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

I. Factors Which Interfere with the Initiation of Infection by Oral Inoculation*

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Described below are observations which seem to account, in large part at least, for the normal resistance of the mouse's intestinal tract to infection with *Salmonella enteritidis* introduced by mouth, the natural route of infection. Thus inoculated, about 10^6 microorganisms are required to infect 50 per cent of young adult CF-1 mice (1). Their resistance, however, can be sharply reduced by the oral administration of a single, large dose of streptomycin, for during the following 24 hours, <10 microorganisms of the same strain suffice to initiate infection (2, 3). Of the changes in the mouse's enteric microflora resulting from streptomycin treatment, the most consequential was thought to be the elimination of certain obligate anaerobes belonging to the genus *Bacteroides* (4, 5).

The experiments to be described demonstrate that multiplication of *S. enteritidis* was inhibited *in vitro* by: (*a*) centrifuged supernatants of heat-killed suspensions of colon content or feces of normal, *i.e.* untreated mice; (*b*) anaerobic cultures of such materials; (*c*) anaerobic cultures of *Bacteroides* isolated from them; (*d*) two fatty acids (acetic and butyric) recovered from bowel content and also from cultures of *Bacteroides*. In all these experiments, the degree of inhibitory activity was always dependent upon pH and was always greater under anaerobic conditions which simulated those within the colon content *in vivo*.

As will be shown in the following communication inhibitory activity was never demonstrable with suspensions of colon content or feces of mice treated by mouth with streptomycin on the preceding day. The following paper (6) also describes changes in the chemical constituents and the pH of colon content which follow such treatment and are associated with enhanced susceptibility.

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Methods

Mice were CF-1 females, approximately 9 weeks old, obtained from the same source as those used in earlier experiments. (1, 3, 5).

Inoculations and *treatments* were *per os*; *i.e.*, by stomach tube.

The *test microorganism* was the same streptomycin-resistant strain of *S. enteritidis* previously used. (1)

In Vitro Inhibition Tests.—

(*a*) *Plate assay*: Inhibition of *S. enteritidis* on surface culture was determined thus: Nutrient agar containing 1 mg/ml of streptomycin[1] was buffered with phosphate to a desired pH and 15 ml pipetted into Petri dishes to insure uniform depth. Each assay was made on 3 agars of different pH, usually 5.5, 6.0, and 6.5. The surface was seeded with approximately 5000 *S. enteritidis* suspended in saline, and 3 or 4 paper discs[2] were placed on the agar and moistened with 0.1 ml of the material to be tested. For anaerobic assay, the plates were put immediately into a Brewer jar. All plates were then kept in a refrigerator 7 hours to permit diffusion of any inhibitory substance before incubation. After 16 hours' incubation, bacteriostatic activity was estimated by measuring the zones of inhibition. These were frequently double: an inner one of complete inhibition surrounded by an outer zone of tiny colonies just visible to the naked eye. To obtain reproducible results, it was necessary to incubate plates for the same length of time in all experiments. Plate assays were used principally as a screening method; *i.e.*, to obtain rough estimates of inhibitory activity. For more precise determinations, the liquid culture method was used.

(*b*) *Assay by liquid culture*: Material to be assayed was titrated by serial 2-fold dilution in 0.05 M phosphate buffer or buffered nutrient broth, usually in 2 ml volumes. In some experiments, several series were set up, each at a different pH. To each tube was added a dilution of broth culture of *S. enteritidis* in its log phase of growth to provide inocula of approximately 10^3 per ml. For anaerobic incubation, the tubes were placed in a Brewer jar. Numbers of *Salmonella* were determined at the start and at several times thereafter by spreading 0.1 ml from each tube and appropriate 10-fold dilutions onto brilliant green agar containing 0.25 mg/ml streptomycin. The pH of each mixture was determined electrometrically before and after incubation.

Determination of pH of Colon Content in Situ.—A mouse was chloroformed and the calomel electrode of a Beckman potentiometer introduced into its pharynx. The abdomen was opened and as quickly as possible, a small (4 mm diameter) glass electrode inserted into material contained within (*a*) the cecum and (*b*) the transverse colon, the segments of the gut in which *Salmonalla* had been found to be most effectively inhibited following oral inoculation into normal, untreated mice; *i.e.*, mice not treated with antibiotic (1, 5). Care was taken to expose the electrode to a sufficient accumulation of bowel content to avoid contact with the mucosa. Before each reading the electrodes were carefully rinsed with distilled water and dried with soft tissue paper.

Eh of Cecal Content in Situ.—This was measured in nembutal anaesthetized or freshly killed mice by inserting a platinum electrode into the cecal content. The electrode was the same one used by Freter (7) which he generously lent to us. It was a platinum wire, the distal 2

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

[2] Half inch diameter, manufactured by Schleicher and Schuell, Keene, New Hampshire, No. 740-E for assay of antibiotics.

cm of which had been flattened to a width of 2 cm and the rest encased in nylon tubing and connected to the Beckman potentiometer.

Chemical Methods: Isolation and Identification of Volatile and Non-Volatile Acids in Colon Content.—Material from cecum and transverse colon of 15 to 20 mice was suspended in as many milliliters of water, acidified to pH 1 with N H_2SO_4 and subjected to continuous ether extraction (18 to 20 hours) or to steam distillation until approximately 200 ml of distillate had been collected. The distillate was neutralized with NaOH and evaporated to dryness. Acids were separated by developing each sample on a celite column with 5 per cent butanol in chloroform by the methods of Phares *et al.* (8) Identification was by paper chromotography, and in the case of the volatile acids confirmed by Duclaux distillation curves.

TABLE I

pH of Colon Content of 70 Mice Determined Electrometrically in Situ Immediately after Death

pH	Cecum		Transverse colon	
	No. mice	Per cent	No. mice	Per cent
5.4–5.8	8	11.4	13	18.5
5.9–6.0	18	25.7	17	24.3
6.1–6.2	19	27.1	18	25.7
6.3–6.4	15	21.4	14	20.0
6.5–6.7	10	14.3	8	11.4
Mean *pH*	6.16		6.10	
Median *pH*	6.1		6.1	

EXPERIMENTAL

Preliminary experiments with the plate assay method showed that under anaerobic conditions, multiplication of *Salmonella enteritidis* was inhibited by saline suspensions of freshly excreted feces or colon content of normal mice, but *not* by those of streptomycin-treated mice. Most effective was the content of the cecum, a large organ in the mouse. The constituent responsible for the inhibition was found to be heat-stable and readily dialyzable. Its activity was sensitive to the pH at which the test was carried out, a characteristic which suggested the importance of determining *in situ* the pH of the material in cecum and transverse colon of normal mice. Eh was also measured because anaerobiosis always favored *in vitro* inhibition of *Salmonella.*

pH and Eh of Colon Content in Situ.—As shown in Table I, pH of cecal and transverse colon content was found to be <6.3 in approximately ⅔ of the 70 mice examined. Essentially the same distribution of readings was obtained on mice fasted for 18 hours. Eh of cecal content ranged from −0.29 to −0.50 mv in 24 mice (mean −0.40 mv). Most of the determinations of Eh were made

immediately after death, but a few measurements on nembutal anaesthetized mice gave similar results.

Inhibitory Activity of Colon Content in Vitro.—The observation that the inhibitory activity of colon content was heat-stable led to the use of heat-killed suspensions in a series of assays made by the liquid culture method. Killing of the microbial population in the test material eliminated the possibility that multiplication of *Salmonella* might have been suppressed simply because the

TABLE II

Effect of pH on Inhibition of S. enteritidis by Heat-Killed Suspensions of Colon Content and by Acetic and Butyric Acids in Broth

pH*	Changes (Log 2) in mean No. of *Salmonella* during 24 hr. anaerobic incubation		
	Pooled colon content	Broth containing	
		0.04 M acetic acid 0.02 M butyric acid	0.02 M acetic acid 0.01 M butyric acid
5.9	−0.7 (8)‡	−0.8 (7)	
6.0	+1.1 (12)	−0.8 (7)	+2.0 (6)
6.1	+7.2 (15)	−0.2 (11)	7.2 (8)
6.2	11.1 (11)	+3.4 (15)	7.5 (7)
6.3	11.1 (8)	5.4 (21)	11.0 (7)
6.4	17.0 (7)	5.9 (12)	14.5 (7)
6.5	17.0 (4)	9.2 (9)	14.7 (5)
6.6		11.1 (7)	15.0 (3)
6.7		14.4 (7)	

Broth controls at pH 5.7 = 17.0.

* Buffered with 0.05 M phosphate.

‡ Figures in parentheses = No. observations.

available food supply had been too rapidly exhausted by the greater numbers of indigenous microorganisms in the mixture.

In a large number of experiments, material from cecum and transverse colon from several mice was pooled in as many milliliters of phosphate buffer, the centrifuged supernatant heated for 15 minutes in a boiling water bath and centrifuged again to obtain a clear supernatant for assay by the liquid culture method. Electrometric determinations made on aliquots just after inoculation with *Salmonella* and again at the end of each experiment showed only negligible reduction in pH unless considerable growth of *Salmonella* had occurred.

Most assays were incubated aerobically as well as anaerobically. Comparison of the results showed that inhibition of *Salmonella* was always more effective in the anerobic tests.

Plate counts were made immediately after inoculation and again after 6 and 24 hours' incubation. The 24-hour plate counts showed that multiplication

of *Salmonella* had been completely inhibited in the anaerobic cultures of pooled heat-killed suspensions of colon content buffered at pH 5.9 (Table II).

More convincing evidence of the effect of pH on the inhibitory activity of such suspensions was obtained from the 6-hour plate counts which are presented graphically in one of the curves in Fig. 1. Each point plots the mean increase or decrease in numbers of *Salmonella* as per cent of those inoculated (approximately

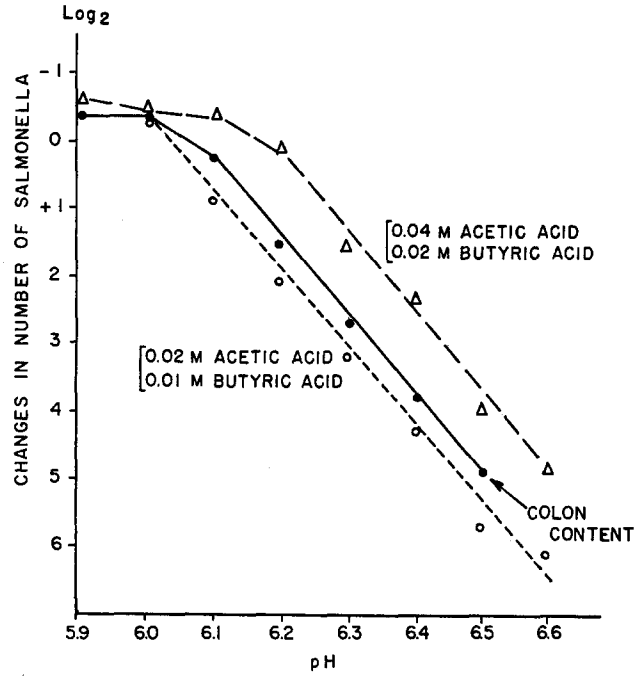

FIG. 1. Effect of pH on inhibitory activity of heat-killed suspensions of colon content and of broth containing acetic and butyric acids. Each point plots the increase or decrease in numbers of *S. enteritidis* after 6 hours' anaerobic incubation as per cent of initial population, approximately 10^3/ml.

10^3/ml). The number of observations from which each mean was derived is given in Table II. Multiplication of *Salmonella* was completely inhibited at or below pH 6.1. As pH was raised above that level, the degree of inhibition steadily declined. Inhibition at the lower pH levels was not due simply to the acidity of the medium because broth controls buffered at pH 5.7 never inhibited multiplication of *Salmonella*.

It should be borne in mind here and in later considerations of inhibition by suspensions of colon content that no allowance was made for the dilution of inhibitory substances present as solutes within the test material, which consisted largely of solid matter.

Colon content of individual mice was also assayed by the same method except that the test material was suspended in distilled water instead of buffer. The results showed that the degree of inhibitory activity of such aqueous suspensions was as closely related to their initial pH as were those of pooled buffered suspensions. After suspension in water the pH of colon content was always higher (by an average of 0.7 pH) than it had been *in situ*; *i.e.*, before removal from the intestine permitted escape of CO_2.

Inhibitory Activity of Anaerobic Cultures of Colon Content.—Freshly prepared meat digest broth (5) inoculated with a dilute suspension of material from cecum and transverse colon developed the same inhibitory properties after several days' anaerobic incubation. No inhibitory activity was produced in aerobic cultures.

Isolation of the Inhibitory Substances Present in Colon Content.—

Material removed from cecum and transverse colon of 15 to 20 mice was pooled and suspended in as many milliliters of water. An aliquot was frozen and stored, the remainder acidified and subjected to steam distillation (see Methods). The distillate and residue were dried, then brought to initial volume and pH of the original suspension. These and the stored aliquots of original suspension were all assayed at the same time by both plate and liquid culture methods.

The results always showed the inhibitory activity of the distillate to be equal to that of the original suspension and the residue to be inactive. All of the activity, therefore, had been carried over into the distillate. Equally effective in recovering inhibitory activity was continuous ether extraction of acidified colon content. Inhibitory activity could not be separated from unacidified colon content by either method.

Identification of the Volatile Acids as Acetic and Butyric.—When the distillate was developed on a celite column with 5 per cent butanol in chloroform, 2 volatile acids were separated which had the same peak effluent volumes as reagent acetic and butyric acids. Their identity was confirmed by isolating each acid and comparing its Duclaux curve with that of authentic acetic and butyric acids. The estimated concentrations in a number of pools of normal colon content were 0.02 to 0.04 M for acetic and 0.008 to 0.02 M for butyric. The ratio of acetic to butyric always approximated 2:1.

Effect of pH on the Inhibitory Activity of Acetic and Butyric Acids in Broth.—In a large number of experiments, these 2 acids were tested in nutrient broth for their ability to inhibit multiplication of *S. enteritidis* under the same conditions and by the same liquid assay method used with heat-killed suspensions of colon content. In each experiment, tubes of buffered broth containing acetic and butyric acids (always in the ratio of 2:1) were adjusted to a series of pH levels and inoculated with approximately 10^3 *Salmonella*/ml. The pH was checked electrometrically, and plate counts were made before and after 6 and 24 hours' anaerobic incubation.

The 24-hour plate counts presented in Table II showed that concentrations of 0.04 M acetic acid:0.02 M butyric acid had completely inhibited multiplication of *Salmonella* at pH 6.1 and that the lower concentrations (0.02 M acetic acid:0.01 M butyric acid) were effective at pH 6.0.

Comparison of Inhibitory Activity of Colon Content and of Acetic and Butyric Acids in Broth.—Results of the 6-hour plate counts in a series of experiments are plotted in Fig. 1, which also shows for comparison, counts obtained with suspensions of colon content. The similarity of the 3 curves shows the same effect of pH on the degree of inhibitory activity, that is, as pH rose activity lessened. In comparing the inhibitory activity of the fatty acids with that of colon content, it should be emphasized that the *in vitro* determinations of the latter almost certainly underestimate by a considerable margin its actual effectiveness *in vivo* owing to its dilution before assay.

Since this study is concerned with the resistance of the mouse's intestinal tract to infection with *S. enteritidis* inoculated by mouth, the most significant data are those which show that multiplication of this strain of *Salmonella* was inhibited by colon content at or below pH 6.2, which was found by electrometric determination *in situ* to be that of material in the cecum and transverse colon in ⅔ of the normal mice examined (Table I).

Impairment of Normal Resistance to Salmonella by Raising the pH of the Intestinal Tract.—The *in vitro* findings just described led to attempts to interfere with the normal resistance of the intestinal tract simply by raising its pH *in vivo*. Among a number of substances tried, the most effective was $MgCO_3$ (U.S.P.) which is relatively insoluble and was non-toxic when administered *per os* in doses of 100 mg suspended in 0.5 ml water. Direct *in situ* measurements on a number of mice showed that within 1½ hours after such treatment, the pH within the cecum and transverse colon had risen to 6.8–7.1 and was maintained at or slightly above this level for another 4 hours.

Mice were therefore treated *per os* with $MgCO_3$ (the controls with saline) 2 hours before and 3½ hours after inoculation *per os* with relatively small numbers of *Salmonella* and were killed the following day for cultures to determine the total numbers of *Salmonella* present in the entire large bowel. The results (Table III) showed that a greater porportion of the $MgCO_3$-treated mice had higher *Salmonella* counts than did the saline controls. Implantation of *S. enteritidis* in the large intestine had, therefore, been facilitated by raising the pH of its content.

The effect of such alteration of pH was confirmed by spleen cultures made 6 days after similar treatments and inoculations. The results presented in Table IV show that a higher incidence of systemic infection had occurred in the $MgCO_3$-treated mice than in the saline-treated controls.

In Vitro Inhibition of Salmonella by Liquid Cultures of Bacteroides.—As reported in an earlier publication (5), the resistance of the normal mouse to infection by oral inoculation with *S. enteritidis* was ascribed to the presence

in its enteric microflora of members of the genus *Bacteroides*. These obligate anaerobes were among the most numerous inhabitants of the cecum and upper colon of normal mice and their elimination by streptomycin treatment was

TABLE III

Effect of $MgCO_3$ on No. of S. enteritidis Present in the Colon 24 Hours after Inoculation

Treatment *per os*	*Salmonella* inoculated *per os*	No. of mice (among 25) having following *Salmonella* counts*				
		None	$<10^2$	10^{2-3}	10^{3-4}	10^{4-5}
$MgCO_3$‡	10^2	15	5	3	1	1
	10^3	0	10	7	5	3
Saline	10^3	23	2	0	0	0
	10^4	17	5	2	1	0

* Plate counts on suspensions of contents of large intestine and appropriate dilutions thereof.

‡ 100 mg suspended in 0.5 ml H_2O, 2 hours before and again 3½ hours after inoculation with *S. enteritidis*.

TABLE IV

Increased Incidence of Systemic Salmonellosis in Mice Treated with $MgCO_3$

Salmonella inoculated *per os*	Spleen cultures 6 days after inoculation			
	$MgCO_3$ *per os*		Saline *per os*	
	Positive	No. mice	Positive	No. mice
	per cent		*per cent*	
10^7			72.2	18
10^6	100	12	33.3	30
10^5	83.3	12	13.3	20
10^4	58.3	12		
10^3	23.8	84		
10^2	16.6	6		

* 100 mg suspended in 0.5 ml H_2O, 2 hours before and again 3½ hours after inoculation with *S. enteritidis*.

always followed by loss of resistance to infection by oral challenge with the same strain of *S. enteritidis*.

Several strains of *Bacteroides* tested by the plate assay method were found to inhibit multiplication of *S. enteritidis in vitro* and, on inoculation into streptomycin-treated mice, partially restored their resistance to *Salmonella* infection, an effect not demonstrable with cultures of any aerobic members of the normal microflora (5).

Supernatants of 5- to 9-day anaerobic broth cultures of *Bacteroides* assayed by the liquid culture method were found to contain a potent inhibitor of *Salmonella in vitro* which was heat-stable, readily dialyzable, ether soluble, and sensitive to the pH of the assay medium,—characteristics similar to those of colon content of normal mice.

Isolation of Acetic and Butyric Acids from Broth Cultures of Bacteroides.—When the supernatant of such a culture was acidified and subjected to steam distillation, the neutralized distillate was found to contain all of the inhibitory activity, and the residue none.

Bioautographs were prepared by developing aliquots of the steam volatile fraction with a butanol solvent system (8). Exposure of the chromatogram for 15 minutes to a large surface of nutrient agar seeded with *S. enteritidis* showed, after 18 hours' incubation, 2 areas of inhibition at Rf's comparable with those of authentic acetic and butyric acids developed as controls on the same chromatogram.

The demonstration that acetic and butyric acids are produced *in vitro* by *Bacteroides* seems to account for the inhibition of *S. enteritidis* by the content of cecum and transverse colon and therefore for its resistance to the initiation of infection by oral inoculation.

DISCUSSION

Many factors play a role in host resistance to bacterial infection. This study is concerned with resistance to the *initiation* of an enteric infection, specifically those factors which interfere with the establishment of *S. enteritidis* in the mouse's gastrointestinal tract following oral inoculation. As previously reported (1), inocula introduced by mouth leave the stomach without multiplying, owing to the acidity of gastric juice. In the small intestine, they fail to multiply because they pass through it too quickly, not because its content exerts any inhibitory activity. When detained there by peristaltic arrest, they do multiply (1).

Upon entry into the large intestine, however, inocula of *Salmonella* encounter an environment demonstrably antagonistic; in fact, sufficiently antagonistic to prevent their multiplication and hence the initiation of infection in most normal mice. The content of the cecum and transverse colon was shown to inhibit multiplication of *Salmonella in vitro* under anaerobic conditions and at the pH demonstrated *in situ* in $\frac{2}{3}$ of the normal mice examined. When the pH was raised *in vivo* by administering $MgCO_3$ by mouth, multiplication of *S. enteritidis* occurred more frequently than in untreated controls and resulted in a higher incidence of infection.

Inability of *Salmonella* to multiply within the large intestine of the normal mouse is therefore conditioned by the pH of its content because the volatile fatty acids present exert their inhibitory activity in their undissociated state

which is determined by pH (9–11). This explanation for the resistance of "the normal mouse" is, to be sure, based upon data which represent mean values for pH and Eh measurements on many animals and determinations of fatty acid concentrations were of necessity made on pooled material obtained from groups of mice. Attention should therefore be drawn to the range of pH levels of intestinal content shown in Table I, a considerable fraction of which were > pH 6.3; *i.e.*, above the level at which the fatty acids are able to act effectively as inhibitors of *Salmonella*. It seems reasonable to assume that the individuals within that fraction would have been susceptible to infection by oral challenge with *S. enteritidis*, a supposition which could not be verified experimentally. It should be recalled that an earlier communication (1) reported a wide range of susceptibility among normal mice to infection with this strain of *Salmonella*, a finding which necessitated the adoption of the ID_{50} as the only reliable index of susceptibility.

The source of acetic and butyric acids in the mouse's colon is thought to be certain members of the genus *Bacteroides* which are among the most numerous inhabitants of the large bowel, particularly of the cecum and transverse colon, where the highest concentrations of the volatile fatty acids are to be found (5). *In vitro*, several strains produced large amounts of the two acids after prolonged incubation. While other microorganisms are known to produce acetic and butyric acids, *Bacteroides* was the group which consistently inhibited *S. enteritidis in vitro*. During the course of very many platings of colon content using a modification of Sieburth's method (12), only 5 colonies of *Escherichia coli* and 2 of enterococci were encountered which showed any inhibitory activity against *Salmonella*.

The presence of acetic and butyric acids in feces has been demonstrated by several investigators. As early as 1878, Brieger (13) reported their recovery from human feces. Bergeim and his collaborators (10, 14) determined the concentrations of these fatty acids in the feces of man and in the colon content and feces of rats. They also studied the inhibitory action of acetic and butyric acids alone and in combination against yeast and various bacteria including 5 strains of *Salmonella*. They emphasized the importance of pH and determined the ranges in which the acids were bactericidal or bacteriostatic.

Recently Meynell (15, 16) reported results similar in many respects to ours. From the cecal content of normal mice he recovered almost the same concentrations of volatile fatty acids which he identified as acetic, butyric, and propionic acids in that order of relative concentration. He also measured pH and Eh of cecal content, but found the former to be higher than our determinations which were made *in situ*. Using a strain of *Salmonella typhimurium*, he demonstrated inhibition anaerobically *in vitro* although at somewhat higher concentrations of the fatty acids and at a slightly higher pH. Meynell concludes that the outcome of oral challenge with *S. typhimurium* is determined by pH, Eh, and the presence of fatty acids in cecal content.

Freter (7) has suggested that unsuccessful competition by *Shigella flexneri* for carbon sources in a sufficiently reduced medium accounts for its inhibition *in vivo* and *in vitro*, an explanation inapplicable to our inhibition experiments with heat-killed suspensions of colon content.

Of interest are the observations of Sieburth (17) who demonstrated that the absence of *E. coli* in the gastrointestinal tract of polar birds was due to the presence of acrylic acid derived from the algae in their diet.

The possibility cannot be denied that other, unconsidered factors may be concerned with the resistance of the mouse's intestinal tract to the initiation of infection with *S. enteritidis*. However, the observations herein described seem to demonstrate that under relatively anaerobic conditions and at the pH of colon content of most normal mice, the concentrations of acetic and butyric acids there present quite adequately account for its resistance to the implantation of moderate inocula of *S. enteritidis*.

SUMMARY

Multiplication of *Salmonella enteritidis* was inhibited *in vitro* by buffered suspensions of fecal material freshly removed from the large intestine of normal mice. Most effective was material obtained from cecum and transverse colon. Inhibitory activity was not impaired by sterilization by heat or filtration. From such materials were isolated acetic and butyric acids in concentrations which inhibited *Salmonella in vitro*. The degree of inhibitory activity of suspensions of colon content and of mixtures of the two fatty acids was conditioned by pH and favored by anaerobiosis. Effective inhibition occurred at or slightly below the pH of colon content of most normal mice as determined *in situ* by direct measurement. Acetic and butyric acids were isolated from anaerobic cultures of several strains of *Bacteroides* previously demonstrated to be one of the most numerous inhabitants of the large intestine of the normal mouse.

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