EXPERIMENTS ON ADAPTIVE VARIATION AND REJUVENATION

BACILLUS DYSENTERIAE FLEXNER, GROWN ON MEDIA CONTAINING SULPHANILAMIDE, ACRIFLAVINE, ARGYROL, NEOSALVARSAN AND NO DRUG

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I. INTRODUCTION

The work on which the following paper is based was done in the laboratory of the Department of Pathology, Cambridge, by kind permission of Prof. H. R. Dean, and in the Memorial Ophthalmic Laboratory, Giza, Egypt. I wish to express my thanks to the staffs of these two laboratories for very great assistance and much patience.

Note on bacterial papillae

Papillae are minute projections which appear on colonies of bacteria growing on agar plates about the 3rd or 4th day of incubation. They occur probably on all bacterial colonies, but have been studied chiefly on members of the coli, typhoid and dysentery groups. If a plate bearing colonies of *Bacillus coli-communis* or *B. dysenteriae* Flexner is held up to the light on the 3rd or 4th day and examined with a hand lens giving × 10 magnification, the papillae can be seen by transmitted light as minute shining bright spots about 0·05 mm. in diameter. They are generally situated toward the centres of the colonies. During the next 2 days these spots increase in size to 0·25 or 0·50 mm. and definitely project above the surface. By transmitted light they are golden, by reflected light they are grey-white projections on the surface. They may grow out over the surface of the colony to its margin. On the other hand, their growth may be arrested earlier, when they measure only 0·10 mm. Papillae vary greatly in number. There may be only one or two on a number of colonies or there may be so many that they cover half the surface of any colony.

In some bacterial species obvious variations take place in the papillae if the agar plate contains a sugar such as lactose or saccharose. In fact, it was this power of variation which first drew attention to these minute structures. This happened in the case of a non-lactose fermenter, *B. coli-mutabile*, which was growing on a plate containing lactose with litmus as an indicator. The alkaline colonies were of course tinged blue, and on them minute red papillae appeared which were found to consist of lactose fermenters. Subcultures from these red papillae on to lactose plates gave rise to colonies of two kinds, blue which again formed red papillae, and red which gave origin to a permanently lactose-fermenting strain. With these observations, which were published by Massini in 1907, began the scientific study of bacterial variation.

After this first paper the subject of bacterial papillae has been studied by the following:*

Burck (1908) repeated and confirmed Massini's work on *B. coli-mutabile*.

Reiner Müller (1909*a*, *b*) also worked at the same species. In addition, he found three strains of coliform bacteria which formed papillae with variation on arabinose agar plates, and he discovered that *B. typhosus*, if grown on agar with isodulcite, formed white colonies with red papillae which on subculture gave red and white colonies. In a later paper (1911) he stated that the reddening of the papillae in this latter case was not due to acid formation; neverthe-

* Early work by Twort and Goodman was conducted by culture in fluid media only.

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less, the parent white colonies were less robust than their red descendants, a fact which Müller attributed to blocking of receptors by isodulcite and consequent starvation of the white strain, while the red strain had overcome this weakness. This explanation appears to be an anticipation of modern views on bacteriostasis by sulphonamides.

Burri & Düggeli (1909) and Burri alone (1910) described a coliform grass bacillus *Bacterium imperfectum* which does not originally ferment saccharose, but which when grown on agar plates with this sugar forms a saccharose-fermenting strain in papillae.

Baerthlein (1911, 1912) again studied *B. coli-mutabile*, confirming previous work. He noted that the red character usually remained constant after growth on non-lactose media, but that atavistic regression to white might occur after growth on agar plates for a week or more.

Penfold (1911), continuing work which Twort had begun in fluid culture media, found that if *B. typhosus* is grown on plates containing dulcite, a red dulcite-fermenting strain arises in the papillae.

Bernhardt & Markoff (1912), investigating a strain of *B. dysenteriae* Flexner from monkeys, found that it did not originally ferment maltose but acquired the power to do so by variation in papillae when grown on maltose agar. This red-to-maltose strain reverted to white after passage through a monkey. They also found that *B. coli-mutabile* did occasionally revert from red to white on animal passage.

Baerthlein (1918) summarized the papillary variations which he found under the following heads:

(1) Variation in size and shape of colonies, their pigmentation and gelatinous character.

(2) Variation in form of the individual bacteria—length, breadth, staining power, etc.

(3) Variation in their powers of fermentation.

(4) Variation in serum reaction.

(5) Variation in virulence.

He also found that irreversibility is never absolute, since return can generally be caused by prolonged stimulation or animal passage.

Toeniessen (1920, 1921) studied variation in Friedlander's bacillus.

The present writer (Stewart, 1924) studied papillary variation in *B. coli-mutabile*, *B. paracolon*, *B. typhosus*, *B. dysenteriae* Flexner, and *B. acidi-lactici* Hüppe. He found *B. coli-mutabile* variable to lactose, *B. paracolon* variable to lactose and to saccharose, *B. typhosus* and *B. acidi-lactici* to dulcite, and *B. dysenteriae* Flexner to maltose. The variations fall into two very definite groups: (1) In *B. coli-mutabile*, *B. acidi-lactici* and *B. typhosus* they take place constantly after a regular short exposure (of two to five days) to the sugar in question. (2) In *B. paracolon* and *B. dysenteriae*, on the other hand, the first occurrence of the variation takes place rarely, in perhaps one single papilla after long exposure (of 3–6 weeks). But descendants of this first variant papilla are regular in frequent variation. Stewart also described a race of *B. coli-mutabile* which formed a yellow pigmented variant in addition to the lactose fermenter.

Stewart (1926) suggested that the white or non-lactose-fermenting form of *B. coli-mutabile* fails to ferment lactose not through absence of a ferment but through the action of an inhibitory factor (since the negative character is dominant over the positive in the white form). This view has been upheld by the work of Deere, Dulaney & Michelson (1936) and Deere (1939), who found that the ferment lactase is present in the white form but that it is inhibited by some unknown cause.

The entire subject of bacterial papillae, their biological significance and the variations which arise in them has, however, received little attention from bacteriologists. They are not mentioned in any textbook in the English language and only in two of the larger works of reference.

Previous paper on sulphonamide-resistant strains

In a previous paper the present writer (Stewart, 1947) has shown that *B. dysenteriae* Flexner, in developing resistant strains during culture on sulphonamide media, conforms to the pattern of Massini's variation in *B. coli-mutabile* (Massini, 1907) in the following manner:

Colonies of bacilli grown on agar plates which contain sulphanilamide (SA) 1/20,000 form papillae or secondary colonies from the 3rd day onward. If subcultures are made from these papillae on to plates containing SA 1/10,000, colonies of two types result: (1) large and resistant, composed of bacilli which are able to grow on plates of sodium sulphacetamide (SSAC) 1/20,000; (2) small and non-resistant, not able to grow on SSAC 1/20,000.* On the other hand, subcultures not from papillae but from the growing margins of the primary colonies on SA 1/20,000 contain no resistant forms. Resistant strains keep their character after long growth on drug-free media. The change from non-resistant to resistant takes place by discontinuous variation, the new, variant type making its appearance suddenly in company with the original unmodified type, from which it is at once sharply distinguished.

Purpose of the present paper

In the present paper further observations in support of these views will be described, and it will be shown that there is no gradual and equal increase of resistance in the whole of a bacterial population exposed to sulphonamides, and that any appearance

* Experience has shown that ability to grow freely on SSAC 1/20,000 after 24 hr. incubation is a good test of increased resistance to sulphonamides.

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of such a gradual increase, which can be charted as a curve, is due to an increasing proportion of resistant cells in a mixed population. [Compare and contrast Kirby & Rantz (1943) and McIntosh & Selbie (1943).]

Prefatory note on abbreviations used in the text

Sulphanilamide: SA.

Sodium sulphacetamide: SSAC.

Satellite action (Pike & Foster, 1944). A bacterium if sown *thickly* on a plate containing SA may be able to grow in a concentration of the drug which would prevent growth of a thinner inoculum. A non-resistant strain may also be able to grow if sown in company with resistant cells.

No-contact designates a strain of bacterium which has never before been in contact with the drug under experiment.

Ringer/4 = Ringer's solution quarter strength.

Technical methods

(1) *Special culture media for experiments with sulphonamides*

I have found that the omission of peptone from the usual culture media avoids anti-SA action without seriously affecting their power to nourish *B. dysenteriae* Flexner. Peptone-free media have therefore been used whenever SA was to be added.

Lemco broth is the basis of such media. It consists of: Lab. Lemco 10; sodium chloride 5; water 1000. Heat at 100° C. for 30 min. Neutralize at 37° C. Heat again 30 min. Filter through paper and adjust to pH 7·4. Autoclave 30 min. at 10 lb. pressure.

Lemco agar—Lemco broth with the addition of agar 2 %.

(2) *Plating*

Agar medium for plating in all experiments, both with SA and other drugs, was put up in 18 c.c. quantities in screw-cap bottles. It was thus easy to calculate the amount of drug which had to be added. The drugs, SA, acriflavine and argyrol, were made up in strengths 1/500 or 1/1000 in glass-distilled water and autoclaved at 10 lb. pressure for 30 min. Neosalvarsan, 0·6 g., was dissolved in 15 c.c. of sterile distilled water, giving a strength of 1/25. This solution kept well in a frigidaire without precipitating and was diluted when required, 0·2 c.c. in 2·5 c.c. of distilled water giving a concentration of 1/322·5. The drugs were added to the melted agar at a temperature of 50° C., and the resultant medium was poured and dried in the usual way. For inoculation of plates bacteria were taken from 24 hr. cultures in drug-free broth, 5 loops were added to 2·5 c.c. of Ringer/4 in a screw-cap bottle and well shaken. Two loops of this suspension were then spread on each plate with a right-angled glass spreader.

The loop which I used contained an average of 0·0016 c.c., therefore the Ringer suspension was a dilution of 1/300 of the broth culture.

(3) *Pure lines*

Pure lines can be established beyond doubt in the *coli* and dysentery groups by dilution of a broth culture in Ringer/4 and vigorous shaking, followed by plating on a non-selective medium (Stewart, 1942, 1944). Two loops of the Ringer suspension are spread with a right-angled glass spreader on a pair of plates. All strains used by me were purified by plating on at least two successive plates of drug-free nutrient agar.

(4) *Test for resistance to sulphonamides*

A. Experience has shown that a good test for increased resistance after culture in SA media consists of plating the bacteria on agar plates containing: (*a*) SA 1/10,000, (*b*) SSAC 1/20,000 and (*c*) SSAC 1/10,000. Strains of increased resistance will grow on (*a*) and (*b*) and possibly on (*c*), while strains which have not previously been in contact with SA or which have not increased in resistance in spite of contact with SA may grow on (*a*) but will not grow on (*b*) or (*c*).

B. *Titration.* A series of plugged test-tubes measuring (internal diameter) 13 × 125 mm., or of centrifuge tubes 21 × 93 mm., are put up, each containing 5·5 c.c. of broth, which has a depth in the test-tubes of 40 mm. (Lemco broth is used for SA experiments, ordinary broth for others.) These tubes are autoclaved at 10 lb. pressure for 30 min., and the contained broth is then reckoned as 5 c.c. The drug to be used is added from a sterilized solution (1/250, 1/500 or 1/1000) kept in a screw-cap bottle. The series used for SA was generally 1/34,000, 1/21,000, 1/15,000, 1/13,000, 1/10,500, 1/8800, 1/7600, 1/6700, 1/6000, 1/5500. Bacteria were added from a suspension in Ringer/4 containing 5 loops of a 24 hr. broth culture in 2·5 c.c. of Ringer. Two loops of this suspension were added to each titration tube. The series was then incubated for 24 hr., read and recorded as visible growth (a moving shimmer when shaken in light against a black background) marked + or no growth (0).*

II. EXPERIMENTS WITH *BACILLUS DYSENTERIAE* FLEXNER GROWN ON SULPHONAMIDES

(A) Single cultures on plates containing SA.

(B) Can a resistant strain be raised to a higher level of resistance by growth on plates containing a higher percentage of SA?

(C) Single cultures in broth tubes containing SA.

(D) Chain cultures in broth tubes with SA (Tables 1 and 2).

* Note that the appearance of visible growth at 24 hr. depends to some extent on the numbers of viable cells inoculated. A mixture of resistant and non-resistant cells, as in the unanalysed descendants of a papilla, may therefore give an intermediate reading. Different readings may also result from difference of thickness of growth in 24 hr. broth cultures of the same organism.

A. *Single cultures on plates*

I shall record two examples of experiments out of a number performed.

Experiment 1

A race of *B. dysenteriae* Flexner, isolated in Cairo and purified by plating, gave typical sugar reactions, viz. lactose O, glucose A, maltose O, mannite A.

It was numbered R 8.

(*a*) *Primary plate.* A no-contact strain (see p. 333) was sown on a plate of Lemco agar containing SA to a strength of 1/20,000. After 24 hr. incubation about 250 delicate colourless colonies appeared, measuring 0·50–0·75 mm., with markedly irregular star-shaped outlines. (This form of colony is not the rough variation, since descendants on drug-free media resume smooth round outlines.)

(*b*) *Separation of strains from papillae and from the non-papillary margin.* On the 3rd day of incubation the star-shaped colonies were studded with papillae, golden by transmitted light, measuring 0·25–0·30 mm. Subcultures into drug-free broth were made from these papillae and from the margins of colonies as far removed from the papillae as possible. These two strains were purified by plating on agar with SA 1/20,000. On this medium colonies of the papillary strain were robust and circular, measuring 0·5–1·0 mm. Colonies of the marginal strain were again delicate and star-shaped, 0·25–0·75 mm.

(*c*) *Titration of no-contact, marginal and papillary strains of* R 8. These three strains were then titrated in tubes of Lemco broth containing SA in concentration from 1/34,000 to 1/5000. The titration tubes were read after incubation for 24 hr. with the following results:

The no-contact and marginal strains grew up to, but not beyond, 1/34,000.

The papillary strain R 831 grew up to 1/5800.

The papillae therefore contained resistant cells while the cells from the margins were still non-resistant. The variation to increased resistance had taken place only in the papillae and was obviously discontinuous. There was no general increase of resistance of all cells exposed to the drug.

Experiment 2

(*a*) *Primary plate. B. dysenteriae* Flexner W.Y. Race 18, was sown on a plate of Lemco agar containing SA 1/20,000. The colonies at 48 hr. incubation were rounded and smooth and measured 0·1–2·0 mm.

(*b*) *Isolation of strains from papillae and from the non-papillary margin of colonies.* Papillae appeared on the primary plate on the 5th day of incubation. Papillae and points of the non-papillary margin of colonies were picked off into drug-free broth and then plated on Lemco agar containing SA 1/10,000. (This strength would differentiate resistant from non-resistant strains.) The plates from both papillary and marginal strains showed large colonies of 0·5 mm. diameter and small colonies 0·1 mm. after 24 hr. The numerical proportion of large to small was, however, very different on the two plates, on the papillary plate large to small were as 20:1, on the marginal plate as 1:100.

(*c*) *Test for resistance.* Four purified strains were then established by picking off separate colonies into drug-free broth, viz. papillary large and small, and marginal large and small, and these four strains were then sown on plates containing SSAC 1/20,000 and 1/10,000. Papillary large grew on the former but not on the latter, while the other three strains did not grow on either. Therefore the papillae on the primary plate had developed a resistant strain alongside the original non-resistant strain.

Note. This case differs from that described in Exp. 1, where the subcultures from papillae contained resistant cells only. The difference between resistant and non-resistant cells was so great in Exp. 1 that the non-resistant were completely overgrown on a medium containing SA, i.e. in the papillae of the primary plate.

B. *Can a strain of Flexner's bacillus which has become resistant to SA develop a higher degree of resistance in a second step if it is grown on agar containing a higher percentage of SA?*

The possibility of such an occurrence is suggested by the behaviour of certain strains of *B. typhosus* if they are sown on plates of McConkey's medium containing dulcite instead of lactose (Stewart, 1927, p. 69). On the first sowing they form white colonies which after some days bear pink papillae. Subcultures on to the same medium from these papillae give separate white and pink colonies, the latter bearing dark red papillae. Subcultures from these dark red papillae give pink and dark red colonies. If the three strains white, pink and dark red are mixed and sown on a dulcite plate, the three types of colony arise perfectly distinct and with no intermediate gradation of colour. Thus full fermentation of dulcite is achieved in two distinct steps.

Experiment 3

To test whether *B. dysenteriae* Flexner could achieve a second step exaltation of resistance to SA, a strain of R 8 was grown on agar containing SA 1/20,000 (see Exp. 1 above). It developed a resistant strain (R 831), which on titration grew in SA 1/5800, but not in 1/4417. (The original no-contact strain grew in SA 1/34,000 and failed in 1/21,000.) R 831 was then plated on agar containing SA 1/6500, the colonies on which developed papillae on the 4th day. These were picked off into broth which was sown on agar containing the same strength of SA, viz. 1/6500.

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The largest colony which appeared on this medium was picked off to give a pure highly resistant strain (R 966) if such a one had been formed, while the unanalysed descendants of the papillae were kept in culture (R 955) in case the largest colony (R 966) should not prove most resistant.

The two strains, R 966 and R 955, were then titrated (in centrifuge tubes 21 × 93 mm.) in comparison with the original resistant strain (R 831). In one experiment both R 966 and R 831 grew in SA 1/6055 but not in 1/5045, showing that R 966 had not achieved a second increase of resistance. In another experiment R 955 (the unanalysed papillary mixed strain) and R 831 both grew in 1/5045. I found that titration of resistant strains in Lemco broth containing high concentrations of SA did not always give an upper limit. I therefore titrated the mixed papillary strain R 955 and the original resistant strain R 831 on plates containing SSAC which gives more clear-cut results than SA. The series of plates contained SSAC in the following concentrations: 1/20,000, 1/15,500, 1/10,500, 1/8000, 1/7400, 1/6500, 1/5800. There was no consistent difference of growth in the two strains; the size of the colonies was approximately equal up to 1/8000 and both strains produced only minute irregular scraps of growth measuring 0·05 mm. on SSAC 1/6500 and 1/5800.

Experiment 4

Another resistant strain of Flexner's bacillus which was able to grow on plates containing SSAC 1/20,000 was sown on a plate containing SA 1/7000. Papillae appeared on the 4th day and were picked off into broth. The strain thus formed failed to grow on plates containing SSAC 1/10,000.

Therefore neither of these resistant races appeared to be able to form a second-step strain of higher resisting power.

C. *Single cultures in tubes of Lemco broth*

Experiment 5

(*a*) The *primary culture* consisted of Lemco broth 10 c.c. containing SA 1/10,000 in a boiling tube 22 × 175 mm. (as advocated by Kirby & Rantz, 1943). The same race, R 18, as in Exp. 2 was inoculated and the tubes incubated for 5 days.

(*b*) *Separation of a resisting variant from an unmodified non-resistant form.* The primary culture after suitable dilution was sown on a plate of Lemco agar containing SA 1/20,000. Colonies which appeared were large type 1·0–2·0 mm. and small type 0·1–0·2 mm. The large type proved itself capable of growing on plates containing SA 1/10,000 and SSAC 1/20,000 but not on SSAC 1/10,000, while the small type grew on SA 1/10,000 but not on SSAC 1/20,000 or 1/10,000. Therefore the large type was a resisting variant while the small type was the unmodified original strain.

Experiment 6

Flexner's bacillus was grown in Lemco bouillon, 10 c.c. in a boiling tube measuring 22 × 175 mm., with SA added to give a concentration of 1/10,000. On the 5th day a subculture was made on a drug-free nutrient agar slope which was then plated on Lemco agar with SA 1/20,000. Colonies of large type (1·0–2·0 mm.) and of small type (0·1–0·2 mm.) resulted. The large type proved resistant by growing on SA 1/10,000 and on SSAC 1/20,000, while the small type grew on SA 1/10,000 but not on SSAC 1/20,000.

D. *Chain cultures in Lemco broth*

Kirby & Rantz (1943) have worked out a method of estimating resistance to SA by the opacity of standard broth cultures read by means of a spectrophotometer at different times during 24 hr. incubation. Each culture consisted of 10 c.c. of standard broth (containing ammonium sulphate, glucose and casaminoacids) in a boiling tube 22 × 175 mm., with the addition of SA to a concentration of 1/10,000. With these tubes the authors made chain cultures of a strain of *Escherichia coli* (sive *B. coli-communis*) from the blood in a case of pyelonephritis. Each link of the chain was incubated 24 hr. and then subcultured into the next link.

The authors report as follows: 'During the first day, following the initial lag period, there was marked inhibition of growth in all tubes containing sulphonamides. Thereafter there was a gradual daily development of resistance the progress of which is indicated at intervals of 2–3 days on the charts. The organisms became maximally resistant about the 10th or 12th day. Thereafter transferring them for 20 more days did not produce any greater degree of resistance. Further, no loss of resistance was observed after transferring resistant organisms to drug-free media.'

The authors do not appear to have plated the links of the chain on SA 1/20,000 or 1/10,000 to find out the composition of the bacterial population at successive stages. I have therefore made similar chain cultures and plated each successive link, at the end of its 24 hr. incubation, on to Lemco agar containing SA 1/10,000. Strains isolated were then tested (*a*) by titration in tubes of Lemco broth containing SA in concentrations from 1/13,000 to 1/5000 (Table 1) or (*b*) by plating on agar containing SSAC 1/20,000 (Table 2).

The plan and the results of these experiments can be followed most readily by referring to Tables 1 and 2.

Experiment 7 (Table 1)

In this experiment the chain consisted of boiling tubes each containing 10 c.c. of Lemco broth of

pH 7·2 with SA to 1/10,000. These daily links are shown in column (1) marked I–VII.

Link I was inoculated with two loops of a 24 hr. broth culture of *B. dysenteriae* Flexner, and was then incubated for 24 hr. One loop was then transferred to link II and one to a tube of drug-free peptone broth marked I*a* in column 2. I*a* was incubated for 24 hr. and then plated after dilution in Ringer/4 (2 loops in 2·5 c.c.). Two loops of this Ringer dilution were spread on a plate of Lemco agar containing SA 1/10,000 marked I*b* in column 3. This plate was examined after 24 hr. incubation, the colonies being counted and classed as large (L) or small (S). The numerical ratio of L to S is shown in column 5. At least one colony of each type was then picked off into a tube of drug-free broth which was incubated for 24 hr. and the result, growth (+) or no growth (0), recorded in column 6. It will be seen that the small colonies of links IV, V and VII failed to grow in drug-free broth although as many as six colonies were picked off from each plate of column 3. It was therefore not possible to titrate them, but there can be no doubt that they were non-resistant since their vitality had been so reduced on the plates containing SA 1/10,000 that they could not furnish viable subcultures. The strains thus established in drug-free broth, column 6, were then titrated in tubes each containing 5 c.c. of Lemco broth to which had been added SA to the strength shown in columns 7–12. The results were read after 24 hr.

In addition to the pure strains L and S isolated on the plates of column 3, the mixed cultures I*a* and II*a* were also titrated, since I realized that if only a few cells of links I and II had become resistant they would probably not be picked out on the plates of column 3, while they might be able to show themselves if I*a* and II*a* were titrated in their crude state.

Results

The figures from the strains L and S, I–VII, should be compared with the no-contact strain (line 1) which is not able to grow in 1/8800 (column 9).

Link I. There were very few L colonies on the plate I*b* (line 3), and the L strain which was titrated proved non-resistant (unable to grow in 1/7650). On the other hand, I*a* unanalysed (line 2) can grow in 1/7650, showing that a few cells had become resistant in I*a*.

S colonies were very numerous and the strain titrated was non-resistant, i.e. unable to grow in 1/10,500. Therefore the great bulk of the cells remained unmodified in link I.

Link II. L colonies were more numerous on the plate II*b* (line 6), but the L colony which was picked out still proved non-resistant, i.e. unable to grow in SA 1/7650, while II*a* unanalysed grew in this strength (line 5). (The question whether the colony chosen as L would or would not be representative is of course one of arithmetical chance.) S was still predominant (line 7) and non-resistant, i.e. unable to grow in SA 1/8800.

Link III. L and S colonies (column 4) were now equal in number (column 5). The L colony chosen was emphatically resistant (line 8, column 11). S forms were still readily isolated (line 9) and proved non-resistant (column 9).

Link IV. L colonies predominated in column 5, line 10, and grew in SA 1/5050 (column 12). S colonies, although still present (line 11, column 5), could not be isolated. They were so weak that they failed to grow in drug-free broth (column 6, line 11).

Link V. L colonies were numerous and resistant (line 12, column 12). S, although apparently more numerous, failed in drug-free subculture (line 13, column 6).

Link VI. L colonies were numerous and resistant. The S strain was again isolated (line 15, column 6) and proved non-resistant (column 8).

Link VII. L colonies were numerous and resistant, S colonies were present but were not isolated (line 17, column 6).

Summary of Table 1. Resistant forms appeared in III*a* after 3 days' growth in SA 1/10,000, but were actually present in small numbers at the end of the first day.

Non-resistant forms have been demonstrated by titration in link VI after 6 days' growth in SA 1/10,000. They are unable at any link stage (except II, column 8, line 7) to effect lodgement (when isolated) in SA 1/10,000, therefore they are emphatically non-resistant. Their persistence in the link tubes (column 1) was due to the support given by satellite action of the mixture in which they are growing.

Note. The link tubes II–VII were each inoculated with one loop undiluted of its predecessor, while the titration tubes were inoculated with one loop of a dilution in Ringer amounting to 1/3000 (2 loops in 2·5 c.c.).

Experiment 8 (Table 2)

Columns 1–6 of Table 2 are the same as in Table 1, except that the link tubes contained SA 1/20,000. The L and S strains were then tested by plating on SSAC 1/20,000 (column 7 and 8) after diluting a 24 hr. broth culture in Ringer/4.

The results were the same as in Table 1. Resistant forms did not appear in the first 2 days. Both I*b* L and S and II*b* L were unable to grow on SSAC 1/20,000, column 7, while the colony chosen for II*b* S was not viable in drug-free broth. Resistant forms did appear however on plates III*b*, IV*b* and V*b* as L colonies which, after growing readily in drug-free broth (column 6), grow also on plates of SSAC 1/20,000 (column 7) to colonies measuring 0·5 mm. at 24 hr.

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Table 1

For explanation see text, p. 335. Experiment 7.

1	2	3	4	5	6	7	8	9	10	11	12	
						Broth+SA						
Lemco broth +SA 1/10,000	Drug-free broth	Lemco agar plate SA 1/10,000	Colonies	Numerical proportion	Broth from single colony	1/13,000	1/10,500	1/8800	1/7650	1/6050	1/5050	Lines
No-contact strain		.	.	.	.	.	+	0	0	.	.	1
Link I	I*a*	Not analysed	.	.	.	.	+	+	+	0	.	2
		I*b*	L	1	+	.	+	.	0	0	0	3
			S	400	+	.	0	0	.	.	.	4
Link II	II*a*	Not analysed	.	.	.	.	+	+	+	0	.	5
		II*b*	L	1	+	.	+	+	0	0	0	6
			S	15	+	+	±	0	.	.	.	7
Link III	III*a*	III*b*	L	1	+	.	+	.	.	+	0	8
			S	1	+	+	.	0	.	.	.	9
Link IV	IV*a*	IV*b*	L	2	+	.	+	.	.	+	+	10
			S	1	0	.	.	.	.	.	.	11
Link V	V*a*	V*b*	L	1	+	.	+	.	.	+	+	12
			S	2	0	.	.	.	.	.	.	13
Link VI	VI*a*	VI*b*	L	.	+	.	+	.	+	+	0	14
			S	.	+	.	0	0	.	.	.	15
Link VII	VII*a*	VII*b*	L	3	+	.	+	.	+	+	0	16
			S	1	0	.	.	.	.	.	.	17

Table 2

For explanation see text, p. 336. Experiment 8.

1	2	3	4	5	6	7	8	9	
						Lemco agar plate SSAC 1/20,000			
Lemco broth +SA 1/20,000	Drug-free broth	Lemco plate SA 1/10,000	Colonies	Numerical proportion	Drug-free broth	24 hr.	48 hr.	Drug-free broth	Lines
Link I	I*a*	I*b*	L	1	+	0	0	+	1
			S	100	+	0	0	+	2
Link II	II*a*	II*b*	L	1	+	0	0	+	3
			S	10	0	.	.	.	4
Link III	III*a*	III*b*	L	4	+	0·5+	1·25+	.	5
			S	1	+	0·08±	0	.	6
Link IV	IV*a*	IV*b*	L	1	+	+	+	.	7
			S	1	+	+	+	.	8
Link V	V*a*	V*b*	L	2	+	+	+	.	9
			S	1	+	0	0	.	10
No-contact strain		.	.	.	.	0	0	.	11

Among L and S colonies on an SA plate sown from a mixture of resistant and non-resistant cells, the L colonies will generally contain resistant and the S colonies non-resistant cells, especially if the two types are nearly equal in number. But this rule is not absolute, since the size of a colony may be determined by other factors as well as resistance. For example, non-resistant cells may form an L colony by satellite action (Table 1, I*b*, L) and resistants may form an S colony for some other reason (Table 2, IV*b*, S).

In judging titration results the caution given in the introduction (footnote, p. 333) should be borne in mind, especially in regard to unanalysed strains and intermediate readings. The latter do not belie discontinuity of variation.

There is no reason to assume that bacteria in papillae undergoing discontinuous variation under the impact of a drug will necessarily all achieve resistance at precisely the same level. Differences in titration value of different newly formed resistant strains in Table 1, lines 6, 8, 10, 12, 14 and 16, and Table 3, lines 10, 11, 14 suggest that they reach different levels.

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Non-resistant forms were clearly present on I*b* and II*b*, growing in drug-free broth (column 6, lines 1–3, I*b* L and S, and II*b* L) but failing to grow on SSAC 1/20,000 (column 7). They were also present in III*b* S (line 6, columns 7 and 8), since, although minute colonies appeared on SSAC 1/20,000 at 24 hr., they were dead at 48 hr., while resistant colonies

There is no general and equal increase of resistance affecting all the cells exposed to sulphanilamide, but a discontinuous variation to resistance affecting only a proportion of the cells. These resistant cells then slowly outgrow in number the non-resistant cells, although the latter can still survive by satellite action.

Table 3. B. dysenteriae *Flexner grown on acriflavine*

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	
		Subcultures			Titration in acriflavine										
Primary plates	From	Into drug-free broth	Colonies on agar plate with acriflavine 1/60,000	Drug-free broth	1/48,000	1/41,000	1/37,000	1/30,000	1/26,000	1/21,000	1/17,000	1/13,000	1/11,000	1/9300	Line
					Plates										
Agar plate + acriflavine 1/60,000	48 hr. colonies	+	L	+	.	.	.	.	.	+	.	.	.	.	1
			S	0	.	.	.	.	.	.	.	.	.	.	2
	Papilla 3rd day	+	L	+	.	.	.	.	.	+	.	.	.	.	3
			S	0	.	.	.	.	.	.	.	.	.	.	4
	Margin 3rd day	0	.	.	.	.	.	.	.	.	.	.	.	.	5
	Papilla 5th day	+	L	+	.	.	.	.	.	+	.	.	.	.	6
			S	0	.	.	.	.	.	.	.	.	.	.	7
	Margin 5th day	0	.	.	.	.	.	.	.	.	.	.	.	.	8
	No-contact strain	+	.	.	.	+	.	+	.	0	.	.	.	.	9
					Broth tubes										
Agar plate with acriflavine 1/60,000	Papilla 4th day	+	L	+	.	+	.	.	+	.	+	+	0	0	10
			S	+	.	+	.	.	+	.	+	0	.	.	11
	Margin 4th day	0	.	.	.	.	.	.	.	.	.	.	.	.	12
	No-contact strain	+	.	.	.	±	.	.	0	.	0	0	.	.	13
Agar plate with acriflavine 1/60,000	Papilla 7th day	+	Not analysed	.	+	+	+	+	.	+	.	0	0	.	14
	No-contact strain	+	.	.	+	+	0	0	.	0	.	.	.	.	15

Papillae and marginal non-papillary growth (column 2) were picked off colonies of the plates noted in column 1 and were subcultured into drug-free broth (column 3). No-contact strains were also subcultured in the same way. The broth cultures on lines 1, 3, 6 and 10 were then plated on agar with acriflavine 1/60,000 to get a purified resistant line. The colonies resulting were classed as large and small. Large colonies on lines 1, 3, 6 and 10, and the small colony on line 11 gave pure resistant strains. The no-contact strains were non-resistant. The papillary strain not-analysed on line 14 was possibly a mixture of resistant and non-resistant.

continued (line 5) to grow to 1·25 mm. at 48 hr. They were not demonstrated from IV*b* (columns 7 and 8) but were again present in V*b*, S growing in drug-free broth, column 6, and failing on SSAC 1/20,000, column 7.

Summary

The results in Table 2 agree with those in Table 1, resistant strains appearing after 72 hr. and non-resistant surviving after 5 days in SA 1/20,000.

These results agree also with the time of appearance of papillae on plate cultures and of resistant strains derived from them.

III. EXPERIMENTS WITH *B. DYSENTERIAE* FLEXNER GROWN ON ACRIFLAVINE

(Table 3)

Preliminary experiments showed that Flexner's dysentery bacillus would not grow readily on plates of nutrient agar containing acriflavine in higher concentration than 1/40,000.

If this bacillus is sown on nutrient agar plates containing acriflavine 1/60,000, colonies appear which measure 0·10–0·25 mm. after 24 hr. incubation. After 48 hr. they measure 0·25–0·75 mm., and about

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one-quarter of them carry minute ill-defined papillae. On the 3rd day the colonies measure 0·25–1·00 mm., and many, but not all, carry yellow papillae, while the bodies of the colonies are colourless. On the 5th day colonies without papillae measure 0·25 mm., colonies with papillae 1·00–1·25 mm. The papillae are golden in colour from absorption of acriflavine while the bodies of colonies remain pale grey.

Table 3. If now on the 3rd, 4th or 5th day subcultures are made on to drug-free agar or broth (Table 3, column 2), (1) from papillae and (2) from the margin of colonies, then growth results in all cases from the papillae, but no growth from the margins (column 3). The bodies of the colonies appear to die before the 3rd day.

After 48 hr. growth on the primary plate (Table 3, column 2, line 1) the colonies are too small to allow of papillae being picked off separately, but drug-free subcultures were made from the colonies with papillae (column 3, line 1).

If, further, the bacteria from these drug-free subcultures (column 3, lines 1, 3, 6 and 10) (derived from the papillae bearing colonies at 48 hr. and from the papillae on the 3rd, 4th and 5th days) are plated on acriflavine 1/60,000, the colonies which appear after 24 hr. fall roughly into two groups (column 4), large measuring 0·50–0·75 mm. and small 0·10–0·20 mm. If these two groups are then subcultured into drug-free broth (column 5), the larger colonies give living subcultures, but from the small colonies no growth results in three cases out of four.

Finally, if the descendants of the large colonies are plated on nutrient agar containing acriflavine 1/20,000 and a control strain of the same bacillus which has never been in contact with acriflavine is sown on similar plates, then numerous colonies, measuring 0·05–0·50 mm., appear on the former (Table 3, column 11, lines 1, 3 and 6) descended from papillae and no growth whatever on the latter (line 9).

Therefore papillae formed on acriflavine 1/60,000 contain (1) resistant forms (L) capable of growing on 1/20,000, (2) non-resistant forms (S) which die out even on 1/60,000 (Table 3, columns 4 and 5, lines 2, 4 and 7).

If strains established from the largest and the smallest colonies from papillae (column 5, lines 10 and 11) are titrated in broth tubes containing increasing concentrations of the drug, the former grow in 1/13,000, while the latter grow in 1/17,000, i.e. both are resistant. The no-contact strains will not grow beyond 1/41,000 (lines 13 and 15).

If the descendants of a papilla of the 7th day unanalysed by plating and therefore possibly containing a mixture of resistant and non-resistant cells are titrated in a similar series, they can grow in 1/21,000 (line 14).

IV. EXPERIMENTS WITH *B. DYSENTERIAE* FLEXNER, GROWN ON ARGYROL

(Table 4)

If Flexner's bacillus is sown on plates of nutrient agar containing argyrol, it will certainly grow when the strength of the drug is 1/120,000 or 1/90,000 but may fail on any higher concentration.

Table 4. On agar plates containing argyrol 1/120,000 or 1/90,000 after 24 hr. incubation the colonies measure 1·0–2·5 mm. Papillae appear on the 4th day. They are largely submarginal in position. If subcultures are made on this day from papillae or from non-papillary areas, growth results in both cases. As the papillae increase in size they become markedly yellow from the drug, while the body of the colony remains pale grey. On the 8th day subcultures on to drug-free media will grow if made from papillae but not if from areas without papillae (Table 6, lines 5 and 6). Secondary papillae may appear on the larger primary papillae on this 8th day.

If strains from papillae and non-papillary areas of the 4th and 8th days on 1/120,000 and of the 6th and 9th days on 1/90,000 (but non-papillary strains are not available on 8th and 9th days), together with a no-contact control, are titrated either on plates (Table 4, lines 1, 2 and 3) or in broth tubes (lines 6–13), all these three, papillary, non-papillary and no-contact, will grow up to the same strength of the drug. Therefore no resistant strain has been formed in the papillae.

V. EXPERIMENTS WITH *B. DYSENTERIAE* FLEXNER ON NEOSALVARSAN

(Table 5)

Flexner's bacillus of a no-contact strain will grow on plates containing neosalvarsan up to *at least* 1/11,500 (Table 5, line 1).

Titrated in broth tubes, papillary, marginal and no-contact strains grow to the same titre (lines 2–4 and 5–7). Therefore no resistant variant is formed in the papillae.

VI. THE BIOLOGICAL FUNCTIONS OF PAPILLAE

(Table 6)

Table 6 shows the results of subculture on successive days from agar plates (both with and without drugs) into tubes of drug-free broth. The subcultures were made in pairs (A) from papillae and (B) from non-papillary areas, of the same or of adjacent colonies. The purpose was to ascertain whether the bacteria in one of these two situations outlived those in the other. It will be seen in Table 6 that in all cases the papillae gave living subcultures after the non-papillary areas had ceased to do so. Papillae are

therefore points of survival amidst adverse conditions.

The conditions to which an organism may be subjected in the course of its life may be divided into (A) ordinary conditions of everyday life, (B) extraordinary conditions. The former include (1) conditions of internal origin which may be loosely covered by the term 'senility', implying the end of a cycle of growth; (2) conditions of external origin such as exhaustion of food supply or accumulation of waste products. The latter include (1) harmful conditions such as the impact of pernicious drugs and (2) beneficial conditions such as the presentation of lactose to a non-lactose-fermenting bacterium.

The final response of a unicellular organism to the sum of ordinary experiences in life is rejuvenation, while the successful response to extraordinary conditions is adaptive variation—for example, the formation of a strain resistant to a drug, or one able to digest a food substance which it had previously not been able to use (e.g. lactose by *B. coli-mutabile* or dulcite by *B. typhosus*).

Taking the plates of various media in Table 6, we find that they can be arranged in two groups, those which do not form resisting variants and those which do. Under the former come drug-free agar, agar containing SA 1/6500 sown with a strain of Flexner's bacillus resistant to SA 1/6000, agar containing argyrol and agar containing neosalvarsan. Under the latter come agar with SA 1/20,000 sown with a no-contact strain and agar with acriflavin.

All the plates were incubated at 37° C. throughout the experiment.

Table 4. B. dysenteriae *Flexner, grown on plates containing argyrol*

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	
	Argyrol															
	1/120,000	1/90,000	1/60,000	1/50,000	1/40,000	1/30,000	1/25,000	1/20,000	1/15,000	1/13,000	1/12,000	1/11,000	1/9000	1/8000	Drug free	Lines
	Plates															
No-contact strain Flexner's bacillus	+	+	.	+	.	+	±	0	0	.	.	.	.	.	.	1
Descendants of papilla 4th day on 1/120,000	.	+	.	+	.	.	±	.	0	.	.	.	.	.	.	2
Non-papillary ditto	.	+	.	+	.	.	±	.	0	.	.	.	.	.	.	3
Papilla 8th day on 1/120,000	+	+	.	0	0	0	.	0	.	.	.	.	.	.	.	4
No-contact strain	+	+	.	0	0	0	.	0	.	.	.	.	.	.	.	5
	Broth tubes															
Papilla 8th day on 1/120,000	.	+	.	+	.	+	+	+	+	.	.	0	.	.	.	6
No-contact	.	+	.	+	.	+	+	+	+	.	.	0	.	.	.	7
Papilla 9th day on 1/90,000, plated on 1/90,000:																
Largest colony	.	.	.	.	.	.	.	.	+	+	+	+	0	0	+	8
Smallest colony	.	.	.	.	.	.	.	.	+	+	+	+	0	0	+	9
No-contact	.	.	.	.	.	.	.	.	+	+	+	+	0	0	+	10
Descendants of papilla 6th day on 1/90,000	.	.	.	+	.	+	.	+	0	0	0	.	.	.	+	11
Ditto from non-papillary area	.	.	.	+	.	+	.	+	0	0	0	.	.	.	+	12
No-contact	.	.	.	+	.	+	.	+	0	0	0	.	.	.	+	13

Column 1. Broth cultures descended from:
Line 1. No-contact strain.
Line 2. Papilla of 4th day on argyrol 1/120,000.
Line 3. Non-papillary area 4th day on same plate.
Line 4. Papilla of 8th day on argyrol 1/120,000.
Line 5. No-contact strain.
Line 6. Papilla of 8th day on argyrol 1/120,000.
Line 7. No-contact strain.
Lines 8–10. Line 8: Largest colony on plate of argyrol 1/90,000 sown from papilla of 9th day on a similar plate. Line 9: Smallest colony on same plate. Line 10: No-contact strain.
Lines 11–13. Line 11: Papilla of 6th day on argyrol 1/90,000. Line 12: Non-papillary area 6th day on same plate. Line 13: No-contact strain.
N.B. Differences of titre between groups 1–3, 4–5, 6–7, 8–10 and 11–13 are due to some accidental cause, probably difference in the medium. The lines of each group are strictly comparable.

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Table 5. B. dysenteriae *Flexner, grown on plates containing neosalvarsan*

	1	2	3	4	5	6	7	8	9	10	11	
	Neosalvarsan											
	1/60,000	1/22,500	1/20,000	1/13,500	1/11,500	1/9600	1/8400	1/7800	1/6800	1/5700	1/4500	Lines
	Plates											
Flexner's bacillus, no-contact	+	+	+	+	+	.	.	.	.	.	0	1
	Broth tubes											
No-contact strain	.	.	.	+	0	0	.	.	.	.	.	2
Largest colony	.	.	.	+	0	0	.	.	.	.	.	3
Smallest colony from 9th day papilla on 1/19,600	.	.	.	+	0	0	.	.	.	.	.	4
Descendants of papilla on 4th day	+	.	.	.	+	.	+	.	.	0	.	5
Descendants of margin 4th day on 1/19,600	+	.	.	.	+	.	+	.	.	0	.	6
No contact	+	.	.	.	+	.	+	.	.	0	.	7

Note. The difference of titre between the group on lines 2, 3 and 4 and that on 5, 6 and 7 is due to some accidental difference in the medium. The members of each group are strictly comparable with each other.

Table 6. *Biological functions of papillae*

	Days																
	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	Lines
Agar plates containing	Plates not forming resistant variants																
No drug:																	
Papilla	.	+	+	+	+	PP	+	.	.	+	.	.	.	.	.	.	1
Non-papilla	+	+	+	+0	+0	0	.	.	0	.	.	.	.	.	.	.	2
SA 1/6500 sown with resistant strain R 831:																	
Papilla	.	.	+	.	.	+	.	PP	+	+	PP	+	+	.	+	.	3
Non-papilla	.	.	+	.	.	+	.	+	0	.	.	+	0	0	.	.	4
Argyrol 1/120,000:																	
Papilla	.	.	PP	+	.	.	.	+	+	+	.	.	.	.	.	.	5
Non-papilla	.	.	+	.	.	.	0	0	0	.	.	.	.	.	.	.	6
Neosalvarsan 1/19,000:																	
Papilla	.	.	+	+	PP	PPP	.	+	.	.	.	.	.	.	.	.	7
Non-papilla	.	.	+	+	.	.	.	0	.	.	.	.	.	.	.	.	8
	Plates forming resistant variants																
SA 1/20,000:																	
Papilla	.	+	+	+	.	.	+	.	.	+	+0	.	.	.	.	.	9
Non-papilla	.	+	+	+	.	+	+	.	.	0	+0	.	.	.	.	.	10
Acriflavin 1/40,000, 1/60,000:																	
Papilla	P	+	+	+	+	.	.	.	.	.	.	.	.	.	.	.	11
Non-papilla	+	+0	0	0	0	.	.	.	.	.	.	.	.	.	.	.	12

Results of subculture on successive days *from* agar plates containing: (1) no drug, (2) SA 1/6500, sown with strain R 831 resistant to SA 1/6000, (3) argyrol, (4) neosalvarsan, (5) SA 1/20,000, (6) acriflavin, *into* tubes of drug-free broth.

Subcultures taken in pairs from: (1) papillae, (2) non-papillary areas.

All plates were sown with no-contact strains except those on lines 3 and 4.

+ = Visible growth at 24 hr. 0 = No growth. PP, PPP = Very great development of papillae.

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Drug-free agar (lines 1 and 2). On this medium papillae are alive after 10 days, while non-papillary areas begin to die on the 5th day. As the substratum is quite neutral the survival of papillary bacteria is due to pure rejuvenation.

Agar with SA 1/6500 (lines 3 and 4). On plates sown with Flexner's bacillus strain 831 (see Exp. 1, p. 334) resistant to 1/6000. Note that both papillary and non-papillary bacteria are resistant (Exp. 1, p. 334) and equally so (Exp. 3, p. 334). But resistance to SA does not imply also resistance to senility and therefore the non-papillary bacteria die about the 10th day while the papillary bacteria are alive on the 15th (and after).

From the plates containing argyrol and neosalvarsan (lines 5–8). No races resistant to these drugs have been isolated, and therefore we ascribe the survival of papillae after the death of non-papillary areas to rejuvenation, and we must include the presence of the drugs in non-poisonous doses among 'ordinary' conditions of life.

Agar with SA 1/20,000 *sown with a no-contact strain* (lines 10 and 11). Papillae are alive on the 11th day, while non-papillary areas have died before this day. Survival of papillary bacteria is due to both variation and rejuvenation, since they contain a variant strain resistant to SA 1/6000 while the original no-contact strain can grow in no solution stronger than 1/34,000.

Agar with acriflavine. Non-papillary bacteria die very early, viz. on the 3rd or 4th day, while papillary bacteria are alive on the 6th (and later). The survival of the papillae is therefore due to adaptive variation acting before the 4th day and possibly to rejuvenation from the 5th or 6th.*

We note that papillae are most numerous on plates which form no variants (Table 6, lines 1, 3, 5 and 7, p. 341).

This may probably be explained as follows, that rejuvenation is a less powerful response than adaptive variation and therefore more centres of rejuvenation are needed to produce survival by the weaker means than by the stronger, i.e. adaptive variation.

The colonies marked PP or PPP in lines 1, 3, 5 and 7 are very striking objects, being covered with papillae which may measure up to 0·3 mm. in diameter. The distribution of these papillae is constantly the same; they appear first on isolated colonies around the outskirts of growth on a plate, and on each individual colony they appear near the margin in that half of the colony which is turned to the unoccupied part of the plate and away from the central accumulation of colonies. At their full development at least 50 % of all colonies carry large numbers of papillae, sufficient in many instances to cover the whole surface of the colony. Some older papillae bear younger (granddaughter) papillae on their surface.

It is interesting to compare this distribution with the distribution on colonies of *B. coli-mutabile* on lactose plates. As Massini pointed out, the red papillae appear near the centre of the colonies and are never seen at the margin.

This remarkable efflorescence of papillae on plates devoid of adaptive variation may be interpreted as an answer to the call for rejuvenation by senescent cultures which have no other means of survival.

The first and most profuse appearance of papillae at the outskirts of growth on a plate, where food is most available and waste products least concentrated, may correspond with the observation that conjugation in *Paramoecium* or *Spirogyra* is most active at the time of highest vitality and before the downward trend of the life cycle begins.

Some writers have asserted that papillae always contain favoured variants of some kind, and that the papilla is merely a successful daughter colony consisting of an improved race. They deny that it has any power to preserve the race in adverse conditions apart from these hypothetical variations, or that its constituent bacteria are in any way analogous functionally with the zygotes of *Paramoecium* or *Spirogyra*. Against this view may be brought the observation that papillae often make their appearance as minute objects 0·05–0·10 mm. in diameter and never grow any larger, which is not the size to be expected in a successful daughter colony.

We should also note that only two or three favourable variations have been found to occur on neutral media (i.e. agar without drugs or sugars) in any strain of bacteria, while the number of successive generations of subculture from successive papillae can be very great indeed before the strain begins to degenerate *in vitro*. It is therefore not possible that these numerous papillae can originate in these few variations, since a variation having once occurred cannot be repeated in the same strain. Bacteria which have achieved an adaptive variation, such as the red race of *B. coli-mutabile*, continue to form papillae either on plain or on lactose agar.

It is certainly true that some change takes place in the bacteria of all papillae, since they survive after the rest of the colony is dead, but this change is analogous to that which takes place after conjugation in *Paramoecium* or *Spirogyra*; in other words, the change is that of rejuvenation (except in those few cases in which adaptive variation also takes place).

* It is a very tempting supposition that the S colonies in Table 1, col. 4, lines 11, 13, 17 and Table 3, col. 4, lines 2, 4, 7 which appear on drug-containing plates after long previous contact with the drug (SA or acriflavine), but are so weakened that they cannot grow when subcultured into drug-free broth, are strains which have survived in papillae by rejuvenation without variation.

This change of rejuvenation is not permanent and irreversible as is adaptive variation, and therefore conjugation or papilla formation does not give perpetual youth, and senility follows again in due course.

It will clarify our ideas if we form a hypothesis about the cytology of these changes, and it may be stated in the following terms. In both rejuvenation and adaptive variation the papillary bacteria carry out an autogamic shuffling of chromatic matter. In adaptive variation this shuffling results in a permanent change in the genetic composition, while in rejuvenation it does not result in this way.

SUMMARY

Bacillus dysenteriae Flexner (*Shigella paradysentriae*) grown on Lemco agar plates (without peptone) containing sulphanilamide 1/20,000 to 1/10,000 forms papillae which on subculture yield two strains: (1) the original strain unmodified and (2) a strain resistant to SA.

The latter has arisen by discontinuous variation. There are no forms intermediate between strain (1) and strain (2).

In one such case (Exp. 1, p. 334) the no-contact race, the unmodified non-resistant strain from the primary sulphanilamide plate and the resisting variant strain were titrated in broth containing sulphanilamide in ten increasing concentrations from 1/34,000 to 1/5000. The first two strains, the no-contact and the unmodified non-resistant from the sulphanilamide plate grew in SA 1/34,000 and failed in 1/21,000, while the resisting variant grew up to the tube containing SA 1/6000 and failed to grow in 1/5000.

On plates containing sulphanilamide this adaptive variation takes place only in the papillae. It can also take place in broth containing, for example, SA 1/20,000 to 1/10,000.

There is no such thing as gradual progressive acquisition of resistance by the whole of a bacterial population exposed to a drug. Such phrases as 'teaching bacteria to resist' are misleading and anthropomorphic and should not be employed.

Fluid cultures which show a progressive gradual increase of resistance do so as a result of an increasing percentage of resistant cells in a mixed population of resistant and non-resistant bacteria. Experiments in fluid media should always be checked and the growth analysed by plating.

Resistant strains maintain this character after prolonged growth on drug-free media.

(2) Flexner's dysentery bacillus grown on plates containing acriflavine 1/60,000 to 1/40,000 forms in papillae strains resistant to this drug. The non-papillary areas of colonies die early, viz. on the 3rd day. The variation in this case also is discontinuous.

(3) On the other hand, if Flexner's bacillus is grown on agar containing organic compounds of silver or arsenic (argyrol or neosalvarsan), it forms no variants resisting these drugs.

(4) Papillae possess two biological functions: (*a*) they are the site of adaptive variation in response to elements in their environment, such as harmful drugs or useful sugars; (*b*) they are also the site of rejuvenation of senile cells and of cells affected by common unfavourable conditions such as exhaustion of food supply and accummulation of excretory substances.

The greatest development of papillae takes place on plates which form no variants, such as plates of drug-free agar, agar plates containing sulphanilamide sown with strains resistant to sulphanilamide and plates containing organic compounds of silver or arsenic.

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