PAPILLARY VARIATION IN COLIFORM BACTERIA

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CONTENTS

Introduction

Papillary variation includes all heritable changes of character which take place in the papillae of bacterial colonies and the corresponding variations in liquid media.

The following is a short summary of what is known about these variations. Papillary variation was first observed in *B. coli-mutabile*, and other coliform bacteria show the same variations. This race forms white colonies on lactose neutral-red plates. After 1–5 days' incubation minute papillae, which become red and increase in size, appear on their surfaces. Subcultures from these papillae give colonies of two kinds, white and red. The white colonies continue to throw red, the reds breed true (Neisser, 1906; Massini, 1907). Similar variation to different sugars occurs in other related bacteria: *B. dysenteriae* Sonne varies to lactose, *B. dysenteriae* Flexner to maltose, *B. paracolon* to saccharose, *B. coli-mutabile*, *B. acidi-lactici* and *B. typhosus* to dulcite (Twort, 1907; Reiner Müller, 1909; Burri, 1910; Stewart, 1926, 1927). Variation takes place only on the sugar concerned, but, once it has taken place, the character remains fixed, even after long growth on other media. Variation is not determined by length of exposure to the sugar. It occurs at (or after) the end of the logarithmic phase. If this phase is kept going by daily subculture (in the appropriate sugar) the bacteria of the chain carried on from culture to culture do not vary, however long they are exposed (Stewart, 1927; Deere, Dulaney & Michelson, 1939). On the other hand, if the logarithmic phase is curtailed by heavy inoculation of a culture, variation occurs early (Stewart, 1927). If the bacterium is grown on a plate without sugar until the colonies are well formed and the sugar is then added, with care not to disturb the colonies, variants appear after very short exposure (Stewart, 1927, 1928). A variation

https://doi.org/10.1017/S0022172400059799 Published online by Cambridge University Press

may be completed in two distinct steps; thus a race of *B. typhosus* formed white colonies on dulcite with pink papillae, subcultures from which gave white and pink colonies; the pink colonies formed dark red papillae from which on subculture came pink and dark red colonies, the latter bred true.

Colonies of the W (white) form do not remain white but become red in the centre as they grow older; subcultures from the red centres again form white colonies. It is therefore probable that the non-lactose-fermenting character is not due to absence of lactase but to a factor which inhibits its action. This factor becomes weaker in old age (Stewart, 1926). The ferment lactase has now been found in the white form of *B. coli-mutabile* (Deere *et al.* 1939; Deere, 1939). It can be extracted from the bodies of the bacteria after drying by vacuum distillation, but while they are alive it is prevented from acting by some cause unknown.

Characters other than sugar reactions are also subject to variation in papillae, such as shape and size of bacteria, virulence, capsulation, shape and consistence of colonies. Papillae do not always contain variant cells. They appear on the colonies of probably all bacteria toward the end of their growth and remain minute unless a favourable variation takes place.

Technique of isolating pure lines

It is imperative that pure lines derived from single cells should be used in the study of these variations.

Regarding the two methods of purification Bernhardt (1915) writes: 'We must state at once that in all our experiments we have always worked with pure lines. In our first experiments with dysentery bacteria we used Burri's single cell method to verify our results but later we have convinced ourselves, in agreement with other workers, that we were able to arrive at the same results by the ordinary methods of bacteriology, viz. sufficient dilution of the original material and plating in series. We have therefore abandoned Burri's method which is wearisome, which takes much time and which is not always applicable.'

The present writer has trusted to plating by the usual two-plate method from well-diluted suspensions in saline. Work with a mixture of lactose-fermenting (red) and non-fermenting (white) bacteria is a good test of the efficiency of this method since mixed colonies can be recognized at once. Red and white bacteria do not mix with each other in a colony but grow in well-defined segments. A red colony never yields white on subculture nor does a white colony yield red if the subculture is made before the time at which variation takes place. Particoloured colonies obviously derived from a red and a white bacterium deposited close together are not common on an uncrowded plate and are very rare on the second of a pair of plates. This is true even when the parent mixture contains roughly equal numbers of red and white bacteria. If colonies arise each from more than one bacterium, then they

should be R (red), W (white), and mixed RW[1] in definite proportions, RW being more numerous than either R or W, unless W tends to cohere to W and R to R. But in a live suspension of coliform bacteria we see under the microscope no cohesion except where bacteria are in the act of dividing, or when they are agglutinated. Furthermore the suspensions used for plating are well diluted in saline and thoroughly shaken (by preference in a screw-capped bottle). Therefore the only condition which would give colonies R and W with few or no RW is that almost all colonies arise from single bacteria. We may therefore feel certain that in coliform bacteria colonies which are well isolated on the second plate each represent a pure line, and if the plating is repeated in series we reach a degree of certainty which is if possible still higher.

MECHANISM OF THE VARIATION WHITE TO RED

There are two hypotheses regarding the mechanism of the variation white to red: (1) that it takes place spontaneously on any medium, lactose or non-lactose, and that a very small proportion of the cells vary, about 1/100,000. On a lactose medium the red variants then outgrow the white; (2) that it takes place on lactose only and in direct adaptation to environment.

The first hypothesis was propounded by Henderson Smith (1913). It has been revived by I. M. Lewis (1934) as follows:

The red variants do not exceed 1/100,000 on non-lactose media. They therefore do not appear in routine plating when only about 200 bacteria are spread and the chance against one being red would be 500 to 1. To demonstrate these hidden reds large numbers of bacteria must be inoculated on a medium which will allow the growth of R but not of W.

Lewis found such a medium in a synthetic lactose agar (K_2HPO_4 3·1 g., KH_2PO_4 0·8 g., KCl 0·2 g., $MgSO_4$ 0·2 g., lactose 10·0 g., agar 20·0 g., water 1000·0 c.c.). One particular strain of *B. coli-mutabile* failed to grow on this medium when small numbers were inoculated, but when several million cells were planted a few colonies appeared, one colony to 100,000 cells of the inoculum. The colonies were all R, while the inoculum came from an agar slant of W. Lewis believes that the ancestors of the R colonies had become R while on non-lactose medium. The facts can, however, be explained in another way, namely, that variation from W to R is accelerated by crowding of the culture (Stewart, 1927, table XXII, p. 97). The strain used by Lewis varied early, forming papillae on the second day in lactose plates, and fermenting lactose peptone water in 36 hr. It is therefore highly probable that variation would occur still earlier under the conditions of the experiment in the film of bacteria surviving on the lactose plates. The cells which varied would then form primary R colonies. It follows that Lewis's experiment did

[1] A single RW colony arising from several bacteria deposited in close contact is a circle divided into two or more sectors bounded by radii of the circle. Such a colony must be distinguished from (1) two colonies in contact whose line of junction is a chord not passing through the centre of either, and (2) from a colony with a wedge of different growth whose apex does not reach its centre. Such a wedge is due to variation at the growing margin of the colony.

not prove that variation had taken place in the non-lactose parent growth. In addition to the factor of number he introduced the two disturbing factors of crowding and starvation into his experiment. To judge the case fairly it is necessary to inoculate large numbers of bacteria into large volumes of nutrient media. This obviously cannot be done on plates but can be done in lactose peptone water. If, for instance, we inoculate 2 million bacteria into 500 c.c. then the logarithmic curve is not affected and R if present in the inoculum will overgrow W ($2\frac{1}{2}$ million cells in 500 c.c. multiply in 11 hr. to 89,130 million). The one essential is to be able to distinguish R cells present in the inoculum[1] and their descendants from those formed by variation from W in the lactose peptone water. Now by regular subculture every 12 or 24 hr. variation can be prevented altogether in the chain of bacteria passed on from culture to culture, while it still takes place in each separate culture at the hour appropriate to the strain, after the next subculture has been made (Stewart, 1927; Deere *et al.* 1939). Chain culture does not interfere with the overgrowing of R over W.[2] Therefore even very small numbers of R in the original inoculum will become sufficiently numerous to appear on a routine plate after one or two subcultures, while if none are present the chain will remain pure white indefinitely. In the experiments to be described one or more plates are spread from each link at 12 or 24 hr. when the subculture is being made, and a control series is put up in which a minute dose of R bacteria is added to the original inoculum.

The strain of *B. coli-mutabile* which I used was one isolated by Mr W. F Gifford in the laboratory of the Cheddleton Mental Hospital in October 1939. I have named it G1 and describe its systematic characters on p. 506. In lactose peptone water flasks R variants appeared after $14\frac{1}{2}$ hr. but not after 12 hr. incubation. The subcultures of the chain therefore had to be made at intervals of 12 hr.

Technique of counting and dilution

In setting up the experiments the numbers of bacteria were estimated roughly by the opacity of the primary suspensions in saline from agar. The final figures were in all cases calculated from the number of colonies on plates sown with measured volumes of decimal dilutions. Anticipation and final figures have agreed closely after some experience. Small volumes were measured by two methods, a standard platinum loop and a pipette. (1) The standard platinum loop delivered 0·00425 c.c. when held horizontally. The volumes of diluent generally used were 4·25 and 42·5 c.c. in screw-capped bottles. One loop of a 12 hr. culture in a lactose peptone water flask contained generally about 400,000 cells, calculated from 400 colonies on a plate sown with one loop of a 10^{-3} dilution. This method was tested as follows: (A) a number of suspensions were plated each on a pair of plates and each plate received one loop. Comparing the number of colonies on the pairs of plates the average

[1] From a non-lactose medium. The present writer believes that there are no such R cells if the W strain has been purified by plating on non-lactose.

[2] See Exp. I, plates C3*a*, C4*a*, Exp. II, plates C3*a*, C4*a*, Exp III, plates B2*a*, B3*a*.

percentage of error came to 6% and the highest error to 13%. (B) A number of dilutions to 10^{-5} or 10^{-6} were made from one suspension and a loop of each was plated. The results agreed closely. (2) The pipettes contained 0·5 or 1·0 c.c. and were used with measured volumes (49·5 or 9·0 c.c.) of saline. In this method the amount plated was 0·5 c.c. of a 10^{-5} dilution distributed over five plates, which had been dried for at least 3 hr.

The salt solutions used for diluting were Pannett and Compton's, or Ringer's without bicarbonate.

EXPERIMENT I (28. xi. 40)

Litre flasks each containing 500 c.c. of lactose peptone water, *p*H 7·4, were put up and numbered A1, B1, C1, D1, for four series. The strain had been purified by plating a W colony on nutrient agar, picking off a well-isolated colony on to agar slopes and testing on lactose MacConkey plates. This procedure guaranteed that no R forms should be brought in from the last contact with lactose. The strain was kept on one agar slope for several days to allow of spontaneous variation and then incubated for 48 hr. on another agar slope. The R strain had been picked off a lactose plate and subcultured on agar slopes, and was also a 48 hr. growth.

Flasks A1 and B1 each received 40 million bacteria W.

Flask C1, 40 million W and 400 R (W : R :: 100,000 : 1).

Flask D1, 40 million W and 40 R (W : R :: 1 million : 1).

The flasks were incubated at 37° C. for 12 hr., and subcultures made into 200 c.c. flasks, each containing 100 c.c. lactose peptone water, which were labelled A2, B2, C2, D2, the volume transferred in each case was one loop delivering 0·00425 c.c. MacConkey plates labelled A1*a*, B1*a*, C1*a*, D1*a*, were spread at the same time, each with one loop of a 10^{-3} dilution. These plates were incubated and examined for R colonies and all colonies were counted to give the bacterial content of the loop. A1*a* had 365 colonies all W, so that the loop transferred to A2 contained 365,000 living cells; B1*a* had 485, C1*a* 395 and D1*a* 390.

Lines A and B were continued to A4 and B4, and were then united in A5 which received one loop from A4 and one from B4 (each containing about 400,000 W bacteria). A5 was continued to A6, from which came plate A6*a* with 204 colonies, all W.

In the same way line C was carried to flask C4 and plate C4*a*. R appeared for the first time on C3*a* with 341 W to 16 R colonies. C4*a* had W and R in equal numbers.

Line D was carried to D8 without R appearing in any flask during the first 12 hr. (plates D1*a* to D8*a*), while in a plate taken after 24 hr. incubation from flask D5 there were 12 R colonies with 492 W (plate D5*b*). These R's had arisen by variation in D5.

The controls therefore showed that from a proportion of 1 to 100,000 R manifested itself in the third culture after a total lactose exposure of 36 hr.,

but from a proportion of 1 to a million it may die out. In the main experiment, A and B, however, a sample of 80 million W showed no R after 3 days' incubation with lactose in a chain of six flasks. We can therefore assume that this sample contained either no R at all or certainly less than 1 in 100,000, and that this hypothetical number of R, if present at all, would die out in competition with W on lactose.

Experiment I

In column (1) the serial number of the flask, the volume of lactose peptone water and the number of cells inoculated are given. In column (2) the number of the MacConkey plate, the nature of the inoculum (thus 1 loop A2, 10^{-3} means one loop delivering 0 00425 c.c. of the contents of flask A2 after 12 hr. incubation diluted to 10^{-3}), and the number of colonies found on the plate after 24 hr. incubation are given. Columns (3) and (4) deal with plates sown from flasks which had been incubated 24 and 36 hr.

		(1)	(2)	(3)	(4)
28. xi. 40	a.m.	A1 (928) 500 c.c. W 40 million			
	p.m.	A2 100 c.c. 365,000 cells from A1	A1*a* 1 loop A1, 10^{-3} W 365 colonies		
29. xi. 40	a.m.	A3 100 c.c. 1 loop A2	A2*a* 1 loop A2, 10^{-3} W colonies only		
	p.m.	A4 100 c.c. 1 loop A3	A3*a* 1 loop A3, 10^{-3} W colonies only		
30. xi. 40	a.m.	A5 100 c.c. 1 loop A4 1 loop B4	A4*a* 1 loop A4, 10^{-3} W colonies only		
	p.m.	A6 100 c.c. 1 loop A5	A5*a* 1 loop A5, 10^{-3} W 400 colonies		
1. xii. 40	a.m.	A7 100 c.c. 1 loop A6	A6*a* 1 loop A6, 10^{-3} W 204 colonies	A5*b* 1 loop A5, 10^{-3} W and R colonies	
28. xi. 40	a.m.	B1 (929) 500 c.c. W 40 million			
	p.m.	B2 100 c.c. 485,000 cells from B1	B1*a* 1 loop B1, 10^{-3} W 485 colonies		
29. xi. 40	a.m.	B3 100 c.c. 1 loop B2	B2*a* 1 loop B2, 10^{-3} W colonies only		
	p.m.	B4 100 c.c. 1 loop B3	B3*a* 1 loop B3, 10^{-3} W colonies only		
30. xi. 40	a.m.	A5 *vide supra*	B4*a* 1 loop B3, 10^{-3} W colonies only		
28. xi. 40	a.m.	C1 (930) 500 c.c. W 40 million R 400			

https://doi.org/10.1017/S0022172400059799 Published online by Cambridge University Press

Experiment I (*continued*)

		(1)	(2)	(3)	(4)
28. xi. 40	p.m.	C2 100 c.c. 395,000 cells from C1	C1*a* 1 loop C1, 10^{-3} W 395 colonies		
29. xi. 40	a.m.	C3 100 c.c. 1 loop C2	C2*a* 1 loop C2, 10^{-3} W colonies only		
	p.m.	C4 100 c.c. 1 loop C3	C3*a* 1 loop C3, 10^{-3} W 341 colonies R 16 colonies		
30. xi. 40	a.m.	C5 100 c.c. 1 loop C4	C4*a* 1 loop C4, 10^{-3} W and R equal numbers		
28. xi. 40	a.m.	D1 (931) 500 c.c. W 40 million R 40			
	p.m.	D2 100 c.c. 390,000 cells from D1	D1*a* 1 loop D1, 10^{-3} W 390 colonies		
29. xi. 40	a.m.	D3 100 c.c. 1 loop D2	D2*a* 1 loop D2, 10^{-3} W colonies only		
	p.m.	D4 100 c.c. 1 loop D3	D3*a* 1 loop D3, 10^{-3} W colonies only		
30. xi. 40	a.m.	D5 100 c.c. 1 loop D4	D4*a* 1 loop D4, 10^{-3} W colonies only		
	p.m.	D6 100 c.c. 1 loop D5	D5*a* 1 loop D5, 10^{-3} W 465 colonies		
1. xii. 40	a.m.	D7 100 c.c. 1 loop D6		D5*b* 1 loop D5, 10^{-3} W 490 colonies R 12 colonies	
	p.m.	D8 100 c.c. 1 loop D7			
2. xii. 40	a.m.		D8*a* 1 loop D8, 10^{-3} W colonies only		

EXPERIMENT II (17. x. 40)

One-litre flasks each containing 500 c.c. of lactose peptone water, *p*H 7·4, were numbered A1, B1, C1.

Flask A1 received 2½ million W cells from a 24 hr. agar slope.

Flask B1 received 2½ million W cells plus 20 R cells (R : W :: 1 : 125,000).

Flask C1 received 2½ million cells plus 10 R cells (R : W :: 1 : 250,000).

Subcultures were made after 12 hr. incubation into 200 c.c. flasks each containing 110 c.c. of lactose peptone water. A1 was continued to A11, B1 to B6 and C1 to C5. Series A showed no R in any flask at 12 hr. (plates A1*a*

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to A 5*a* and A 11*a*) throughout 5½ days' incubation with lactose. But at 24 and 36 hr. incubation R was present (plates A 3*b* and A 2*c*).

In series B, R appeared in the fifth culture, after 2½ days, in proportion R : W :: 433 : 109 (plate B 5*a*).

In series C, R appeared in the third culture, after 1½ days, in proportion R : W :: 3 : 671 (plate C 3*a*). That R should appear earlier in C than in B was unexpected, but was no doubt due to the minuteness of the sample plated.

Therefore R was either absent entirely from a sample of 2½ million W from non-lactose medium or, if present, was in lower proportion than 1 in 250,000.

Experiment II

		(1)	(2)	(3)	(4)
17. x 40	a.m.	A 1 (800) 500 c c. W 2,500,000			
	p.m.	A 2 110 c c. 586,000 cells from A 1	A 1*a* 1 loop A 1, 10^{-3} W 586 colonies		
18 x. 40	a.m.	A 3 110 c.c. 494,000 cells from A 2	A 2*a* 1 loop A 2, 10^{-3} W 494 colonies		
	p m.	A 4 110 c c 400,000 cells from A 3	A 3*a* 1 loop A 3, 10^{-3} W 400 colonies		
19 x 40	a.m	A 5 110 c.c. 320,000 cells from A 4	A 4*a* 1 loop A 4, 10^{-3} W 320 colonies	A 3*b* 1 loop A 3, 10^{-3} W equal number R	A 2*c* 1 loop A 2, 10^{-3} W 500 R 6
	p.m.	A 6 110 c c. 1 loop A 5	A 5*a* 1 loop A 5, 10^{-3} W colonies only		
20. x. 40	a m.	A 7 110 c c. 1 loop A 6			
	p.m	A 8 110 c c. 1 loop A 7			
21. x 40	a m.	A 9 110 c.c. 1 loop A 8			
	p m	A 10 110 c.c. 1 loop A 9			
22 x 40	a m	A 11 110 c c 1 loop A 10			
	p.m.		A 11*a* 1 loop A 11, 10^{-3} W colonies only		
17. x. 40	a m.	B 1 (801) 500 c c. W 2,500,000 R 20			
	p m.	B 2 110 c.c 713,000 cells from B 1	B 1*a* 1 loop B 1, 10^{-3} W 713 colonies		

https://doi.org/10.1017/S0022172400059799 Published online by Cambridge University Press

Experiment II (*continued*)

		(1)	(2)	(3)	(4)
18. x. 40	a m	B3 110 c.c. 400,000 cells from B2	B2*a* 1 loop B2, 10^{-3} W 400 colonies		
	p.m.	B4 110 c.c. 1 loop B3	B3*a* 1 loop B3, 10^{-3} W colonies only		
19. x. 40	a.m.	B5 110 c.c. 1 loop B4	B4*a* 1 loop B5, 10^{-3} W 100 colonies	B3*b* 1 loop B3, 10^{-3} W and R colonies	B2*c* 1 loop B2, 10^{-3} W 60 R 25 colonies
	p.m	B6 110 c.c. W 109,000 R 433,000	B5*a* 1 loop B5, 10^{-3} W 109 R 433 colonies		
17 x. 40	a m	C1 (802) 500 c c W 2,500,000 R 10			
	p.m.	C2 110 c.c. W 578,000	C1*a* 1 loop C1, 10^{-3} W 578		
18. x. 40	a m.	C3 110 c c W 400,000	C2*a* 1 loop C2, 10^{-3} W 400 colonies		
	p.m.	C4 110 c.c. W 671,000 R 3000	C3*a* 1 loop C3, 10^{-3} W 671 R 3 colonies		
19. x 40	a m.	C5 110 c c W 224,000 R 416,000	C4*a* 1 loop C4, 10^{-3} W 224 R 416 colonies	C3*b* 1 loop C3, 10^{-3} W and R colonies	C2*c* 1 loop C2, 10^{-3} W 130 R 23 colonies

EXPERIMENT III (7. v. 40)

Half-litre flasks, each containing 300 c.c. lactose peptone water, *p*H 7·4 were used.

Flask A1 received 2,850,000 W.

Flask B1, 2,850,000 W plus 43 R (R : W :: 1 : 66,270).

In chain A1 to A4, R was absent in all flasks up to 12 hr. (plates A1*a*, A3*a*, A4*a*). Also in plate A4*b* taken after 24 hr. incubation. In A4*c*, however, after 36 hr. incubation there were 500 W colonies and 26 R.

In chain B, R appeared in B2 24 hr. from the start of the chain (plate B2*a*, W colonies 198, R 1) and in B3 (plate B3*a*, W 225, R 56).

Therefore since in the control R was present in the proportion

R : W :. 1 : 66,270

and manifested itself in the second flask of the chain at 24 hr., while in chain A R was absent from the fourth flask at 48 hr., the parent non-lactose culture contained either no R at all, or a hypothetical number less than 1 : 66,270. This hypothetical number died out in competition with W on lactose.

Experiment III

		(1)	(2)	(3)	(4)
7 v. 40	a.m.	A1 (575) 300 c.c. W 2,850,000			
	p.m.	A2 300 c.c. W 434,000	A1*a* 1 loop A1, 10^{-3} W 434		
8. v. 40	a.m.	A3 300 c.c. 1 loop A2, 10^{-3}			
	p.m.	A4 300 c.c. W 351,000	A3*a* 1 loop A3, 10^{-3} W 351 colonies		
9. v. 40	a.m.		A4*a* 1 loop A4, 10^{-3} W colonies only		
	p.m.			A4*b* 1 loop A4, 10^{-3} W colonies only	
10 v. 40	a.m.				A4*c* 1 loop A4, 10^{-3} W 500 R 26 colonies
7. v. 40	a.m.	B1 (576) 300 c.c. W 2,850,000 R 43			
	p.m.	B2 300 c.c. W 272,000	B1*a* 1 loop B1, 10^{-3} W 272 colonies		
8. v. 40	a.m.	B3 300 c.c. W 198,000 R 1000	B2*a* 1 loop B2, 10^{-3} W 198 colonies R 1		
	p.m.		B3*a* 1 loop B3, 10^{-3} W 225 R 56 colonies		

From these experiments we may conclude that if R is present in number not less than 1 in 250,000 it will overgrow W on lactose either in a single culture or in a chain. If, however, the number is as low as one in a million it will probably die out. Now R does not appear in chains started by samples of 2·5, 2·85, and 80 million bacteria from non-lactose media. It is therefore either absent from these samples or is present in a hypothetical number less than 1 in 250,000. This number will not be able to overgrow W on lactose. Therefore the variants which overgrow W and with which we are acquainted have arisen on lactose only.

The race G1; systematic characters and the yellow pigmented variant

The race G1 was isolated from the faeces of a mental patient in October 1939. It consisted of non-motile, Gram-negative bacilli, 1·5–2·0 μ in length. Sugar reactions: lactose 0 for 3 days, A or AG on fourth, glucose AG, saccharose O, maltose AG, mannite AG, dulcite O. Gelatine was not liquefied.

Its colonies on MacConkey plates at 24 hr. were 2 mm. in diameter and smooth, flat, lens-shaped, and colourless; at 48 hr. they were 2·5–4·0 mm. in diameter and pale red in the centre. Papillae appeared as bright, colourless, hemispherical droplets projecting above the surface between 24 and 36 hr., and increased in size and became red between 36 and 48 hr. On a starvation medium consisting of NaCl 0·5 g., lactose 1·0 g., agar 2·0 g., neutral red q.s., water 100·0 c.c. it formed colonies 0·04 to 0·20 mm. in diameter. When not crowded there were no red papillae at 72 hr. but when the number of cells inoculated was so large that a continuous film of growth resulted, red papillae appeared as early as 24 hr. and they may then be indistinguishable from primary reds, having varied prematurely owing to crowding and starvation.

Yellow variant. Yellow and white or unpigmented colonies were noticed on plating about a month after its first isolation. The yellow variant Y arose in papillae on the U (unpigmented) colonies. The papillae made their first appearance after about 36 hr. incubation. They increased in size and became orange yellow by 48 hr. Subcultures from papillae gave white and yellow colonies. The yellow colonies formed no variant papillae on nutrient agar, while the whites continued to cast yellow. The U–Y variation was independent of the W–R, either W or R formed Y from U and either U or Y formed R from W on lactose. We thus got four varieties: (1) WU with R and Y papillae, (2) WY with R papillae, (3) RU with Y papillae on non-lactose, (4) RY without variant papillae. RY on lactose was plain red as the acid from the fermenting sugar prevented the formation of pigment but when put back on nutrient agar it was again bright yellow. U formed Y on the following media, agar with meat extract without peptone, agar with both extract and peptone, agar with peptone only; U formed Y occasionally and without certainty on vegetable proteins. Under the microscope the pigment can be seen in the bodies of the bacteria as subterminal granules 0·25 μ in diameter and outside the bacteria as irregular masses measuring 0·3–0·8 μ.

All the strains W, R, Y and U were serologically identical.

The race G1 with its R and Y variants was exhibited at the Cambridge meeting of the Pathological Society in December 1939. Bacteria of this kind degenerate after one year in culture.

SUMMARY

1. With coliform bacteria careful plating allows the isolation of pure lines with complete certainty.

2. The three experiments here described tend to prove that the variation from non-lactose fermenter to lactose fermenter takes place only on lactose. These experiments should be repeated with other strains which must not be older than one year in culture.

3. A race of *B. coli-mutabile* (G1) forms a yellow pigmented variant in papillae.

I wish to express my thanks to Dr H. B. Fell, Director of the Strangeways Research Laboratory, for kind permission to make use of the laboratory and to the staff for their assistance in the work on which this paper is based. I am also indebted to Prof. H. R. Dean for kind permission to work in his department and to Mr W. F. Gifford for supplying the race G1.

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(*MS. received for publication* 20. IX. 1941.—Ed.)