MODE OF ORIGIN OF SULPHONAMIDE-RESISTANT STRAINS IN *B. DYSENTERIAE* FLEXNER

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INTRODUCTION

The purpose of this paper is to discuss the following question: Do bacteria which become resistant to sulphonamides undergo a variation like that described by R. Massini in *B. coli mutabile*? That is, a heritable change of character in direct response to something in their environment.

PREVIOUS LITERATURE

The facts regarding *B. coli mutabile* are briefly as follows (Massini, 1907):

I. On solid media with lactose and neutral red, variants appear as red, lactose-fermenting, papillae or daughter colonies on the white, non-lactose-fermenting, parent colonies. Variants are not found at the growing margin of the latter.

II. Subcultures from papillae give colonies of two types: (i) white, which again form red papillae, and (ii) red which breed true. The two types are discontinuous.

III. Variation takes place only on the particular sugar involved, i.e. lactose for *B. coli mutabile* or dulcite for *B. typhosus*.

IV. The change is not reversible. A red or fermenting race remains red after many subcultures on lactose-free media.

V. It takes place toward the end of the logarithmic phase, i.e. after one or more days. Therefore if subcultures are made in series every 12 or 24 hr. variation does not take place in the cells handed from culture to culture, although it does take place in each individual culture after the 12 or 24 hr. limit (Stewart, 1928).

If the logarithmic phase is shortened, e.g. by crowding or by starvation, then variation takes place earlier.

VI. The white or non-lactose-fermenting form of *B. coli mutabile* possesses the ferment lactase which is, however, inhibited by some unknown agency (Deere, Dulaney & Michelson, 1936; Deere, 1939*a*, *b*).

One of the curiosities of biological literature is the persistence of the idea that no heritable variation can be caused by direct action of environment, and that any change which appears to be caused in this way has actually arisen spontaneously in the organism, the variant being then selected by its fitness to survive. This is clearly Weissmann's doctrine without the limitation which he set, namely, that it holds good only for multicellular beings in which germplasm is divided from soma. Weissmann himself lays down *a priori* that unicellular beings unconditionally inherit acquired characters. The opposite opinion, to which I have alluded, was first applied to bacterial variation by Henderson Smith (1913) in the case of *B. typhosus* grown on dulcite. It has been revived by Lewis (1934) for *B. coli mutabile*. This author formed the opinion that one in every hundred thousand cells of *B. coli mutabile* (white) on non-lactose media was a lactose fermenter formed by spontaneous variation. To prove this he had to sow a million cells on a lactose plate in order to raise ten red colonies. Now during the 24 hr. which had to elapse between the sowing and the recognition of the red colonies these million cells were of course exposed to the lactose: the bacterial strain chosen was one which formed red variants after exceptionally short contact with lactose; and

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the conditions of the experiment, namely, crowding and starvation on a synthetic medium, were such as are known to accelerate variation. All the conditions, in fact, made it probable that the red cells became red after and not before the plating on lactose.

Nevertheless, Lewis's work has been given an uncritical welcome and has been acclaimed as proving that spontaneous variation does occur, while variation by direct action does not. This view has found its way into at least one text-book. No one doubts, however, that the toxin, for example, of *Streptococcus scarlatinae* produces antitoxin by direct action, nor does anyone believe that acquired immunity is a spontaneous variation.

The present writer has shown that in three large samples of *B. coli mutabile* (white), not one red cell was present in 250,000 (Stewart, 1942).

An interesting link between Massini's account of *B. coli mutabile* (1907) and recent work on sulphonamide action is found in papers by Reiner Müller (1909, 1911) concerning variation in certain coliform bacteria grown on arabinose and in *B. typhosus* grown on rhamnose or isodulcite. The coliform bacteria formed papillae on arabinose, subcultures from which gave colonies no longer fermenting arabinose but growing more lustily on it and forming no papillae. *B. typhosus* behaved in the same way on isodulcite, forming white papillae which in subculture gave white, more robust, colonies. Reiner Müller also obtained some strains of *B. typhosus* which formed a trace of acid in the papillae and in their descendant colonies, but in a later paper (1911) he withdrew this observation and maintained that the more vigorous growth of the papillae and of the rosy daughter colonies was not due to acid fermentation. He considered that it might be explained either by (i) assimilation of isodulcite without acid formation or (ii) *as an adaptation by the bacteria to a substance (isodulcite) which otherwise inhibits growth.* In favour of the latter view is the fact that colonies of *B. typhosus* on isodulcite are very small; against it that mutation can be called out by so low a percentage as 0·01 of isodulcite and that inhibition of growth does not vary with its concentration. Reiner Müller then goes on as follows: 'The side-chain theory of Ehrlich gives the best key to explain these mutations. *Bact. typhosus* has in its body receptors which anchor isodulcite; growth is therefore inhibited because the bacillus is not able to use these receptors for feeding and is consequently starved. But not all receptors are blocked by isodulcite. The mutation consists in the appearance of a race capable of overcoming this block either by reduction of rhamnose or by anti-toxin-like overproduction of receptors for rhamnose.' In another paper (1909*b*) the same author writes: 'For biology in general it may be of the highest significance that we can artificially and with the certainty of a chemical reaction endow a living being with a particular character and that this character is then inherited and remains constant through countless generations.'

FORMATION OF RACES RESISTANT TO SULPHONAMIDES IN *B. DYSENTERIAE* FLEXNER

General technical considerations

I have chosen *B. dysenteriae* Flexner for study in preference to *Streptococcus haemolyticus* because it is more easy to cultivate and will grow on simple media which do not inhibit sulphonamide action. The culture media used were: (A) in the first few weeks of work the fresh rabbit infusion of Pike & Foster (1944), but thereafter I found that (B) a bouillon made of Lab Lemco and common salt without peptone, adjusted to *p*H 7·4, gave better results and was naturally much more easy to prepare. Agar 2% was added for solid media.

All races of bacteria for use in experiments were purified by plating in series on drug-free nutrient agar. Before plating on agar with sulphonamides the bacteria were grown in peptone bouillon for 24 hr. and then two or three loops were transferred to a tube containing 5 c.c. of Ringer's solution (without bicarbonate). This was shaken and two loops were spread with a right-angled glass rod. It is important to avoid crowding a plate since crowding inhibits sulphonamide bacteriostasis, the satellite action of Pike & Foster.

In the following work I have relied largely on the size of the colonies as a guide to the degree of bacteriostasis or conversely to the resistance of the bacteria. This is a reliable criterion in comparing groups of bacteria grown under similar conditions. It needs to be confirmed, however, since colony size depends not only on concentration of sulphonamide and susceptibility of the bacterium but also on factors inhibiting sulphonamide action such as crowding. Crowding also has the opposite action in reducing the size of colonies by starvation. For an absolute test of resistance or susceptibility to sulphonamides bacteria must be sown on cultures of increasing drug concentration until growth is not visible at 24 hr.

The sulphonamides used were the following, in order of potency:

	Molecular weight	Solubility in water at 15° C.	Contraction employed
Sulphanilamide	172	1/240	Sa
Sulphaguanidine	—	1/1000	Sg
Sodium sulphacetamide	—	1/1·5	Ssac
Sodium sulphapyridine	—	1/3	Sspy
Sulphathiazole	255	1/2500	Sth

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Sodium sulphacetamide gives the most clear-cut results regarding the size of colonies.

METHODS FOR DEVELOPING, ISOLATING AND TESTING SULPHONAMIDE-RESISTANT STRAINS OF BACTERIA

The bacteria, of a strain which has never before been in contact with sulphonamide, are sown on a plate of Lemco agar with 1/20,000 of the drug. I used sulphanilamide, sodium sulphacetamide and sulphaguanidine for this purpose. After 2, 3 or more days' incubation, papillae make their appearance on the colonies. These papillae are picked off with a fine-pointed needle into tubes of peptone bouillon. At the same time points on the margin of colonies or other points which appear free of papillae are picked off to represent the original non-papillary mass of the colony. After 24 hr. incubation the bouillon tubes are diluted and plated on Lemco agar with 1/10,000 of the same sulphonamide. On the plates from papillae two discontinuous types of colony will be found: (*a*) large and resistant, (*b*) small and non-resistant. It is, of course, possible that the non-resistant type will not be able to grow at all on 1/10,000. On the plates from non-papillary areas it is probable that only the small, non-resistant type will appear, although there may be a few large colonies also since the early stages of papillae, consisting merely of a few variant cells, are unrecognizable. Such early papillae may occur in the centre of a colony but never at the growing margin. The two strains are then established by picking off large colonies from the plates of papillary origin and small colonies from these with a non-papillary source. They are tested for resistance by sowing on Lemco plates with 1/10,000 and 1/5000 sulphanilamide and with 1/5000 sodium sulphacetamide or with appropriate concentrations of other drugs.

Examples of these methods

Experiment I. *B. dysenteriae* Flexner (race Fl. 4) had been purified by repeated plating on nutrient agar and had no previous contact with sulphonamides.

(1) A minute point from a colony on a nutrient agar plate was emulsified in 5 c.c. Ringer and shaken. Of this suspension two loops were spread on a plate of Lemco agar plus Sa to 1/20,000 (plate 15). The resultant colonies at 24 hr. measured 0·75 mm. in diameter (about 500 colonies).

(2) On these colonies papillae made their appearance on the 5th day and were picked off into bouillon tube no. 33, while on the 6th day a point in the centre of a colony without obvious papillae was picked off into bouillon tube no. 44.

(3) Two loops of each bouillon culture were diluted in 5 c.c. Ringer and sown on plates of Lemco agar with Sa 1/10,000; tube 33 on to plate 53, and tube 44 on to plate 54.

Colonies at 24 hr incubation were:

Plate 53 (papillary):
(*a*) 25 large, 1·25 mm. in diameter.
(*b*) Numerous small, 0·10–0·50 mm. in diameter.

Plate 54 (non-papillary):
(*a*) 2 large, 1·00 mm. in diameter.
(*b*) Numerous small, 0·10–0·20 mm. in diameter.

(4) A large colony was picked off plate 53 and a small colony off plate 54. These two subraces and the original race with no sulphonamide contact were given two subcultures in broth and then sown on Lemco agar plates: (i) with Sa 1/10,000, (ii) with Sa 1/5000, (iii) with sodium Ssac 1/5000. The resulting colonies at 24 hr. measured as follows:

Plates with Sa 1/10,000:
No. 127 sown from no. 53 large subrace, 0·75–1·00 mm. in diameter (120 colonies).
No. 128 sown from no. 54 small subrace, 0·12–0·25 mm. in diameter (160 colonies).
No. 129 sown from no sulpho contact, 0·12–0·33 mm. in diameter (110 colonies).

Plates with Sa 1/5000:
No. 133 sown from no. 53 (large), 0·12–0·50 mm. in diameter (about 100 colonies).
No. 134 sown from no. 54 (small), no growth.
No. 135 sown from no sulpho contact, no growth.

Plates with Ssac 1/5000:
No. 139 sown from no. 53 (large), no growth.
No. 140 sown from no. 54 (small), no growth.
No. 141 sown from no sulpho contact, no growth.

Experiment II. *B. dysenteriae* Newcastle (race New. 6):

(1) Sown on Lemco agar with Sa 1/20,000 (plate 14). Colonies at 24 hr. measured about 1·00 mm. in diameter.

(2) Papillae appeared on the 5th day and were picked off into bouillon tube no. 32, while on the 6th day the non-papillary centre of a colony was picked off into bouillon tube no. 40.

(3) These two bouillon cultures were sown on Lemco agar with Sa 1/10,000, tube 32 on to plate 51 and 40 on to plate 52. Colonies at 24 hr. were:

Plate 51 (papillary):
(*a*) 75 large, 1·25–1·50 mm. in diameter.
(*b*) Very many small, 0·22 mm. in diameter.

Plate 52 (non-papillary):
(*a*) 6 large, 1·25–1·50 mm. in diameter.
(*b*) Many small, 0·12 mm. in diameter.

(4) The large subrace from plate 51, the small subrace from plate 52 and the original race with no sulpho contact were then plated on Sa 1/10,000, Sa 1/5000 and Ssac 1/5000. The resulting colonies at 24 hr. measured:

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Plates with Sa 1/10,000:

No. 130 sown from no. 51 large subrace, 1·00–1·30 mm. in diameter (70 colonies).

No. 131 sown from no. 52 small subrace, 0·10–1·00 mm. in diameter.

No. 132 sown from no sulpho contact, 0·12–1·00 mm. in diameter.

(Nos. 131 and 132 were crowded in parts; this accounts for the larger size of some colonies owing to 'satellite' action, but none were truly resistant; compare the next plates 136–138 and 142–144.)

Plates with Sa 1/5000:

No. 136 sown from no. 51 (large), 0·12–0·25 mm. in diameter.

No. 137 sown from no. 52 (small), no growth.

No. 138 sown from no sulpho contact, no growth.

Plates with Ssac 1/5000:

No. 142 sown from no. 51 (large), 0·05 mm. in diameter? (2 minute evanescent colonies).

No. 143 sown from no. 52 (small), no growth.

No. 144 sown from no sulpho contact, no growth.

Experiment III. *B. dysenteriae* Flexner Z (race Fl. Z 1).

(1) Sown on rabbit agar with Ssac 1/20,000 (plate 16*a*) and on drug-free nutrient agar (plate 18*a*). Colonies at 24 hr. measured:

Plate 16*a*: 0·25 mm. in diameter.

Plate 18*a*: 2·00 mm. in diameter.

(2) Papillae appeared on the 4th day and were picked off into bouillon and later sown on a plate with Ssac 1/10,000 (no. 51). A non-papillary strain was treated in the same way, on to plate 55. Colonies at 24 hr. measured:

Plate 51*a* (papillary strain):

(*a*) Large, 0·75 mm. in diameter.

(*b*) Small, 0·10 mm. in diameter.

Plate 55*a* (non-papillary):

Small, 0·10–0·20 mm. in diameter.

After a succession of subcultures in bouillon the two strains gave the same result again, viz.

Plate 81:

Ssac 1/10,000 sown with large strain from plate 51*a*, colonies at 24 hr. 0·05–0·75 mm. in diameter.

Plate 82:

Ssac 1/10,000 sown with small strain from plate 55*a*, colonies at 24 hr. 0·12–0·25 mm. in diameter.

RESISTANT VARIANTS ARE NOT FOUND IN THE GROWING MARGIN OF A COLONY

Three strains of bacteria were used in this experiment (Flexner 2 and 4, and Newcastle 6). They were sown on Lemco plates with Sa 1/20,000. Papillae appeared between the 4th and 6th days and were picked off into broth, while at the same time cultures were made from the growing margin of colonies. Papillary and marginal cultures were then sown, after dilution with Ringer, on plates containing Sa 1/20,000, 1/10,000 and 1/5000. The resultant colonies from papillary cultures were large type and small, or large alone, but from marginal cultures small only. The large type was resistant.

Experiment IV. *B. dysenteriae* Flexner (Fl. 2) with no previous sulphonamide contact, was plated on Sa 1/20,000 (plate 29). The resultant colonies at 24 hr. measured 0·12 mm. Papillae appeared on the 4th day and were picked off into bouillon tube no. 64. A point on the growing margin of a colony was also picked off into a bouillon tube, no. 63. These two cultures were then sown on Lemco agar plates as follows:

Plate 98: Sa 1/20,000, sown from 64, grew colonies at 24 hr., 0·80 mm. in diameter.

Plate 97: Sa 1/20,000, sown from 63, grew colonies at 24 hr., 0·05–0·08 mm. in diameter.

Plate 100: Sa 1/10,000, sown from 64, grew colonies at 24 hr., 0·50 mm. in diameter.

Plate 99: Sa 1/10,000, sown from 63, at 24 hr., no growth.

Plate 102: Sa 1/5000, sown from 64, grew colonies at 24 hr., 0·168–0·240 mm. in diameter.

Plate 101: Sa 1/5000, sown from 63, at 24 hr., no growth.

Note that this strain Fl. 2 was exceptionally sensitive to sulphanilamide.

Experiment V. *B. dysenteriae* Newcastle (New. 6), no previous sulphonamide contact, plated on 1/20,000 (plate 22). The resultant colonies at 24 hr. measured 0·5–1·0 mm. Papillae on 6th day were picked off into bouillon tube no. 65, and margin into bouillon tube no. 66.

Plate 107: Sa 1/10,000, sown from 65, grew colonies at 24 hr.: (*a*) 0·50–1·00 mm., and (*b*) 0·10–0·12 mm. in diameter.

Plate 108: Sa 1/10,000, sown from 66, grew colonies at 24 hr.: 0·10–0·12 mm. in diameter.

Experiment VI. *B. dysenteriae* Flexner (Fl. 4), no sulpho contact, plated on Sa 1/10,000 (plate 122). Colonies at 24 hr. were 0·25 mm. in diameter. Papillae on 4th day which were picked off into bouillon tube no. 159, and margin into no. 160.

Plate 167: Sa 1/10,000, sown from 159, grew colonies at 24 hr. Smooth, 0·75–2·00 mm. in diameter.

Plate 168: Sa 1/10,000, sown from 160, grew colonies at 24 hr. Smooth, 0·12–0·25 mm.; rough, 0·75 mm. in diameter.

BACTERIOSTASIS BY SULPHATHIAZOLE OF A STRAIN MADE RESISTANT TO SULPHANILAMIDE

A strain of *B. dysenteriae* Flexner Z which had developed resistance to sulphanilamide on plates of

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1/20,000, formed colonies on Sa 1/10,000 which measured 2·00 mm. at 24 hr., while colonies of the non-resistant strain of the same race measured only 0·25 mm. These two strains, Res. and NonR., were sown on plates with Sth 1/1,000,000, 1/100,000, 1/50,000, 1/25,000. The growth of NonR. was completely arrested on 1/100,000 to 1/25,000 but not on 1/1,000,000. The size of colonies of Res. was reduced from 1·50 to 0·25 mm. on Sth 1/100,000, as follows:

B. dysenteriae Flexner Z, grown on Sa 1/20,000, had developed a resistant strain Res. The original strain is NonR. The two strains plated on sulphathiazole grew colonies measuring as follows at 24 hr.:

Plate 167: No drug, sown with Res., colonies 1·50–2·00 mm. in diameter; sown with NonR., colonies 1·50–2·00 mm. in diameter.

Plate 166: Sth 1/1,000,000 sown with Res., colonies 0·50–1·50 mm. in diameter; sown with NonR., colonies 1·00–1·50 mm. in diameter.

Plate 165: Sth 1/100,000 sown with Res., colonies 0·20–0·30 mm. in diameter; sown with NonR., no growth.

Plate 158: Sth 1/50,000 sown with Res., colonies 0·36 mm. in diameter; sown with NonR., no growth.

Plate 159: Sth 1/25,000 sown with Res., colonies 0·25 mm. in diameter; sown with NonR., no growth.

ACTION OF SODIUM SULPHAPYRIDINE ON A STRAIN MADE RESISTANT TO SULPHAGUANIDINE

A strain of *B. dysenteriae* Flexner Z had developed resistance to sulphaguanidine and was then plated on sodium sulphapyridine. The colonies on the latter were greatly reduced in size:

Plate 108: Sg 1/10,000 sown with Res. to Sg., colonies at 24 hr. 1·50–2·00 mm. in diameter.

Plate 109: Sspy 1/10,000 sown with Res. to Sg., colonies at 24 hr. 0·17 mm. in diameter.

PAPILLAE ON COLONIES OF *STREPTOCOCCUS HAEMOLYTICUS*

If *Str. haemolyticus* is grown on plates of serum agar papillae make their appearance on the 5th day.

SUMMARY

The change by which a strain of *B. dysenteriae* Flexner becomes resistant to sulphonamides is similar in character to the change in *B. coli mutabile* from white (non-lactose-fermenting) to red (lactose-fermenting).

The highest proportion of variants are found in papillae, while no variants are found in the growing margin of colonies.

Reversion does not take place during growth on sulphonamide-free media.

The variation is a direct and heritable response to a chemical stimulus and in adaptation to this part of the environment.

The variation described by Reiner Müller of *B. typhosus* on rhamnose and of certain coliform bacteria on arabinose may also be of the same nature.

Strains of *B. dysenteriae* Flexner resistant to sulphanilamide or sulphaguanidine 1/10,000 are only partially resistant to sulphathiazole 1/100,000 and to sodium sulphapyridine 1/10,000.

Technique. The size of (uncrowded) colonies on a plate containing 1/10,000 sulphanilamide or sodium sulphacetamide is a useful criterion of resistance or non-resistance, but a resistant strain must be able to grow on 1/5000 Sa and will not grow on 1/5000 Ssac.

The best culture medium for these tests is Lab. Lemco bouillon with agar, but without peptone.

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REFERENCES

DEERE, C. J. (1939*a*). *J. Bact.* **37**, 355–63.
DEERE, C. J. (1939*b*). *J. Bact.* **37**, 473–83.
DEERE, C. J., DULANEY, D. & MICHELSON, I. D. (1936). *J. Bact.* **31**, 625–33.
LEWIS, I. M. (1934). *J. Bact.* **28**, 619–38.
MASSINI, R. (1907). *Arch. Hyg., Berl.*, **61**, 250.
MÜLLER, REINER (1909*a*). *Zbl. Bakt.* Abt. I, Ref. 42, Beihft. p. 57.
MÜLLER, REINER (1909*b*). *Umschau*, **13**, 397. (Quoted by Burri (1910). *Zbl. Bakt.* Abt. II, Orig. **28**, 321.)
MÜLLER, REINER (1911). *Zbl. Bakt.* Abt. I, Orig. **58**, 97.
PIKE, R. M. & FOSTER, A. Z. (1944). *J. Bact.* **47**, 97.
SMITH, HENDERSON (1913). *Zbl. Bakt.* Abt. I, Orig. **68**, 151.
STEWART, F. H. (1928). *J. Hyg., Camb.*, **27**, 379–95.
STEWART, F. H. (1942). *J. Hyg., Camb.*, **41**, 497–508.
STEWART, F. H. (1944). *J. Hyg., Camb.*, **43**, 136–41.

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