RESISTANCE OF THE MOUSE'S INTESTINAL TRACT TO EXPERIMENTAL SALMONELLA INFECTION

II. Factors Responsible for Its Loss Following Streptomycin Treatment*

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The preceding communication (1) presents a series of observations believed to provide a satisfactory explanation for the resistance of normal (CF-1) mice to initiation of infection with *Salmonella enteritidis* by oral inoculation. Acetic and butyric acids were shown to be present in content of the large intestine in concentrations which inhibited *Salmonella in vitro* at pH 6.2 or below; *i.e.*, at pH levels found in colon content of ⅔ of normal mice examined. Both fatty acids were produced in high concentrations by anaerobic cultures of several strains of *Bacteroides* selected for their inhibitory activity against *Salmonella in vitro*. Members of this genus are among the most numerous constituents of the enteric microflora of normal mice, but are completely eliminated by the oral administration of streptomycin (2). Following such medication, resistance of the colon to infection with *S. enteritidis* is promptly reduced to such an extent that <10 microorganisms inoculated *per os* are able to initiate infection, whereas approximately 10^6 are required to infect 50 per cent of untreated controls (3–5).

Described below are (*a*) changes in pH and in concentrations of acetic, butyric, and lactic acids in contents of the mouse's large intestine following oral streptomycin treatment and associated with the alteration in enteric microflora previously reported; and (*b*) *in vitro* experiments on growth inhibition of *S. enteritidis* in colon content of streptomycin-treated mice and in broth containing varying concentrations of the acids mentioned above.

Materials and Methods

The following experiments were carried out in conjunction with those reported in the preceding communication (1) in which most of the methods have been described, except that

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for lactic acid determination which was the colorimetric method of Barker and Summerson (6). Streptomycin[1] was administered as a single dose of 50 mg in 0.5 ml water *per os*; *i.e.*, by stomach tube as previously described (4).

EXPERIMENTAL

Pertinent Chemical Changes in Colon Content Following Streptomycin Treatment

Mice were treated with streptomycin and killed 1, 3, and 5 days thereafter for the following examinations of content of cecum and transverse colon. pH and Eh were determined on individual mice, other determinations were made on pools of material from 15 to 20 animals.

TABLE I

Effect of Streptomycin Treatment on pH of the Mouse's Colon Content

Content of:	Determinations *in situ* immediately postmortem			
	Untreated controls	Days after streptomycin (50 mg *per os*)		
		1	3	5
	pH	*pH*	*pH*	*pH*
Cecum				
Mean	6.16	6.42*	6.02*	6.09
Median	6.1	6.4	6.0	6.1
Range	5.7–6.7	5.8–7.3	5.3–7.1	5.5–6.8
Transverse colon				
Mean	6.10	6.40*	6.16	6.08
Median	6.1	6.4	6.1	6.1
Range	5.6–6.7	5.7–7.2	5.5–6.9	5.4–6.7

* Significantly different from untreated controls according to Dunnett's method of multiple comparison (7). Based on 70 mice in each group.

The data on normal controls (except for lactic acid) were presented in the preceding paper, but are included here for comparison with those on streptomycin-treated mice.

pH.—The greatest change was observed on the 1st day after treatment as an increase of pH in content of cecum and transverse colon. This rise was followed by a decrease on the 3rd day, particularly in cecal content (Table I).

Eh.—No consistent changes were observed.

Acetic, Butyric, and Lactic Acids.—Concentrations in colon content of both acetic and butyric acids, especially the latter, were reduced on the 1st day after treatment, but both returned to about normal levels within the next few days. During this period, however, the concentrations of lactic acid increased (Table II). The significance of this observation will be discussed below.

[1] Streptomycin was generously supplied by Abbott Laboratories, North Chicago, Lederle Laboratories, Pearl River, New York, Eli Lilly & Co., Indianapolis, Merck & Company, Rahway, New Jersey, Chas. Pfizer & Company, Brooklyn, E. R. Squibb & Sons, New York and the Upjohn Company, Kalamazoo, Michigan.

Growth Inhibition of S. enteritidis by Varied Concentrations of Acetic and Butyric Acids in Broth.—Using the liquid culture method already described (1) the inhibitory activity of acetic and butyric acids was assayed in broth at a number of concentrations and pH levels including those found in colon content on the 1st, 3rd, and 5th days after oral streptomycin treatment. Duplicate assays were incubated aerobically and anaerobically, but only the results of the latter are reported herein because anaerobic conditions more nearly simulate those within the large bowel.

No inhibition of *Salmonella* was demonstrable in broth buffered at pH 6.4 and containing 0.02 M acetic acid and 0.001 M butyric acid,—the mean concentrations found in colon content on the 1st day after streptomycin. This failure

TABLE II

Effect of Streptomycin Treatment on Concentrations of Acetic, Butyric, and Lactic Acids in Colon Content

Colon content* obtained:	Concentration		
	Acetic	Butyric	Lactic
	M	M	M
1 day after treatment‡	0.02	0.001	0.02
3 " " "	0.03	0.005	0.05
5 " " "	0.05	0.01	0.1
Untreated controls	0.05	0.03	0.005

* Means of determinations on 3 or more pools of material from cecum and transverse colon of 15 to 20 mice.

‡ 50 mg streptomycin *per os*.

to inhibit *in vitro* is consistent with the *in vivo* observation that the mouse's intestine is highly susceptible to initiation of infection with *Salmonella* introduced by mouth on the 1st day after treatment.

Inhibition of *Salmonella* did occur, however, in broth containing concentrations of the fatty acids and at the pH simulating those found in the mouse's colon content on the 3rd and 5th days after streptomycin treatment, a time when the intestine is still relatively susceptible to infection. This inconsistency of results,—*in vitro versus in vivo*,—was resolved by the finding that on the 3rd and 5th days, lactic acid was present in colon content in concentrations which interfered with the inhibitory activity of acetic and/or butyric acids in broth.

Antagonistic Effect of Lactic Acid on Inhibitory Activity of Acetic and Butyric Acids in Vitro.—Inhibition of broth cultures of *Salmonella* by acetic and butyric acids, alone or in combination, was counteracted by the addition of lactic acid, most effectively by 0.1 M, the mean concentration found in colon content on the 5th day after treatment (Tables III and IV). This was also the concentra-

tion which augmented growth of *Salmonella* to the greatest degree in control medium, nutrient broth at pH 6.0 (Table V). At higher pH levels, the antagonistic effect of lactic acid was still demonstrable but less striking because the fatty acids became relatively less inhibitory as the pH was increased.

TABLE III

Effect of Lactic Acid on Inhibitory Activity of Acetic and Butyric Acids in Nutrient Broth Buffered at pH 6.0

Concentrations		Changes (log 2) in No. of *Salmonella* at:			
		6 hrs.		24 hrs.	
Acetic acid	Butyric acid	Lactic acid 0.1 M			
		Omitted	Added	Omitted	Added
M	M				
0.04	0.02	d	1	d	11
0.03	0.015	d	1	0	11
0.02	0.01	0	4	1	14
0.04	Nil	d	3	d	13
0.03	"	0	3	1	14
0.02	"	0	5	7	15
Nil	0.03	0	3	2	14
"	0.02	0	5	3	15
"	0.01	1	7	5	16
"	Nil	8	11	16	15

1 = 50 to 150 per cent increase from initial number (approximately 10^8/ml); 2 = 150 to 250 per cent increase from initial number, etc.

0 = <50 per cent change from initial number; d = >50 per cent decrease from initial number.

Plate counts were made after 6 and 24 hours' anaerobic incubation. From these counts, the changes in *Salmonella* population were calculated as percentage increase (or decrease) rounded to the nearest log 2 in this and the following tables to facilitate comparison of the experimental results.

Antagonism of Inhibitory Activity by Pyruvic and Other Cocarboxylic Acids and by Glucose.—Pyruvic acid was even more effective than lactic in counteracting the inhibitory activity of acetic and butyric acids. Addition of 0.1 M to broth containing inhibitory concentrations of the fatty acids resulted in abundant growth of *Salmonella* under aerobic as well as anaerobic conditions.

Succinic and citric acids in equivalent concentrations were about as effective

as lactic acid in counteracting inhibition by acetic and butyric acids and also in augmenting growth of *Salmonella* in control medium. Glucose, on the other hand, failed to antagonize the fatty acids although it did increase growth of the test microorganism in control medium.

TABLE IV

Effect of Lactic Acid on Inhibitory Activity of Acetic and Butyric Acids in Nutrient Broth Buffered at pH 6.0

Concentrations			Changes (log 2) in No. of *Salmonella* at:	
Acetic acid	Butyric acid	Lactic acid	6 hrs.	24 hrs.
M	M	M		
0.04	0.02	0.3	0	4
		0.15	0	8
		0.1	1	11
		0.07	0	7
		0.03	0	4
		0.01	0	0
		Nil	0	d
0.03	0.015	0.3	0	9
		0.15	0	10
		0.1	1	11
		Nil	0	d
0.02	0.01	0.3	0	12
		0.15	0	12
		0.1	4	14
		Nil	0	1

Legend as in Table III.

Inhibitory Activity of Colon Content Obtained from Streptomycin-Treated Mice.—

Contents of cecum and transverse colon were removed from mice killed 1, 3, or 5 days after oral administration of 50 mg streptomycin. Material from 15 to 20 mice was pooled in as many milliliters of phosphate buffer, centrifuged, the supernatant heated at 100°C for 15 minutes, and recentrifuged. The clear supernatant was assayed by the liquid culture method by inoculating aliquots with *S. enteritidis* to provide approximately 10^8 microorganisms/ml. The pH was checked before and at the end of each experiment and found to be unchanged unless considerable growth of *Salmonella* had occurred.

Plate counts made before and after 6 and 24 hours' anaerobic incubation showed that at each pH, inhibitory activity of colon content was minimal the day after streptomycin treatment, but increased as the interval following treat-

TABLE V

Growth of S. enteritidis in Different Concentrations of Lactic Acid in Nutrient Broth at pH 6.0

Concentration of lactic acid	Changes (log 2) in No. of *Salmonella* at:	
	6 hrs.	24 hrs.
M		
1.0	0	d
0.5	0	8
0.3	8	12
0.25	10	12
0.1	12	16
0.07	10	15
0.05	9	16
0.01	8	16
Nil	8	16

Legend as in Table III.

TABLE VI

Growth of Salmonella in Colon Content of Streptomycin-Treated and Untreated Mice

Colon content* obtained:	Increase (log 2) in No. of *Salmonella*							
	pH 6.0		pH 6.1		pH 6.2		pH 6.4	
	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.	6 hrs.	24 hrs.
1 day after treatment‡	4.3	17.6 (5)	5.4	18.0 (15)	5.7	18.2 (5)	5.9	19.0 (5)
3 " " "	2.7	15.4 (6)	3.7	17.6 (14)	5.1	18.0 (4)		
5 " " "	1.5	12.3 (4)	3.0	17.0 (14)	4.8	17.6 (6)		
Untreated controls	−0.4	1.1 (12)	0.3	7.2 (15)	1.4	11.1 (11)		

Results given as means of the number of observations shown in parenthesis.

* Heat-Killed supernatants of buffered suspensions of pooled content of cecum and transverse colon from 15 to 20 mice.

‡ 50 mg streptomycin *per os*.

ment was lengthened (Table VI). By the 5th day, however, inhibitory activity was still below the normal level.

Antagonism of Inhibitory Activity of Colon Content of Untreated Mice.—*Lactic*, *pyruvic*, *succinic*, and *citric* acids in appropriate concentrations counteracted the inhibitory activity of colon content obtained from untreated mice, as they did in broth containing inhibitory concentrations of acetic and butyric acids. *Glucose* failed to do so.

Of the 4 acids which successfully antagonized inhibitory activity, pyruvic

TABLE VII

Effect of Lactic and Pyruvic Acids on Inhibitory Activity of Colon Content from Untreated Mice in Phosphate Buffer at pH 6.0 under Aerobic and Anaerobic Incubation

Concentration	Changes (log 2) in No. of *Salmonella* at:			
	6 hrs.		24 hrs.	
	Aerobic	Anaerobic	Aerobic	Anaerobic
M				
Lactic				
0.3	d	d	8	11
0.15	d	0	8	13
0.07	d	d	7	13
0.03	d	d	2	8
0.01	d	d	d	3
Pyruvic				
0.2	6	6	17	17
0.1	4	4	17	17
0.05	1	1	14	15
0.02	0	d	8	5
0.01	d	d	3	2
Untreated colon content	d	d	d	d

Legend as in Table III.

TABLE VIII

Partial Restoration of Inhibitory Activity of Colon Content from Streptomycin-Treated Mice by Addition of Acetic and Butyric Acids

Colon content* obtained:	Changes (log 2) in No. of *Salmonella* at:			
	6 hrs.		24 hrs.	
	Acetic acid 0.03 M and butyric acid 0.015 M			
	Omitted	Added	Omitted	Added
1 day after treatment‡	6	0	19	3
3 " " "	4	0	18	6
5 " " "	3	0	17	5
Buffered nutrient broth	8	d	16	1

Legend for log 2 figures same as in Table III.

* Heat-killed supernatants of buffered suspensions (pH 6.1) of pooled content of cecum and transverse colon from 15 to 20 mice.

‡ 50 mg streptomycin *per os*.

acid was the most effective and, unlike the others, was as active under aerobic as under anaerobic conditions (Table VII).

Addition of Acetic and Butyric Acids to Colon Content of Streptomycin-Treated Mice.—Comparison of results in Tables II, III, and VI show that colon content obtained from mice 3 and 5 days after streptomycin treatment was less inhibitory than broth containing acetic and butyric acids in concentrations observed in colon content on those days. Moreover, acetic and butyric acids added to such colon content failed to increase its activity to the same inhibitory level as identical concentrations in broth (Table VIII) presumably because antagonistic concentrations of lactic acid were present in colon content on the 3rd and 5th days after streptomycin treatment.

DISCUSSION

Earlier publications (1, 4) have described the various factors which in the normal, untreated mouse are responsible for the resistance of its intestinal tract to infection with *Salmonella enteritidis* introduced by mouth. Most important are those factors which inhibit multiplication of the microorganism within the lumen of the large intestine. Conditions necessary for effective inhibition include anaerobiosis, appropriate pH and adequate concentrations of acetic and butyric acids in the content of the large bowel, particularly cecum and transverse colon. The present report describes how these conditions are affected by oral administration of a single, large dose of streptomycin and result in loss of resistance to this experimental infection. The chemical changes produced by such antibiotic therapy are summarized in Fig. 1 which plots mean values for pH and concentrations of acetic-butyric and lactic acids in colon content of untreated and of mice treated 1, 3, and 5 days previously with 50 mg streptomycin *per os*. Fig. 2 shows the inhibitory activity for *Salmonella* demonstrable in broth with the combinations of pH and acids plotted above the four abscissae in Fig. 1, and for comparison, the inhibitory activity of heated supernatants of colon content.

These *in vitro* findings are also compared with the *in vivo* effect of streptomycin treatment on the resistance of mice to oral challenge with *S. enteritidis* (shown as a series of columns in Fig. 2). The results are plotted as ID_{50}, the numbers of microorganisms required to infect approximately 50 per cent of mice inoculated by mouth (5). Resistance to infection was lowest during the first 24 hours following treatment, but was steadily regained on succeeding days and approached normal by the 5th day. From the data shown in Fig. 2, it may be concluded (*a*) that inhibition or growth of *Salmonella* in colon content *in vitro* is determined by its pH and by the concentrations of acetic, butyric, and lactic acids present, and (*b*) that *in vivo*, the same factors determine resistance or susceptibility of untreated or streptomycin-treated mice to oral challenge with this strain of *S. enteritidis*.

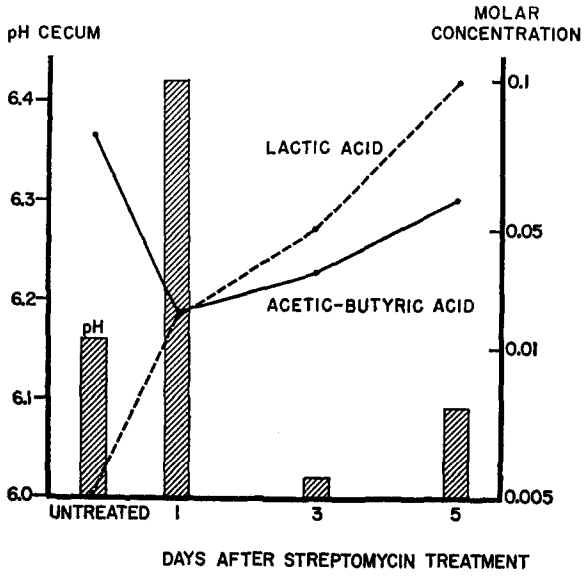

FIG. 1. Effect of streptomycin treatment (50 mg *per os*) on chemical constituents of colon content.

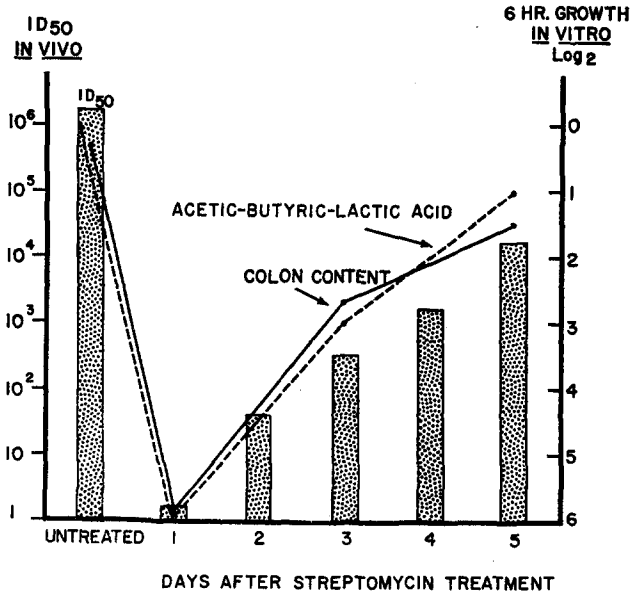

FIG. 2. Growth inhibition of *S. enteritidis in vitro* by heat-killed suspensions of colon content and by broth containing the chemical constituents shown in Fig. 1, and, for comparison, resistance of mice to oral challenge. ID_{50} = numbers of *S. enteritidis* required to initiate infection in 50 per cent of mice.

The chemical changes in the content of the mouse's colon are ascribed to the changes in its indigenous microflora caused by the antibacterial action of the drug. The initial effect is a virtual elimination of all Gram-negative bacilli, the most important of which seems to be the removal of members of the genus *Bacteroides* which have been shown to produce acetic and butyric acids in high concentrations during anaerobic growth in pure cultures (2). *Bacteroides* were found to be among the most numerous inhabitants of the contents of cecum and transverse colon, the segments of large bowel from which material was obtained for the chemical studies and tests of inhibitory activity described in this and the preceding paper.

On the 1st day after streptomycin treatment, inhibitory activity of colon content is reduced not only because the concentrations of acetic and butyric acids are lower, but also and more importantly, because its pH is higher. At the pH levels found in colon content, a very small rise sharply increases the dissociation of the fatty acids which exert their antibacterial action in the undissociated state (8–10). Each rise in pH, therefore, decreases by a large factor their effectiveness in inhibiting multiplication of *Salmonella*.

Meynell's (11) recent report describes similar observations on mice treated 1 day before with streptomycin and explains in much the same way their loss of resistance to *Salmonella* infection. He used a strain of *Salmonella typhimurium* which produced more lethal infections than our strain of *S. enteritidis*. He found reduced concentrations of acetic, butyric, and propionic acids and a rise in pH and Eh in cecal content of mice, changes which he considered responsible for their increased susceptibility to oral challenge with *S. typhimurium*. His observations, however, did not extend beyond the 1st day after streptomycin treatment.

As shown in Fig. 1, the explanation which accounts for the susceptibility of mice on the 1st day after streptomycin treatment does not explain their susceptibility on the 3rd and 5th days when the pH and fatty acid concentrations are at levels which should be inhibitory. Failure to inhibit *S. enteritidis* beyond the 1st day is due to increasing concentrations of lactic acid which counteract the inhibitory activity of the fatty acids in colon content. Lactic acid presumably accumulates because it is not utilized as it is when the enteric microflora is intact, but continues to be produced by the large numbers of lactobacilli and enterococci which survive the action of streptomycin.

The antagonistic effect of lactic acid on the antibacterial activity of the fatty acids is not understood. The same effect was obtained *in vitro* with pyruvic, succinic, and citric acids, but not with glucose. Of possible significance is the fact that each of these substances can serve as the sole carbon source for growth of this strain of *S. enteritidis*. Accumulation of lactic acid in the content of the mouse's cecum and transverse colon follows a series of chemical and microbial changes initiated by the action of streptomycin on the normal enteric micro-

flora. Their sequence indicates the close interdependence of the various chemical and microbial factors within the lumen of the gut. Of the possible effects of such changes, we are here concerned solely with those which provide an environment favorable to the multiplication of *S. enteritidis*, for the purpose of this study was an explanation of the means by which the antimicrobial action of streptomycin renders the mouse's intestinal tract susceptible to the implantation of that microorganism.

SUMMARY

Determinations of pH, Eh, and concentrations of acetic, butyric and lactic acids were made on the content of cecum and transverse colon of groups of mice killed 1, 3, and 5 days after oral administration of 50 mg streptomycin. Control observations on untreated mice are reported in the preceding communication. Heat-killed supernatants of suspensions of bowel content were tested *in vitro* for their ability to inhibit multiplication of our standard streptomycin-resistant strain of *Salmonella enteritidis* during aerobic and anaerobic incubation. Also tested in like fashion were series of cultures in broth buffered at various pH levels and containing acetic, butyric, and lactic acids in varying concentrations.

In colon content of mice on the 1st day after streptomycin treatment, the pH had risen and the concentrations of the fatty acids fallen, a combination of effects which adequately accounts for its inability to inhibit multiplication of *Salmonella in vitro* and *in vivo*. By the 3rd day after streptomycin treatment, pH and fatty acid concentrations had returned to normal levels. The susceptibility of mice to oral challenge on the 3rd day was explained by the finding that lactic acid had accumulated in colon content to levels which, in broth, effectively counteracted the activity of inhibitory concentrations of the fatty acids. Other cocarboxylic acids also antagonized the inhibitory activity of the fatty acids; glucose did not.

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