

INDIGENOUS, NORMAL, AND AUTOCHTHONOUS FLORA
OF THE GASTROINTESTINAL TRACT*

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PLATE 10

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Comparative studies of albino mice obtained from various colonies have revealed that the so called indigenous (or normal) microbiota of these animals exerts a profound influence on their rate of growth, their efficiency in the utilization of food, and their resistance to infection, toxic substances, and other stressful agencies (1-4). Indeed, many attributes of mice which are characteristic of the colonies from which the animals were derived are in reality determined not by genetic endowment, but by the microbiota prevailing in the colony. The very statement of this fact illustrates the ambiguity of the phrase "normal microbiota". This phrase merely denotes a multiplicity of microbial types which happen to be associated with a given animal population, but it does not imply that such organisms are necessarily present in other populations of the same animal species. As we shall see later, the same kind of ambiguity applies to the phrase normal microbiota when applied to man.

The study of the intestinal bacterial flora has necessitated the development of special bacteriological techniques, designed for the quantitative enumeration of bacterial types which are extremely abundant *in vivo*, but which commonly fail to grow on the usual culture media under aerobic conditions. These techniques, which are described in the preceding paper, have enabled us to follow the trend of the bacterial population in the gastrointestinal tract of mice from the time of birth (5).

In the present paper, we shall focus attention on some unexpected findings concerning the composition and distribution of the indigenous flora in adult animals. We shall show in particular that (a) the whole gastrointestinal tract harbors throughout life an abundant bacterial flora, the composition of which is characteristic for each section of the tract; and (b) some bacterial species are intimately associated with the wall of the various organs.

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The experimental methods used in the present study were the same as those described in the preceding paper (5).

Comparative Composition of the Fecal Flora of Swiss Mice from Various Colonies.—We have reported earlier that there exist profound differences in the composition of the fecal flora of various strains of mice (1–3). Further evidence of this fact has been obtained from more recent studies involving the use of culture media and incubation techniques permitting the growth of anaerobic bacteria (5).

TABLE I
Comparative Fecal Flora of Three Different Colonies of Swiss Mice

	Mouse Colony		
	SS	CFW	NCS
Lactobacilli	10 ⁸ *	10 ⁸	10 ⁹
Bacteroides	10 ⁹	10 ⁹	10 ⁹
<i>E. coli</i>	10 ⁷	10 ⁸	0
Coliforms	10 ⁹	10 ⁸	10 ³
Pseudomonas	Present	Present	Not detectable
Proteus	“	“	“ “
Clostridia	“	“	“ “

* The figures represent the numbers of colonies obtained per gram of fresh fecal material.

In Table I, the mice of the NCS colony are compared with other Swiss mice from two other colonies, one (SS Lynch) raised at The Rockefeller Institute under conventional conditions, the other (CFW) produced commercially by Carworth Farm Laboratories, New City, New York. In all cases, the animals were males 6 to 7 weeks old, and had been given pasteurized pellets and sterile acidified water *ad lib.* for at least 10 days before the test.

Several experiments carried out over a period of 2 years gave uniform results; these results have been summarized in Table I, which gives the approximate number of colonies of each bacterial group obtained per gram of stool.

Comparative Composition of the Flora in the Various Parts of the Gastrointestinal Tract.—While it is well known that the large intestine harbors an abundant bacterial population, it is generally believed that the stomach and small intestine of adults are practically free of living microorganisms. This belief, however, originated from the use of cultural methods designed for the growth of ordinary enterobacteria and enterococci, organisms which are erroneously assumed to constitute the typical flora of the intestinal tract. The availability of new cultural media and incubation techniques has led us to reexamine bacteriologically the various parts of the gastrointestinal tract. We shall limit our presentation here to the results obtained with the stomach, the middle half of the small intestine, and the large intestine.

The findings reported in Table II refer to male and female NCS mice ranging in age from 4 weeks to 18 months. The animals were fed either D & G pellets or a semisynthetic casein diet for at least 1 week before the experiment. The results presented are expressed in terms of approximate numbers of colonies of various bacterial types recovered per gram of organ.

While the results differed markedly depending upon the composition of the diet, they were essentially unaffected by the sex or age of the animal. In all cases, the lactobacilli and anaerobic streptococci (probably of Group N) were extremely numerous in the stomach and the large intestine, and also present, but in smaller numbers, in the small intestine. Organisms of the bacteroides group (at least two different colonial types) constituted the most numerous population in the large intestine, but were hardly detectable in the other parts of the gastrointestinal tract. As to the ordinary enterobacteria and enterococci,

TABLE II
Effect of Diet on Digestive Flora of NCS Mice

	Pellets			Casein diet		
	Stomach	Small intestine	Large intestine	Stomach	Small intestine	Large intestine
Lactobacilli (total)	10 ^{9*}	10 ⁷	10 ⁸	10 ⁷	10 ⁴	10 ⁷
Lactobacilli (rhizoid)	10 ⁸	10 ⁶	10 ⁸	0	0	0
Streptococci N	10 ⁸	10 ⁶	10 ⁸	10 ⁷	?	10 ⁶
Bacteroides	0	0	10 ⁹	0	0	10 ¹⁰
Enterococci	0	0	10 ³	0	0	10 ⁴
Enterobacteria	0	0	10 ³	0	0	10 ³

* The figures represent the numbers of colonies obtained per gram of whole organ.

they were found only in the large intestine, and even there they were always much less numerous (often a millionfold less) than the other bacterial groups mentioned above.

These findings are of course in agreement with those reported in the preceding paper for young animals. The additional fact of importance to be emphasized here is that the bacterial flora remained essentially the same throughout the life span of the animals, as long as these were maintained under favorable physiological conditions. Indeed, the numbers of enterobacteria and enterococci tended to fall progressively, whereas the populations of lactobacilli, anaerobic streptococci, and bacteroides remained at the high levels indicated in Table II.

It should be mentioned here that treatment with penicillin or tetracyclines brought about a rapid and often lasting disappearance of these three dominant bacterial groups, but further description of this effect and its consequences must be postponed for later publications.

We have reported earlier that the composition of the diet affects profoundly

the fecal population of lactobacilli, both quantitatively and qualitatively (4). The results presented in Table II indicate that the diet also affects the lactobacillus population in the stomach and the intestine. In both these organs, the casein diet brought about a sharp decrease in the numbers of lactobacilli, and complete disappearance of the rhizoid type of this species.

Association of the Bacterial Flora with the Gastrointestinal Organs.—While studying the bacterial flora of the gastrointestinal tract, it became apparent that a large percentage of the microorganisms could not be dislodged from the walls of the organs even by repeated washings. This phenomenon was particularly striking in the case of the stomach, as shown by the following experiment.

The stomachs of NCS mice were opened, and samples of the lumen contents were removed. The stomach was then cut up in small fragments which were washed with vigorous agitation in 10 ml of Hanks solution containing 0.1 per cent bovine serum albumin. The fluid was decanted and the stomach fragments subjected to two further washings. A sample was taken of the fluid after the third washing. The stomach fragments were finally homogenized in a teflon grinder as usual. The stomach contents, the third washing, and the homogenate of washed tissue were then plated in appropriate dilutions of various culture media (Table III), Fig. 1.

Similar experiments were carried out with the stomach of 6 white rats and of 4 young swine (Table III).

TABLE III
The Lactobacillus Flora of Three Animal Species

	NCS mice	White rats	Swine*
Stomach contents.....	$10^8 \ddagger$	10^7	+
Third washing.....	10^7	10^5	—
Washed stomach homogenate.....	10^8	10^7	+

* Because of the large variability of results among the different swine tested, quantitative enumeration of bacterial colonies was not possible. (See text page).

‡ The figures indicate the number of colonies calculated per gram of original tissue.

The results presented in Table III reveal that lactobacilli are present in large numbers in the stomach wall of mice and rats and cannot be dislodged from this tissue even by repeated washings. The results with swine were less clear cut as only 1 of the animals yielded large numbers of lactobacillus colonies and another 1 yielded none. It may be relevant to these findings that the four stomachs were from swine which had been experimentally infected with the virus of hog influenza and that the animal which did not yield lactobacilli was in an advanced state of disease at the time of sacrifice. Worth noting also is the fact that its stomach contents harbored large numbers of various microorganisms other than lactobacilli.

A few preliminary observations have been made in an attempt to determine more precisely the mode of association of the lactobacilli with the stomach wall. Sections prepared and stained by ordinary techniques do not reveal any obvious sign of the organisms. However, frozen sections of the stomach of NCS mice show in the mucous layer a thick palisade of Gram-positive rods tightly packed and having a size and shape compatible with that of lactobacilli, Fig. 2. Streptococci, in smaller numbers, can also be detected by this technique. On the basis of these observations, it seems worth postulating that the bacteria are embedded in the mucous layer which lines the stomach, and are progressively shed into the lumen, thus eventually reaching the fecal material. Similar, but less extensive, observations have been made with regard to the association of lactobacilli, anaerobic streptococci (Group N), and bacteroides with the walls of the intestine. However, it seems best to illustrate these findings by presenting the results of experiments carried out in germfree animals re-associated with various bacterial species. These experiments are described in the following paper (6).

DISCUSSION

Animals and human beings have evolved in intimate and constant association with a complex microflora and microfauna. Under natural conditions, the development and functions of their tissues are influenced by countless microorganisms which are always present in the digestive and respiratory tracts, and probably also in other organs. It is to be expected, therefore, that anatomical structures and physiological needs have been determined in part by the microbiota which prevailed during evolutionary development, and that many manifestations of the body at any given time are influenced by the microbiota now present. It can be taken for granted, in other words, that the microbiota is part of the environment to which animals and man have had to become adapted, and on which they have come to depend.

Unfortunately, studies of the bacterial components of the indigenous microbiota which inhabits the gastrointestinal tract have been concerned chiefly with the common Gram-negative enterobacilli, enterococci, Gram-positive aerobic spore formers, and to some extent clostridia. The emphasis placed on these organisms is explainable in large part, if not completely, by the fact that they lend themselves to *in vitro* studies, because they grow readily on artificial culture media. Yet it is certain that they represent but a very small part of the total indigenous microbiota, and not the most important.

Numerous experiments with various colonies of mice, and more limited observations with other mammalian species, leave no doubt that under normal conditions the predominant flora of the gastrointestinal tract consists of species which escape cultivation by ordinary bacteriological techniques. Our findings agree with those of other investigators who have reported large populations of

various strains of lactobacilli and bacteroides in fecal contents. References 6-8 present recent reviews of studies dealing with this problem. Furthermore, as shown in the present paper, anaerobic streptococci (probably of Group N) constitute another bacterial group which is consistently numerous in normal mice.

Interestingly enough, the species which are the most abundant in the gastrointestinal tract have exacting nutritional requirements and multiply best under anaerobic conditions. Some indeed are strictly anaerobic. It is of interest in this regard that certain streptococci require an anaerobic environment for primary isolation from the tissues, although they rapidly become more tolerant of ordinary atmosphere when subcultured *in vitro*.

The difficulty experienced in isolating from the tissues some of the most important components of the gastrointestinal flora accounts of course for the paucity of knowledge concerning its composition and activities. In fact, there is much reason to believe that certain important indigenous species have not yet been recognized. Evidence for this is found in the failure to cultivate by the *in vitro* culture methods presently available some of the morphological types which are revealed by direct staining of the content of the intestinal tract. Furthermore, as will be pointed out in the following paper, the introduction into germfree mice of known bacterial cultures isolated from normal mice fails to restore to the former animals some of the biological properties associated with life under usual non-germfree conditions.

The availability of the new culture media and of the convenient incubation techniques described in the preceding paper has enabled us to establish that the stomach and the small intestine normally harbor a large bacterial population. Even more important, probably, is the fact that each part of the gastrointestinal tract seems to have a flora peculiarly its own. In NCS mice, for example, lactobacilli and Group N streptococci are especially numerous in the stomach (although they are found also in other organs); flavobacteria multiply most abundantly in the small intestine early in life; and bacteroides colonize the large intestine when the animals are 12 to 16 days of age and from then on remain localized in this organ. There is strong evidence, furthermore, that several bacterial species are extremely abundant in the walls of the organs which they inhabit and indeed that they multiply selectively in the mucous layer, rather than in the lumen. This anatomical distribution suggests that the bacteria are intimately associated with, rather than mere contaminants of, the respective organs.

By following the dynamics of the bacterial population from the time of birth throughout the life span of the animals, it has been possible to recognize two types of behavior among the components of the gastrointestinal flora. The populations of some species increase rapidly, reach extremely high levels, but then fall sharply within a few days after reaching their maximum, and may

even disappear almost completely. By and large, this type of population dynamics applies to flavobacteria, enterococci, *Escherichia coli*, the coliform bacilli (SLF) and other enterobacteria, and probably the clostridia. In contrast, the populations of lactobacilli, anaerobic streptococci, and bacteroides persist throughout life at an approximately constant level, reached before weaning. Whatever the precise mechanisms involved in determining these differences in behavior, it is obvious that the bacteria of the latter group do not call forth an effective protective response from the animal, whereas those of the former group are promptly brought under control after their population has reached a high level.

On the basis of these facts, and of others which cannot be discussed here, we would like to suggest that the indigenous flora is made up of microorganisms exhibiting two very different types of relationships with their host. According to our hypothesis, some types of microorganisms have achieved a state of symbiosis with their host, probably through a long period of evolutionary adaptation. Because they persist at a constant high level throughout the life span, it seems appropriate to refer to them as constituting the autochthonous biota. Lactobacilli are typical of this group. Other microorganisms possess some degree of infectivity and for this reason can become established in the tissues, but they elicit a protective response and therefore are either eliminated or kept at a low population level under normal circumstances. *Escherichia coli* illustrates this type of relationship.

The expression "indigenous microbiota," as commonly used, thus includes both symbiotic and potentially pathogenic organisms. It would seem useful to reserve the expression "normal microbiota" for those microorganisms which are so ubiquitous in a given community that they become established in practically all its members. In summary, the indigenous microbiota of each animal species is made up of microorganisms which have been present during its evolution (the autochthonous microbiota), of those which are ubiquitous in the community (the normal microbiota), and of true pathogens which have been accidentally acquired and are capable of persisting in the tissues.

Although the facts and views discussed above are derived from the study of mice, they are probably applicable to human beings as well. Many studies carried out during the past 50 years since Tissier's original observations have confirmed that lactobacilli of several species become established immediately after birth; from then on they constitute one of the dominant microbial groups in the intestine and the mouth, not only in the infant, but also in children and in adults (11, 12). Furthermore, it is certain that anaerobic bacilli of the bacteroides group are normally extremely numerous in the large intestine. In contrast, the numbers of *E. coli* and other enterobacteria, as well as of enterococci, are extremely erratic although the fecal flora of most persons appear to exhibit a fairly characteristic pattern (unpublished observations).

Many facts, not to be described here, have revealed that the indigenous microbiota can influence the morphological and physiological characteristics of its host to such an extent that traits assumed to be the unavoidable consequences of the genetic endowment are determined in reality by the microbial environment. Unfortunately, little is known of the comparative biochemical activities of the different bacterial species present in the gastrointestinal tract, but a few facts may serve as guide posts for the development of working hypotheses in this field. The species which are the most numerous under normal conditions, such as the lactobacilli, anaerobic streptococci, and bacteroides, have a much narrower range of biochemical activities than the coliform bacilli, enterococci, and clostridia which multiply abundantly as a result of various types of disturbances. It is likely, therefore, that the chemical transformation of metabolites in the intestine is less pronounced when the autochthonous organisms predominate than when other species gain the upper hand.

There is good reason to believe that indigenous bacteria can affect, favorably and unfavorably, the nutritional state of their host, and also its resistance to infection. We shall see later that the flora of the gastrointestinal tract can exert dramatic morphogenetic effects which are essential for normal histological development and function (6). Furthermore, this flora can attack many substances such as amino acids and bile acids, and the products of this attack differ according to the type of microbial species which predominate.

The composition of the microbial flora, which depends upon environmental and internal factors, consequently determines the kind of biologically active substances which are released from the digestive tract into the general circulation. Thus, interference with any one of these components is likely to disturb the equilibrium between them and to affect thereby the functions of the system as a whole. In other words, the different parts of the digestive tract, the microorganisms that it harbors, and the conditions which govern the interplay between these various components constitute a highly integrated ecosystem.

SUMMARY

The bacterial flora of the gastrointestinal tract differs qualitatively and quantitatively from one colony of mice to another. Certain components of this flora, however, are always present in large and approximately constant numbers in healthy adult mice, irrespective of the colony from which the animals are derived.

Lactobacilli and anaerobic streptococci are extremely numerous in the stomach, the small intestine, and the large intestine. In contrast, organisms of the bacteroides group proliferate only in the large intestine. These three bacterial species persist at approximately constant levels in their characteristic localization throughout the life span of healthy animals. They are closely associated with the walls of the digestive organs, and are probably concentrated in the mucous layer.

A few experiments carried out with rats and young swine indicate that lactobacilli are also present in large numbers in the stomach of these animal species.

It is suggested that some of the components of the gastrointestinal flora have become symbiotic with their hosts in the course of evolutionary development and thus constitute a true autochthonous flora. The other components of the indigenous flora are acquired early in life either through accidental contact or because they are ubiquitous in the environment. The "normal" flora is that which is always present in the environment of the animal colony under consideration.

BIBLIOGRAPHY

1. Dubos, R., and Schaedler, R. W., The effect of the intestinal flora on the growth rate of mice, and on their susceptibility to experimental infections, *J. Exp. Med.*, 1960, **111**, 407.
2. Schaedler, R. W., and Dubos, R., The fecal flora of various strains of mice. Its bearing on their susceptibility to endotoxin, *J. Exp. Med.*, 1962, **115**, 1149.
3. Dubos, R., Schaedler, R. W., and Costello, R., Composition, alteration, and effects of the intestinal flora, *Fed. Proc.*, 1963, **22**, 1322.
4. Dubos, R., and Schaedler, R. W., The effect of diet on the fecal bacterial flora of mice and on their resistance to infection, *J. Exp. Med.*, 1962, **115**, 1161.
5. Schaedler, R. W., Dubos, R., and Costello, R., The development of the bacterial flora in the gastrointestinal tract of mice, *J. Exp. Med.*, 1965, **122**, 59.
6. Schaedler, R. W., Dubos, R., and Costello, R., Association of germfree mice with bacterial isolated from normal mice, *J. Exp. Med.*, 1965, **122**, 77.
7. Haenel, H., Aspekte der mikroökologischen Beziehungen des Makroorganismus, *Zbl. Bakt. I Ref.*, 1960, **176**, 1.
8. Smith, H. W., and Crabb, W. E., The faecal bacterial flora of animals and man. Its development in the young, *J. Path. and Bact.*, 1961, **82**, 53.
9. Rosebury, T., *Microorganisms Indigenous to Man*, New York, McGraw Hill, 1962, 435.
10. Petuely, F., and Lindner, G., Ernährung und Darmflora, *Deut. Med. J.*, 1962, **13**, 116.
11. Tissier, H., Repartition des microbes dans l'infection intest. chez le nourrisson, *Ann. Inst. Pasteur*, 1905, **29**, 109.
12. Gyllenberg, H., and Roine, P., The value of colony counts in evaluating the abundance of "Lactobacillus" bifidus in infant faeces, *Acta Path. et Microbiol. Scand.*, 1957, **41**, 144.

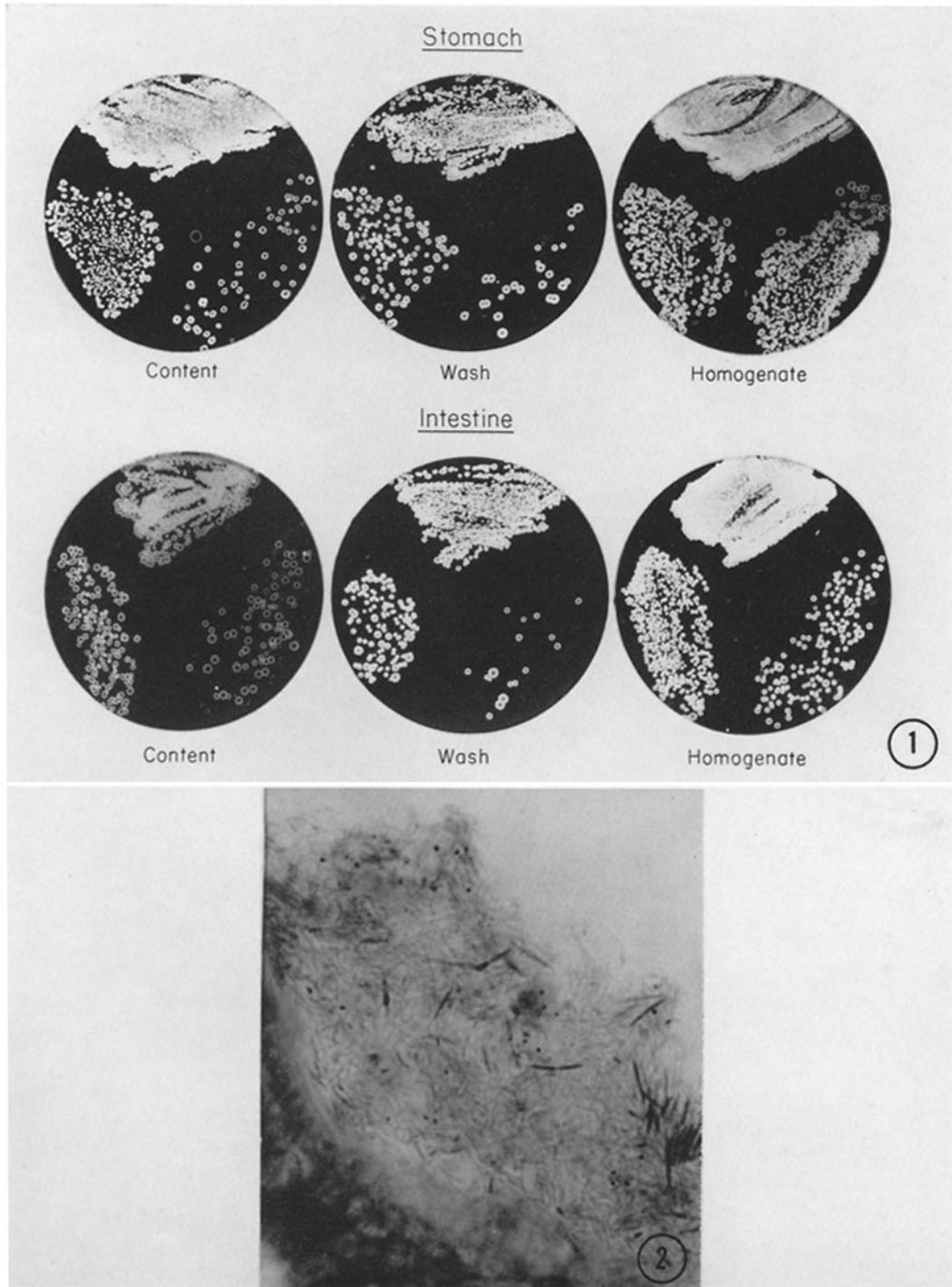
EXPLANATION OF PLATE 10

FIG. 1. Colonies of lactobacilli recovered from the stomach and large intestine of mice. Each photograph shows the surface of an agar plate containing a selective medium for lactobacillus. In all cases, the three sections of the plate were inoculated with corresponding dilutions of material obtained from the stomach or the large intestine. "Content" refers to the content of the stomach or of the large intestine. "Wash" refers to the third washing. "Homogenate" refers to the washed organ homogenized in a teflon grinder.

The upper section of each plate shows the numbers of colonies obtained from one loopful of a suspension of the organ in 5 ml of diluent. The left and right sections show the numbers of colonies from one loopful of the same materials diluted 1:10, and 1:100 respectively.

It will be noted that the numbers of colonies recovered from the homogenate of the washed organ were as large as, or larger than, that recovered from the contents. $\times 0.44$.

FIG. 2. Photomicrograph of a frozen section of the stomach of a normal NCS mouse stained by Weigert-Gram technique. The edge of the stomach mucosa runs from the lower right corner to the upper left corner. Numerous rod shaped bacteria having the appearance of lactobacilli, as well as cocci, can be seen in close association with the mucosa. $\times 1200$.



(Dubos *et al.*: Indigenous, normal, and autochthonous flora)