STUDIES OF BACTERIA FROM FERMENTING EGG WHITE AND THE PRODUCTION OF PURE CULTURE FERMENTATIONS[1]

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Received for publication April 3, 1942

In a previous study (1942) an investigation was made of the bacterial and chemical changes taking place during the natural fermentation of egg white, a preliminary step in the commercial processes for preparing dried egg albumen. This study established rather conclusively that predominance of bacteria of either the *Aerobacter* or *Escherichia* genus is necessary from the standpoint of the production of a high quality dried albumen from naturally fermented egg white. Other bacterial types were occasionally encountered in experimental laboratory fermentations but it was found that when species of *Proteus* or *Pseudomonas* were present in large numbers during such fermentations, a dried albumen of inferior quality was usually obtained.

The cultures isolated in this investigation have been studied more thoroughly with the object of confirming the tentative classifications originally made, and determining their effects on sterile egg white in pure culture.

The results of microscopic and cultural studies on twenty strains tentatively classified in the genus *Aerobacter* are given in Table 1.

From the data given in this table it appears that 12 of these cultures are strains of the species *Aerobacter aerogenes* (Bergey, 1939). They produce acid and gas from glycerol and do not liquefy gelatin. Seven strains were motile and five were non-motile. Eight strains were encapsulated. They all fermented glucose, mannose, and galactose, the three sugars known to be present in egg white. They also fermented sucrose, maltose, arabinose, raffinose, cellobiose, mannitol and starch, although six strains failed to ferment dulcitol. They all brought about acid coagulation in litmus milk and produced nitrites from nitrates. Only one strain produced indole in tryptone broth.

The remaining eight strains may also be *Aerobacter aerogenes* since they are not exactly typical of the other species in this genus, namely, *Aerobacter cloacae* (Bergey, 1939). They do not liquefy gelatin. Neither do they ferment glycerol, starch or dulcitol. They are all actively motile by virtue of peritrichous flagella and none have capsules. It is believed, therefore, that they resemble *Aerobacter cloacae* more closely than *Aerobacter aerogenes* and should be classified as strains of the former. All eight ferment glucose, mannose and galactose, with the production of acid and gas, fully as vigorously as the 12 typical strains of *Aerobacter aerogenes*.

The results of similar studies with 13 isolations tentatively classified as belonging to the genus *Escherichia* are given in table 2.

[1] Agricultural Chemical Research Division Contribution no. 63.

The data recorded in table 2 show that all 13 strains of this group utilized citric acid as a sole source of carbon. Only one produced gas in Eijkman's broth at 45°C. This particular culture did not produce indole in tryptone broth. Seven of the 13 strains did produce indole and nine of them produced acetyl-methyl-carbinol. The production of acetyl-methyl-carbinol is not commonly encountered with methyl-red positive cultures but has been reported by Parr (1938) for strains of *Escherichia fruendii*. Three of the 13 strains had well-developed capsules. All were motile. There would seem to be little doubt that all of the 13 cultures should be classified as strains of *Escherichia freundii*. It should be noted that all strains fermented glucose, mannose and galactose.

One surprising feature of these studies was the absence of true fecal types of the *Escherichia* genus in this group. These investigations were not extensive enought to say at this time that these species are consistently absent in naturally fermented egg white; it is possible that under conditions resulting in this fermen-

TABLE 1

*Aerobacter group, colonies on eosin-methylene-blue agar consistently of the Aerobacter type, non-spore-forming, gram-negative, short rods**

SPECIES	FLAGELLA STAIN GRAY'S	MOTILITY HANGING DROP	CAPSULE STAIN ANTHONY'S	GLYCEROL BROTH	STARCH BROTH	DULCITOL BROTH	INDOLE TEST
A. aerogenes (12 cultures)	Not made	7+ 5−	8+ 4−	+	+	6+ 6−	1+ 11−
A. cloacae (8 cultures)	+	+	−	−	−	−	−

* All cultures fermented lactose with the production of acid and gas, utilized citric acid as the sole source of carbon; were methyl-red negative, Voges-Proskauer positive, reduced nitrates, produced an acid curd in litmus milk and a yellowish growth on potato slants. They fermented with the production of acid and gas, glucose, mannose, galactose, maltose, sucrose, cellobiose, raffinose, arabinose and mannitol broths.

tation they are completely overgrown by the strains of *Citrobacter* (*Escherichia freundii*) and *Aerobacter*. This possibility is being investigated.

Five cultures were isolated that were tentatively classified as belonging to the genus *Proteus*. Further studies on these organisms have failed to reveal clearly the exact species with which they should be identified. However, the results obtained tend to confirm the initial presumption that they resemble the genus *Proteus* more closely than any other genus now recognized. The results of these studies are given in table 3.

The data shown for the five strains would indicate that there may exist a heretofore undescribed group of organisms intermediary between *Aerobacter* and *Proteus*, for all five cultures have characteristics in common with both genera. In appearance, their agar colonies and their growths on agar slants resemble *Aerobacter cloacae*. They produce acetyl-methyl-carbinol, a characteristic more commonly associated with *Aerobacter* than *Proteus*. This property has been assigned to *Proteus bombycis*, a species listed in the appendix, for the genus *Pro-*

teus, in Bergey's Manual (1939), but, unlike *Proteus bombycis*, these cultures do not have well-defined capsules. All five of the strains fermented mannitol, although two of them did not produce gas. Since they also ferment sucrose it may be that they should be identified as strains of *Proteus hydrophilus* or *Proteus ichthyosmius*; however, they did not produce indole in tryptone broth, or fishy odors in milk, and did not grow on potato slants according to the descriptions

TABLE 2

*Escherichia group, colonies on eosin-methylene-blue agar originally coli-like, non-spore forming, gram-negative, short rods**

SPECIES	CAPSULE STAIN ANTHONY'S	EIJKMANS BROTH AT 45° C.	VOGES-PROSKAUER TEST	INDOLE TEST	DULCITOL BROTH	STARCH BROTH
E. freundii (13 cultures)	3-encapsulated	12−	8+ 1 weakly +	7+	5+	5+
	10-no capsules found	1+	4−	6−	8−	8−

* All cultures appeared to be actively motile in hanging drop, fermented lactose producing acid and gas, utilized citric acid as a sole source of carbon, reduced nitrates and were methyl-red test positive. None liquefied gelatin. They all produced an acid curd in litmus milk and abundant yellow growths on potato slants. All produced acid and gas in glucose, mannose, galactose, sucrose, maltose, cellobiose, raffinose, arabinose, mannitol and glycerol broths.

TABLE 3

*Proteus group, pleomorphic, actively motile, gram-negatiue rods, fermenting glucose and sucrose but not lactose**

SPECIES	GELATIN STAB.	VOGES-PROSKAUER	METHYL RED TEST	MANNITOL BROTH	GALACTOSE BROTH	MANNOSE BROTH	ARABINOSE BROTH	RAFFINOSE BROTH	GLYCEROL BROH	SALICIN BROTH
Proteus sp. (5 cultures)	3-infundibuliform and 2-stratiform liquefaction	3+ 2 weakly +	3− 2+	3+ with acid and gas 2+ with acid but no gas	3+ with acid and gas 2+ with acid but no gas	3+ with acid and gas 2+ with acid but no gas	2− 2+ with acid & gas 1+ with acid but no gas	2− 3+ with acid and gas	1+ with acid and gas 4+ with acid but no gas	2+ with acid & gas 3+ with acid but no gas

* All cultures showed peritrichous flagella with Gray's stain and produced pearl white spreading growths on agar plates and slants and creamy white spreading growths on potato slants. They all decomposed urea, reduced nitrates, and were indole negative. None showed capsules with Anthony's stain or fermented dulcitol broth. All decolorized and peptonized litmus milk and fermented maltose broth with the production of acid and gas.

given for these species. Two of the cultures did not ferment arabinose, raffinose, or dulcitol. The other three fermented arabinose and raffinose but not dulcitol. All five fermented glucose, mannose and galactose.

Two strains of *Serratia* were isolated. These possessed all of the characteristics assigned to *Serratia marcescens*. The results of the studies on these two isolations, along with data compiled on a number of strains of *Pseudomonas* of

the fluorescent type isolated also from samples of fermenting egg white, are given in table 4.

From table 4 it can be seen that there were quite a diverse number of species of the genus *Pseudomonas* encountered. The strains listed represent only those isolations made at the conclusion of the periods employed in experimental fermentations. If the isolations made from all lots of freshly broken-out egg whites studied had been listed here, the diversification of species would have reached formidable proportions. Species of this genus seem to be very prevalent in egg

TABLE 4

Serratia and Pseudomonas group, gram-negative motile rods (usually occurring singly) or cocco-bacteria, indole negative

PROBABLE SEECIES	AGAR SLANT	GELATIN STAB.	LITMUS MILK	NITRATE REDUCTION	GLUCOSE BROTH	POTATO SLANT	FLUORESCENCE IN ULTRAVIOLET LIGHT WHEN GROWN IN STERILE EGG WHITE
Serratia marcesens* (2-cultures)	2 bright fuchsin color	2 infundibuliform liquefaction	2 acid curd	2+	2+ (acid but no gas)	Profuse dark red	2−
Pseudomonas ovalis† (2-cultures)	1 greyish white, 1 greenish white	2 no liquefaction	2 slight alkaline curd	2−	2−	1 dirty white 1 yellow to brown	1+ (blue), 1+ (blue green)
Pseudomonas fluorescens† (2-cultures)	2 olive green to white	2 infundibuliform liquefaction	2 slight alkaline curd	2+	2+ (acid but no gas)	1 yellow to brown, 1 dirty cream	2+ (bright green)
Pseudomonas aeruginosa† (1-culture)	Olive green	Infundibuliform liquefaction	Acid curd peptonized	+	+ (acid but no gas)	Yellow	+ (bright green)
Pseudomonas jaegeri† (1-culture)	Greenish white	Saccate liquefaction	Alkaline curd peptonized	−	+ (acid but no gas)	Brown to orange	+ (bright green)
Pseudomonas chlororaphis† (1-culture)	Greenish white	No liquefaction	Coagulated with green pellicle	+	Pigment crystals produced	Brown to orange	+ (bright green)
Pseudomonas schuylkilliensis† (1-culture)	Bluish white	Stratiform liquefaction	Alkaline curd peptonized	−	+ (acid but no gas)	Orange to brown	+ (blue green)

* Peritrichous flagella with Gray's stain.
† Polar flagella with Gray's stain.

white separated from storage eggs and a description of the various species encountered appears to be a task far too large to be included herein.

Since it is quite easy, with freshly laid eggs, to obtain sterile egg white in any quantity desired, if asceptic technique is employed in breaking the shell and separating the white from the yolk, a study was made to determine the changes in sterile egg white brought about by representative strains of the groups of cultures listed in tables 1, 2, 3, and 4.

These fermentations were carried out with 250 ml. quantities of sterile egg white in individual sterile glass jars of 500 ml. capacity. Inoculations were made from 48-hour starter cultures growing in sterile tubes of egg white at 30°C. Di-

lution plate counts were made on the starter cultures at the time of inoculation, using glucose agar. By using this count and the volumes of starter added, it is possible to calculate the approximate number of bacterial cells per ml. at the beginning of the fermentation period. This, of course, makes it possible to determine the influence of the size of the inoculum on the rate of fermentation by any selected strain of bacteria.

Using a selected strain of *Aerobacter aerogenes* five different volumes of inocula were employed and samples were removed aseptically at periodic intervals during 168 hours of incubation at 30°C. for pH measurements, sugar determinations by the method of Stiles, Peterson and Fred (1926) and formol titrations for combined amide and amino nitrogen.

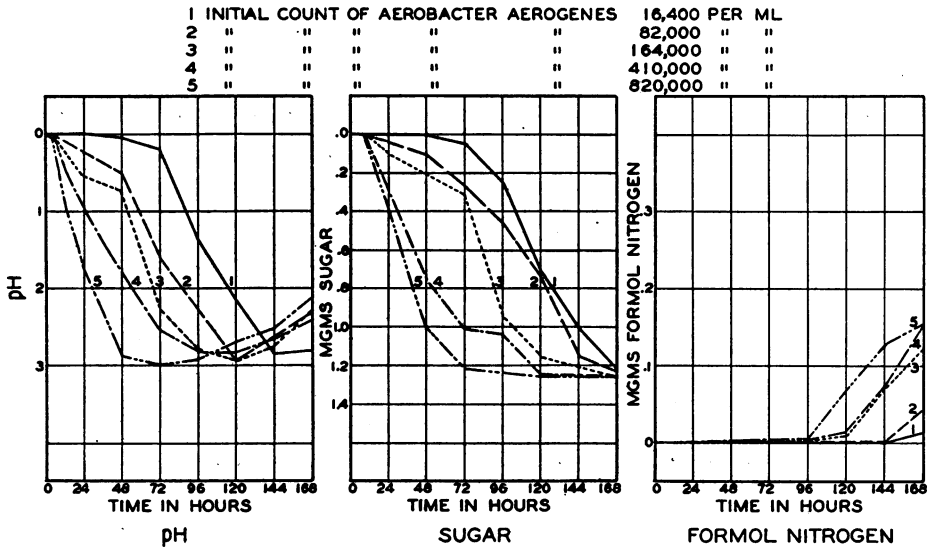

FIG. 1. CHANGES OVER THE INCUBATED STERILE CONTROL IN EGG WHITE INOCULATED WITH VARYING NUMBERS OF CELLS OF AEROBACTER AEROGENES

The rate and extent of change in pH, sugar, and formol nitrogen in these inoculated samples was determined by comparing the values found in these determinations at the different time intervals with the values obtained at the corresponding time intervals for incubated sterile control samples. The calculated deviations from the control are presented graphically in figure 1, in which values for the control are indicated by 0.

From figure 1 it can be seen that the rate of change in pH is directly proportional to the number of cells of *Aerobacter aerogenes* present at the beginning of the incubation period. With an initial count of 820,000 cells per ml., there is a change in pH of 3.0 in 72 hours; with 410,000 cells per ml., 96 hours are required for a change of a comparable size; with 164,000 cells 120 hours are required; with 82,000 cells 120 hours; and with 16,400 cells 144 hours. There is a subsequent change in the opposite direction in pH with each inoculated sample and the

amount of this change at the 168-hour interval appears also to be directly proportional to the number of cells initially present and thus directly proportional to the initial rate of change in pH.

The changes in sugar content over the uninoculated control parallel very closely the changes in pH with each sample. Thus, the rate of sugar utilization is also directly proportional to the number of bacteria initially present. That is, the larger the inoculum, the more rapid the utilization of the available sugar. The term "available sugar" is used here since it would appear that not all of the sugar is removed by the bacteria. The amount of sugar appears to decrease only to a relatively low constant level.

No changes in the formol nitrogen titration values over the sterile incubated control sample were found during the initial stages of fermentation. However, changes did occur in the latter stages of the fermentations and the extent of these appears to be directly proportional to the increase in the pH values after the available sugar had been utilized. Thus, it appears that the amount of change in the combined amide and amino nitrogen in 168 hours is directly related to the initial rate of fermentation.

Practically no measurable changes occurred in the sterile control egg white incubated for 168 hours. Dilution plates made from this control white at the 168-hour period showed that some slight contamination had occurred during sampling since there was a count of 213 bacteria per ml. at that time. This figure is the average figure for three one-ml. platings. It would appear, however, that this contamination was not great enough to have had any appreciable biochemical significance.

A study similar to the one just described was made to determine comparatively the magnitude and rate of change in sterile egg white brought about by substantially the same numbers of cells of *Aerobacter aerogenes*, *Escherichia freundii*, *Serratia marcescens*, *Proteus sp.* and *Pseudomonas aeruginosa* when added in pure culture.

By comparing the values obtained with the inoculated samples of egg white for pH, sugar and formol nitrogen with those found for sterile, uninoculated, incubated control egg white, comparative values were obtained for the five organisms with regard to the extent and rate of change brought about in the egg white. The deviations from the control are presented graphically in figure 2, for each organism in which values for the control are indicated by 0.

From figure 2 it can be seen that the number of cells present at the beginning of the fermentation period was, within the range of experimental error in dilution plate counting, the same for all of the five species of bacteria employed in this study.

From this figure it is apparent that the change in pH with *Escherichia freundii* is somewhat greater and more rapid than with *Aerobacter aerogenes*. The extreme change produced (in 96 hours) tended to persist longer in the case of *Escherichia freundii* than in the case of *Aerobacter aerogenes*. Sugar utilization on the other hand appeared to be as great although somewhat slower with *Aerobacter aerogenes* than with *Escherichia freundii*. It is probable, therefore, that more of the sugar was converted into gas with the former organism than with the latter. There

was no significant change in the formol nitrogen values with either of these organisms within 120 hours.

With the other three organisms the pH changes over the control sample were neither so rapid nor so great as those brought about by *Aerobacter aerogenes* and *Escherichia freundii*. Sugar utilization was not so complete nor so rapid. There were pronounced changes in the formol nitrogen values as early as 24 hours with *Serratia marcescens*, 48 hours with *Proteus sp.* and 48 hours with *Pseudomonas aeruginosa*. With *Serratia marcescens* and *Proteus sp.* the amounts of combined amide and amino nitrogen continued to increase rapidly and were apparently still on the increase at the conclusion of the incubation period, or at 120 hours. However, with *Pseudomonas aeruginosa* the formol nitrogen value increased up to the 72-hour period and apparently remained constant thereafter.

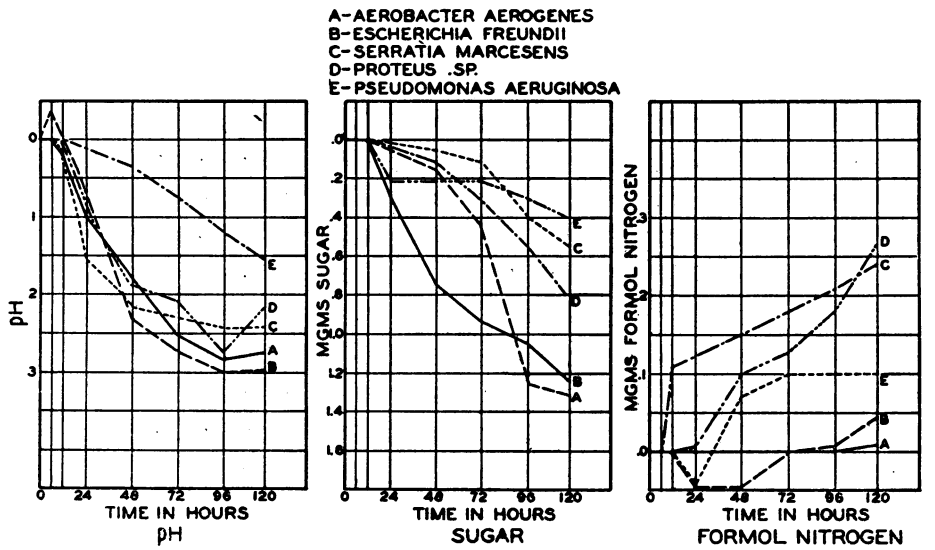

FIG. 2. CHANGES OVER THE INCUBATED STERILE CONTROL IN EGG WHITE INOCULATED WITH COMPARABLE NUMBERS OF BACTERIAL CELLS OF VARIOUS BACTERIAL SPECIES

The changes in pH and sugar values and formol titrations with egg white brought about by pure cultures of *Aerobacter aerogenes* and *Escherichia freundii* are the same as those found previously for naturally fermenting egg white. There can be no doubt, therefore, that these are the important organisms from the standpoint of normal, natural commercial fermentations.

SUMMARY

1. Twenty strains of bacteria isolated from fermenting egg white and previously identified as belonging to the genus *Aerobacter* were studied with the objective of establishing species identity. Twelve of these 20 isolations have been shown to be strains of *Aerobacter aerogenes*. The remaining eight strains were found to resemble *Aerobacter cloacae* more closely than *Aerobacter aerogenes* although they do not conform exactly with the descriptions given for either species.

2. Thirteen isolations from fermenting egg white previously identified as

belonging to the genus *Escherichia* have been shown through similar studies to be strains of *Escherichia freundii.*

3. Five isolations tentatively classified as belonging to the genus *Proteus* have not been identified with any recognized species since the results of microscopic and cultural studies do not clearly conform with the characteristics listed for recognized species.

4. Two red chromogenic isolations were studied and found to be strains of *Serratia marcescens.*

5. Eight isolations tentatively classified as belonging to the genus *Pseudomonas* have been studied and given tentative species identifications. Two were identified as strains of *Pseudomonas ovalis,* two as strains of *Pseudomonas fluorescens,* one as *Pseudomonas aeruginosa,* another as *Pseudomonas jaegeri,* another as *Pseudomonas chlororaphis* and the other as *Pseudomonas schuylkillensis.*

6. Lots of sterile egg white inoculated with varying numbers of cells of a selected strain of *Aerobacter aerogenes* fermented similarly to natural normal fermentations and the rate of fermentation was directly proportional to the number of bacterial cells added in the inoculum. That is, as the size of the inoculum increased, so did the rate of fermentation. In this study the course of fermentation was followed by pH measurement, sugar determinations and formol titrations, made at periodic intervals of time.

7. Sterile egg white inoculated with a selected strain of *Escherichia freundii* fermented in the same manner as sterile egg white fermented with *Aerobacter aerogenes.* The minor differences observed were a tendency for *Escherichia freundii* to produce acid more rapidly and maintain a lower pH longer than *Aerobacter aerogenes.*

8. Sterile egg white samples inoculated with selected strains of *Serratia marcescens, Proteus sp.,* and *Pseudomonas aeruginosa* and allowed to ferment did not show changes in pH, sugar content and formol titration values corresponding to normal natural fermentations. Decreases in pH and sugar content were neither so great nor so rapid as with *Aerobacter aerogenes* and *Escherichia freundii.* On the other hand, the fermentations with all three of these organisms were characterized by rapid and marked increases in the amount of formol nitrogen, indicating strong proteolytic action on the part of these species.

ACKNOWLEDGMENT

The authors are indebted to Dr. G. G. Slocum of the Food and Drug Administration, Federal Security Agency, for assistance in the species identification of the cultures reported.

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