Three "natural" internal defences in the fresh egg are known, only one of which is capable of being controlled artificially at present. First, although the *p*H of the white of the fresh egg just from the oviduct is about 7·6 it rapidly changes to about 9·5, and becomes therefore unfavourable for bacterial growth. Apart, however, from effects of *p*H and the factors mentioned below, egg-white is apparently inhibitory towards certain organisms (Sharp & Whittaker, 1927). The *p*H can be controlled by storage in different partial pressures of carbon dioxide (Moran, 1937). Secondly, egg white contains lysozyme which inhibits the growth of, and may destroy, certain organisms (Fleming, 1929). Thirdly, the native proteins of

[1] *Proteus melanovogenes* itself has not so far been found in the Australian or New Zealand imported eggs examined here. The strain used was supplied by Dr Miles. For discussion of the relationship of that organism to the above strains of *Proteus* see page 350.

egg-white are very resistant towards bacterial attack, and it is only when protein breakdown material or other simple source of nitrogen is added that bacteria flourish and produce further decomposition (Bainbridge, 1911). Should any enzymic breakdown of the proteins occur, it will therefore make bacterial growth easier. The above are complex processes and nothing is known of the way in which they may operate in the commercial handling of eggs. Three obvious external factors which lend themselves to simple experimentation are (1) the effect of the coating of mucin (Moran & Hale, 1936) deposited round the shell during laying, (2) the effect of the deposition of moisture on the shell, (3) variation in porosity of the shell. Experiments have been carried out on the first two of these points. In addition, in dealing with cold or refrigerated gas-stored eggs, it is necessary to consider the temperature relationships of the rot-producing organisms, and the concentrations of carbon dioxide inhibiting their growth.

The effect of washing on the bacterial penetration of eggs

Examination of the treatment given to dirty eggs in various packing stations, whether by hand or machine, showed so much variation that it was clearly impossible to reproduce a representative technique in the laboratory. Attention has therefore been confined to an attempt to answer specific questions.

Two experiments were carried out to investigate the effect of washing: one a small scale experiment with 140 first class quality clean Rhode Island White × Sussex eggs, the other a larger scale experiment with 1000 soiled eggs of second quality. In each the eggs were first washed under defined conditions, then exposed to a known bacterial infection for a given time, and finally incubated and examined. In certain cases some were opened immediately and plate cultures made.

Experiment 1

Washing consisted of putting in warm water for one minute, scrubbing for one and a half minutes with brush and soap, rinsing well in distilled water, and wiping dry with a clean glass-cloth. Soaking consisted of immersing for a stated period in an aqueous suspension containing about 5×10^4 *Pseudomonas* bacteria per c.c., after which the eggs were drained and dried on a towel.

The results are summarized in Table V.

Table V. *Effect of washing on bacterial penetration of eggs*

First class quality Rhode Island White × Sussex eggs, clean and taken direct from farm. Soaking treatment immersion in bacterial suspension, 5×10^4 per c.c.

Treatment	Plate cultures made immediately after treatment (% positive)	% rots after 14 days incubation at 20° C. (80% relative humidity)
Untreated controls	0	0
Washed controls	0	0
1 hr. soaking, washed	10	40
1 hr. soaking, unwashed	0	0
12 hr. soaking, washed	10	70
12 hr. soaking, unwashed	0	0

Although the numbers of eggs used above were too small for final conclusions, the results indicate that the fresh egg possesses a coating which is extraordinarily resistant to bacterial penetration, since such eggs can be soaked for a period of 12 hr. in a dense bacterial suspension, without any bacterial penetration. Washing the egg apparently removes this coating, so that even one hour's soaking leads to a large percentage of rots. The difference between the figures for the eggs examined immediately (plate cultures) and those incubated is interpreted to mean that the bacteria had penetrated the shell, but had not got through the shell membranes into the white, when the examination was made. Another interpretation is possible, namely, that further penetration of the washed shells, by bacteria left on the shell, occurred during storage, but this is unlikely since the eggs were stored at a low humidity ($\leqslant 80\,\%$).

Experiment 2

The experiment was repeated, using 1000 soiled eggs of second class quality. Several types of washing treatment were given, viz., wiping quickly with water and cloth, water and soda (ordinary washing soda solution *p*H 10), rubbing with steel wool + sand + water, and steel wool + sand + soda. In all cases clean running water was used, and the cloth was thoroughly rinsed and cleansed at every third egg. The results are summarized in Table VI. No plate cultures were made in this case, but the eggs were incubated for 1 month at 20° C. and 80 % relative humidity before being candled and opened.

Table VI. *Effect of washing on bacterial penetration of eggs*

Second quality soiled eggs. Soaking treatment 20 sec. immersion in bacterial and fungal spore suspension. (10^6 bacteria per c.c. and $0{\cdot}5 \times 10^6$ fungal spores per c.c.)

Treatment	Not soaked % rots	Soaked % rots
Not washed	1	13
Washed water + cloth	0	19
Washed soda + cloth	5	19
Washed steel wool + sand + water	0	31
Washed steel wool + sand + soda	1	31

These results support those obtained previously, that washing under clean conditions has no effect on immediate bacterial penetration by bacteria already present on the shell, even with dirty eggs. Washing does, however, remove a protective coating, rendering subsequent bacterial penetration very much easier when the washed egg is exposed to any further bacterial infection. The more vigorous the washing the greater the effect. The quality of the egg is also an important factor. Even 24 hr. soaking in a dense bacterial suspension failed to effect any bacterial entry into first quality unwashed eggs. 20 sec. soaking, on the other hand, with second quality eggs, gave an infection such that 13 % of the eggs rotted in incubation. Although the eggs are not, of course, graded on the quality of the shell, it is possible that variation in the porosity of the shell is the factor here implicated.

In these experiments, to make the results as clear-cut as possible, the resistance of the washed egg to subsequent bacterial infection, as compared with the unwashed egg, was tested by immersing the egg in aqueous suspensions of the organisms. It seems likely that in commercial practice "sweating" has a similar effect. Jenkins, Hepburn, Swan & Sherwood (1920) in fact found that in certain cases "sweating" raised the percentage of rotting from 2·4 to 12·5.

The effect of carbon dioxide on the growth of *Pseudomonas* sp.

Previous experiments with strains isolated from other animal tissues indicated that the genera *Achromobacter* and *Pseudomonas* were fairly susceptible towards carbon dioxide, *Proteus* and *Coli* being comparatively resistant (Haines, 1933*b*). Certain eggs examined in the present experiments suggested that some strains of *Pseudomonas* might be more resistant. Accordingly a series of subcultures on the surface of agar in Petri dishes were set up in desiccators containing water and the required concentration of carbon dioxide, checked by daily analysis. The concentrations of carbon dioxide used were 60 and 80%, and tests have been made at 0, +5, +10, +15, and +20° C. Two strains of *B. pyocyaneus*, sixteen strains of *Pseudomonas* isolated from eggs, one strain of *Pseudomonas* isolated from dung, and stock strains of *B. fluorescens liquefaciens* and *non-liquefaciens* were used as the test organisms.

At 20° C. most of the controls had grown fully in 1 day and all in 2 days, while in 80% carbon dioxide fair growth was obtained in 4 days and good growth in 6 days with all the organisms except *B. fluorescens liquefaciens* and *non-liquefaciens*. These data suggested that growth might occur at lower temperatures in atmospheres enriched with carbon dioxide. A complete analysis has not yet been made, but so far growth has not been obtained, at 0, +5, or +10° C., with the above species, in either 60 or 80% carbon dioxide, in periods up to two months. Growth has, so far, been completely inhibited at those temperatures, but the organisms were not destroyed. After 2 months in 80% carbon dioxide at 0° C., all the strains tested grew well on putting the plates into air at 20° C. At +15° C., on the other hand, all the organisms grew well in 4 days in both 60 and 80% carbon dioxide, excepting *B. fluorescens liquefaciens* and *non-liquefaciens*. The corresponding controls were fully grown in 1–2 days.

Since organisms of the genus *Pseudomonas* are one of the most prolific causes of rotting in eggs, it follows that careful temperature control in addition to gas storage is required for the large-scale preservation of eggs.

The growth of rot-producing organisms at low temperatures

All the strains of *Pseudomonas* isolated from eggs grew well on artificial media at 0° C. Injected into eggs, the early stages of rotting could be detected after 3–4 weeks at 0° C. In 3 months at 0° C. marked decomposition had taken

place with some strains, leading to offensive odours, liquefied white and decomposed yolk. In 6 months at 0° C. an advanced stage of decomposition had been reached, the white being entirely liquefied, turbid, the yolk broken down and in some cases partly blackened or greenish black.

On agar *Proteus melanovogenes* sometimes grew scantily at 0° C., at other times not at all. It did not grow well at +5, but fairly well at +10° C. Inoculated into eggs, slight blackening on the periphery of the yolk was obtained after 6 months' storage, but the rotting so obtained was very different from the hard black yolk given by this organism at 20° C. The other strains of *Proteus* isolated all grew fairly well at 0° C. on agar; definite rotting was produced at 0° C. in 3 months, but the amount of rotting was slight compared with that obtained in a few weeks at 20° C.

Eight out of the eleven coliform strains isolated grew well on agar at 0° C.: several of these multiplied in eggs at that temperature. No definite rotting was produced: the white became turbid and a strong "fishy" odour developed.

On balance the most troublesome organisms in eggs stored at 0° C. belong to the genus *Pseudomonas*: whenever pronounced black rotting of the *Proteus* type is found in cold-stored eggs it is probable that at some stage the eggs were left for a considerable period at too high a temperature, during which *Proteus* bacteria were able to multiply.

CLASSIFICATION OF THE ORGANISMS ISOLATED FROM ROTS

The coliform, *Proteus* and *Pseudomonas* organisms isolated from rots have been studied in detail. The coliform and *Proteus* organisms, all of which were atypical, gave reproducible results in the usual biochemical tests, and the reactions of each representative type are given in Table VII (p. 14). Cultures of each of these have been deposited with the Lister Institute.

All sugar reactions have been carried out at least in duplicate, and when negative results were obtained the tubes were incubated for at least 14 days before being discarded.

Coliform organisms

Eleven cultures were examined. In the first instance, Gram-negative, non-sporing organisms, not liquefying gelatin, and giving acid and gas in lactose, dextrose, maltose and laevulose, were provisionally taken to belong to this group. Further differentiation was sought with the Voges-Proskauer, citrate and methyl red tests. Two strains only, SE 15 and 50, gave positive Voges-Proskauer reactions, and they also fermented sucrose. According to Bergey (1934) these organisms should therefore be placed in the *aerogenes* (*Bacterium lactis aerogenes*) group. Strain SE 15 gave, however, a positive methyl red test, and thus does not fall into Koser's *aerogenes* group (Koser, 1924). Strain SE 15 was also peculiar in that it grew scantily at both 37 and 0° C.: of the others SE 80 did not grow well at 37° C., SE 50 did not grow at all, and strains SE 40, 50, 69, 72, 80, 83, and 94 grew at 0° C.

In view of the relative unimportance of these organisms in connexion with the present investigation, and the impossibility of arriving at a final classification, they have not been studied further. Their characteristics are given in Table VII.

None of the coliform organisms produced a definite rot on inoculation into fresh eggs. Most of them grew quite well, producing a definite turbidity in the white. Several strains, notably SE 54, produced a marked "fishy" odour. This smell was also apparent in artificial cultures of this organism.

Proteus *bacteria*

Gram-negative, non-sporing, motile rods, liquefying gelatin, not fermenting lactose, but giving acid and gas in dextrose, sucrose and maltose, were provisionally assigned to the genus *Proteus*. Seven strains, in addition to *P. melanovogenes*, which has not been found in the present series of rots, have been studied. Of the strains isolated in the present work, SE 45, 50, 60 produce marked blackening in 14 days at 20° C. after inoculation into fresh eggs, and strains SE 66, 68 produce some blackening, the white becoming viscous and "stringy". *P. melanovogenes* and strains SE 66, 68 and 74 did not grow at 37° C., strains SE 26, 30, 45 and 60 did. *P. melanovogenes* grows scantily at 0° C. on nutrient agar, better but not well at +5° C.; all the other strains of *Proteus* grew fairly well at 0° C. in about 14 days. The seven strains of *Proteus* isolated from these eggs are distinguishable biochemically from *P. melanovogenes* in that they all ferment raffinose and xylose, neither of which is attacked by the former organism, and they none of them produce haemolysis on blood agar, which *P. melanovogenes* does. Among themselves, strains SE 45, 60, 66, 68 and 74 are apparently similar biochemically, but the first two grow at 37° C. and the last three do not. The type of rot produced is not the same for these five organisms. Strain SE 30 differs from all the others in fermenting dulcitol. It is doubtful if this organism really belongs to the genus *Proteus*: its liquefaction of gelatin is slight, and slight acidity, but no gas, is produced in lactose. *P. melanovogenes* itself, however, sometimes gives slight acidity in lactose, strain SE 26 liquefies gelatin but slowly, and this strain together with SE 30 differ from all the others in fermenting sorbose. Strains SE 45, 60, 66, 68 and 74 give rapid and marked liquefaction of gelatin, as does *P. melanovogenes*.

Biochemically, *P. melanovogenes* can be distinguished from the other strains of *Proteus* studied. In order to see whether there was any antigenic relationship between it and them, the H and O antigens of strains SE 74, 68, 66, 60, and 45 were tested against *P. melanovogenes* (strain ER 20) antiserum.[1] The assumption was made that the alcohol and formalization treatments used for the *Salmonella* group for somatic and flagellar antigens respectively could be used also with these organisms. The results are summarized in Table VIII (p. 16).

[1] Kindly supplied by Dr Miles.

Table VII. *Cultural characteristics of coliform and* Proteus *bacteria isolated from eggs*

Coliform organisms

No.	Source	Gelatin	Growth at 0° C.	Growth at 37° C.	Morphology	Nitrates	V.-P.	H_2S	Indole	M.R.	Citrate	Dextrose	Lactose	Sucrose	Maltose	Galactose
SE 15	Yolk, English stored egg	N.L.	0	0	Gram −, motile	R	+	+	0	+	0	AG	AG	AG	AG	AG
SE 40	Yolk, rot, English egg	N.L.	+	+	Gram −, motile 20, not at 37° C.	R	0	0	+	+	+	AG	A	AG	AG	AG
SE 50	Red rot, Australian egg	N.L.	+	0		R	+	0	0	0	0	AG	AG	AG	AG	AG
SE 54	Black rot, Australian egg	N.L.	0	+	Gram −, motile	R	0	0	+		0	AG	A	0	AG	AG
SE 69	,, ,,	N.L.	+	+	,, ,,	R	0	0	0	+	0	AG	AG	AG	AG	AG
SE 72	,, ,,	N.L.	+	+	,, ,,	R	0	0	0	+	0	AG	AG	0	AG	AG
SE 78	,, ,,	N.L.	0	+	,, ,,	R	0	0	0	+	0	AG	AG	0	AG	AG
SE 80	,, ,,	N.L.	+	±	,, ,,	R	0	0		+	0	AG	AG	0	AG	AG
SE 83	,, ,,	N.L.	+	+	,, ,,	R	0	0		+	0	AG	AG	0	AG	AG
SE 84	,, ,,	N.L.	0	+	,, ,,	R	0	0	0	+	0	AG	AG	0	AG	AG
SE 94	,, ,,	N.L.	+	+	,, ,,	R	0	0	+	+	+	AG	A	AG	AG	AG

No.	Source	Laevulose	Raffinose	Dulcitol	Sorbitol	Mannitol	Inositol	Sorbose	Mannose	Xylose	Arabinose	Rhamnose	Dextrin	Salicin	Litmus milk	Agar
SE 15	Yolk, English stored egg	AG	AG	AG	AG	AG		0	AG	AG	AG	AG	AG	AG	AC	Thick, smooth, glistening, white
SE 40	Yolk, rot, English egg	A	AG	AG	AG	AG		AG	AG	AG	AG	AG	AG	AG	A	,,
SE 50	Red rot, Australian egg	AG	AG	AG	AG	AG	0		AG	AG	AG	AG	AG	AG	A	,,
SE 54	Black rot, Australian egg	AG	0	0	AG	AG	0	0	AG	AG	AG	AG	AG	0	Alk.	Flat, leaden, white, smooth
SE 69	,, ,,	AG	AG	0	0	AG		0	AG	AG	AG	AG	AG	AG	A	,, ,,
SE 72	,, ,,	AG	AG	0	0	AG	0		AG	AG	AG	AG	AG	AG	A	,, ,,
SE 78	,, ,,	AG	0	AG	AG	AG			AG	AG	AG	AG	AG	AG	A	,, ,,
SE 80	,, ,,	AG	AG	0	0	AG			AG	AG	AG	AG	AG	AG	A	,, ,,
SE 83	,, ,,	AG	AG	0	0	AG		0	AG	AG	AG	AG	AG	AG	A	
SE 84	,, ,,	AG	0	AG	AG	AG		0	AG			AG	AG	AG	A	
SE 94	,, ,,	AG	AG	AG	AG	AG		A	AG			AG	AG	AG		

Table VII (*contd.*)

Proteus bacteria

No	Source	Gelatin	Growth at 0° C.	Growth at 37° C.	Morphology	Nitrates	V.-P.	H_2S	Indole	Ammonia	Urease	Dextrose	Lactose	Sucrose	Maltose	Galactose
ER 20 *Proteus melanovogenes*	Black rot, South African eggs. Miles and Halnan	Saccate L	±	0	Gram −, motile filamentous	R	+	+	+	0	±	AG	SA	AG	AG	AG
SE 26	Yolk, English egg	Slow crater L	+	+	,, ,,	R	0	0	0	0	0	A	0	AG	AG	AG
SE 30	Black rot, English egg	Slight L	+	+		R	0	0	+	0	0	AG	SA	AG	AG	AG
SE 45	Red rot, English egg	Rapid crater L	+	+		R	+	+	+	0	0	AG	0	AG	A	AG
SE 60	Black rot, Australian egg	Rapid saccate L	+	+		R	+	+	0	0	0	AG	0	AG	AG	AG
SE 66	Black rot, Australian egg	Rapid saccate L	+	0		R	+	+	0	0	0	AG	0	AG	AG	AG
SE 68	,, ,,	,,	+	0		R		+		0	±	AG	0	AG	AG	AG
SE 74	,, ,,	,,	+	0		R		+		0	0	AG	0	AG	AG	AG

No.	Source	Laevulose	Raffinose	Dulcitol	Sorbitol	Mannitol	Inositol	Sorbose	Mannose	Xylose	Arabinose	Dextrin	Salicin	Adonitol	Litmus milk	Agar
ER 20 *Proteus melanovogenes*	Black rot, South African eggs Miles and Halnan	AG	0	0	AG	AG	0	0	AG	0	AG	AG	AG	0		Thick glossy, white
SE 26	Yolk, English egg	A	AG	0	0	AG		AG	AG	AG	AG	AG	A	0	SC	White, scanty
SE 30	Black rot, English egg	A	AG	AG	AG	AG		AG	AG	AG	AG	AG	AG	AG	LR	Thick, china white
SE 45	Red rot, English egg	AG	AG	0	AG	AG		0	AG	AG	AG	SA	AG	0	AC	Thick, glossy, white
SE 60	Black rot, Australian egg	AG	AG	0	AG	AG	A	0	AG	AG	AG	AG	AG	0	AC P	Thick, china white, glossy
SE 66	Black rot, Australian egg	AG	AG	0	AG	AG	A	0	AG	AG	AG	AG	AG	0	AC	,, ,,
SE 68	,, ,,	AG	AG	0	AG	AG	0	0	AG	AG	AG	AG	AG	0	AC	Smooth, flat, china white
SE 74	,, ,,	AG	AG	0	AG	AG	A	0	AG	AG	AG	AG	AG	0	AC	,, ,,

N.L. = not liquefied. L = liquefaction. R = reduced. + = positive. ± = doubtful. 0 = negative. AG = acid and gas. A = acid. AC = acid and coagulation. P = peptonisation. LR = litmus reduced. SC = slight coagulation.

Table VIII. *Agglutination titres of* Proteus *strains against* Proteus melanovogenes (*ER* 20) *antiserum*

	H	O
	1½ and 4 hr.	24 hr.
ER 20	1/3200	1/500
SE 74	all negative at 1/50	negative at 1/50
SE 68		
SE 66		
SE 60		
SE 45		

It seems clear, therefore, that the *Proteus* strains isolated from Australian eggs are not related in any way antigenically to *P. melanovogenes*.

Pseudomonas *bacteria*

Twenty-nine strains of *Pseudomonas* from eggs, all producing a fluorescent pigment on nutrient agar, have been studied and compared with a number of other strains from different sources.

It is difficult to devise a classification within this genus, partly owing to the scanty literature, and partly due to the lack of any pronounced characteristics of the organisms. Bergey (1934) lists thirty-one species, but he bases his primary divisions on the position of the flagella. It has, however, been shown with other organisms that flagellation varies with the method of culture (Colquhoun & Kirkpatrick, 1932) and this, coupled with the technical difficulty of obtaining reproducible results with flagella stains, makes this characteristic a doubtful criterion. All the strains so far examined in the present work possessed a clump of flagella, apparently at least three, at one pole only, and yet were clearly not all identical. The routine biochemical tests are not of much help, since gas is never produced from any sugar, and while acidity is obtained in some cases it is generally slight and the test may be either positive or negative according to the composition of the basal medium (Sears & Gourly, 1928). All the strains examined consistently produced acid in a medium containing 1% dextrose, 1% peptone, 0·5% sodium chloride + Andrade's indicator, a few consistently produced acid from sucrose and several from galactose, but with xylose, arabinose and mannose, although these were sterilized by passage through a Seitz filter to avoid steaming, the results were too variable to be of any value. Such differences as were obtained in the ordinary biochemical tests are summarized in Table IX.

It seemed better to attempt a provisional classification based on the utilization of a sugar or other carbon source when this formed the sole source of carbon in a medium of known composition. The basal medium of Robinson (1932) consisting of excess (a spatula full) of magnesium ammonium phosphate distributed into boiling tubes with 10 c.c. of a 0·5–1% solution of the carbon compound was used in some preliminary tests. A standard inoculum (about 10^5 organisms per c.c.) was added. Heavy turbidity after incubation indicated utilization of the substance, since control tubes without the carbon source

never developed turbidity. The results in this medium were not however entirely consistent, and growth is slow. A number of factors are suspected of being responsible for this. These include, variation of the *p*H (media made up according to Robinson's method by autoclaving with different compounds have given figures from *p*H 6 to 8) and absorption of substances from the cotton-wool plugs of the tubes. A better medium for these organisms consists of diammonium hydrogen phosphate 1 %, carbon source 1 %, and magnesium sulphate 0·1 %, the solutions being autoclaved separately with tinfoil-coated plugs, and diluted down aseptically for use. In Robinson's medium it was possible to arrange the strains of *Pseudomonas* isolated, together with certain stock strains, in six groups according to whether they did, or did not, utilize sucrose, ethyl alcohol, erythritol, and sorbitol. Most of the other substances investigated (other sugars, fatty acids, alcohols) either were all utilized, or all non-utilized, non-selectively. The classification arrived at does not, however, agree with that subsequently obtained in the second medium using the same carbon sources. If these difficulties can be overcome, the method might be promising, and is being further investigated.

Table IX. *Some characteristics of the* Pseudomonas *sp.*

All Gram-negative rods, actively motile.
Strains SE 2–47 possess 2–3 polar flagella, others not yet examined.
All strains liquefied gelatin, mostly rapidly.
All strains digested litmus milk, generally with alkalinity at first.
Nitrates reduced by strains SE 2, 4, 6, 10, 11, 16, 18, 36, 38, 47, 48, 49, 63, 64, 65, 67, 75, 82, *fluorescens liquefaciens*, *fluorescens non-liquefaciens*, *Pyocyaneus* 13, *Pyocyaneus* 14, *Ps.* 1, 104, E.
Nitrates not reduced by strains SE 8, 28, 29, 34, 86, 87, 88, 91, 103, D.
Hydrogen sulphide formed by strains SE 8, 63, 64, 65, 67, 82, 91.

Sources of organisms

Pyocyaneus 13 = *B. pyocyaneus*, Fildes III, Lister No. 1999.
Pyocyaneus 14 = *B. pyocyaneus*, Goat, Lister 254.
Fluorescens liquefaciens Lister, Chapman No. 964.
Fluorescens non-liquefaciens Lister, Cream 18, 3247.
D and E from putrid pork, *Ps.* 1 from dung, remainder from English, Australian, and New Zealand eggs.

Summary

1. Examination of several hundred eggs suggests that a high proportion (98 %) of the whites of fresh eggs, and a slightly smaller proportion of the yolks (93 %), are sterile.

2. The shell of the egg is heavily infected with a heterogeneous flora, including *Proteus* and *Pseudomonas* bacteria capable of producing rotting.

3. The rots found in imported New Zealand and Australian eggs, and in English stored eggs, may be grouped into black rot, red rot, green rot, and a miscellaneous group.

4. Black rot is brought about chiefly by strains of *Proteus*, but some species of *Pseudomonas* cause some blackening. Red and green rots are due to infection with particular strains of *Pseudomonas*.

5. A "fishy" odour is developed during the multiplication of certain atypical coliform organisms in the white, and a strong "cabbage-water" smell is often found after the growth of *Pseudomonas* species.

6. Washing eggs under clean conditions has no effect on immediate bacterial penetration. Washing removes a protective coating so that if the eggs are subsequently soaked in a bacterial suspension, much more penetration of bacteria occurs than with untreated controls.

7. Detailed descriptions of the coliform and *Proteus* organisms isolated are given. It is shown that the strains of *Proteus* from the eggs here investigated are antigenically not related to *P. melanovogenes* found by Miles and Halnan to be the cause of black rot in South African eggs.

8. It does not at present seem possible to assign specific names to the organisms isolated. The utilization of carbon sources by the species of *Pseudomonas* obtained from eggs, and by certain stock strains, and the possibility of a grouping on that basis, is discussed.

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