ISOLATION OF AN OBLIGATELY ANAEROBIC BACILLUS FROM THE FECES OF NEWBORN INFANTS AND FROM OTHER HUMAN SOURCES, AND ITS PROBABLE IDENTITY WITH THE "KÖPFCHENBAKTERIEN" OF ESCHERICH, RODELLA'S "BACILLUS III," AND BACILLUS PARAPUTRIFICUS (BIENSTOCK)

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Received for publication January 22, 1934

During a preceding study of the intestinal flora of the first ten days in newborn infants, Hall and O'Toole (1934) isolated from six babies out of ten, 18 strains of obligately anaerobic bacilli with oval terminal spores which were tentatively identified as the "*Köpfchenbakterien*" seen by Escherich (1885) (1886) in the feces of newborn infants. A more detailed study of six of these, one from each baby, together with fourteen additional strains which we have isolated from other human materials, showed them to belong to a single species which we regard as the *Bacillus paraputrificus* of Bienstock. Although the probable identity of this species with the "*Köpfchenbakterien*," "*Köpfchensporen*," and "*Köpfchenbacillen*" of the older German writers was clearly established by Bienstock in 1906, modern French, English, and American bacteriologists have generally omitted any accurate description of this bacillus, while modern German writers and a few others have continued to use the term "*Köpfchenbakterien*" with reference either to a species or group of species of anaerobic bacilli with oval terminal spores found in the feces of infants.

[1] This investigation was supported in part by grants from the Commonwealth Fund of New York and the Child Research Council of Denver.

We are indebted to Miss Elisabeth O'Toole for her excellent flagellar stains and to Mr. Glen Mills for the photomicrographs.

HISTORICAL

Bienstock (1884) was probably the first to discover a bacillus with round or oval terminal spores in cultures from feces. These cultures were aerobic and Bienstock referred to the organisms in them as "*Köpfchensporen,*" "*Trommelschlägerform bildenen Bazillus,*" and "*Bazillus der Eiweissfäulniss.*" Bienstock is sometimes cited as having used the specific name *Bacillus putrificus-coli* in 1884 but our examination of his original paper failed to verify this.

Escherich (1885, 1886) described "*Köpfchenbakterien*" in films of feces and in mixed anaerobic cultures as slender rods, 4 to 7μ long, with bright glistening terminal spores which made them resemble spermatozoa, and clearly recognized their probable identity with Bienstock's "*Eiweissfäulniss bacillus.*" Subsequent studies have shown that the "*Köpfchenbakterien*" are obligately anaerobic, but, in spite of the fact that Escherich secured anaerobic cultures under vacuum from the feces of newborn infants, there is nothing to indicate that he ever isolated the "*Köpfchenbakterien*" in pure culture.

For this reason, it is impossible with certainty to identify with the "*Köpfchenbakterien*" any of the microörganisms isolated from infant stools either by ourselves or others, but the high frequency of a single species with the morphology of the "*Köpfchenbakterien*" in the feces of newborn infants is strong presumptive evidence of identity.

Owing to the important significance of the early recognition by both Bienstock and Escherich of bacilli with oval or nearly round spores in the feces, and the absence of their monographs from all but a few libraries in the United States, photographic copies of their illustrations are here reproduced as figures 1 and 2.

Curiously there is no reference to Escherich's "*Köpfchenbakterien*" in the studies on intestinal flora of nursing infants by Gessner (1889), Popoff (1891), Schmidt (1892), Schild (1895), Eberle (1896), Szego (1897), Moro (1900), and Cahn (1901) during a decade and a half following Escherich's first observations. It is equally impossible to identify with the "*Köpfchenbakterien*" any of the oval or round terminally-sporulating anae-

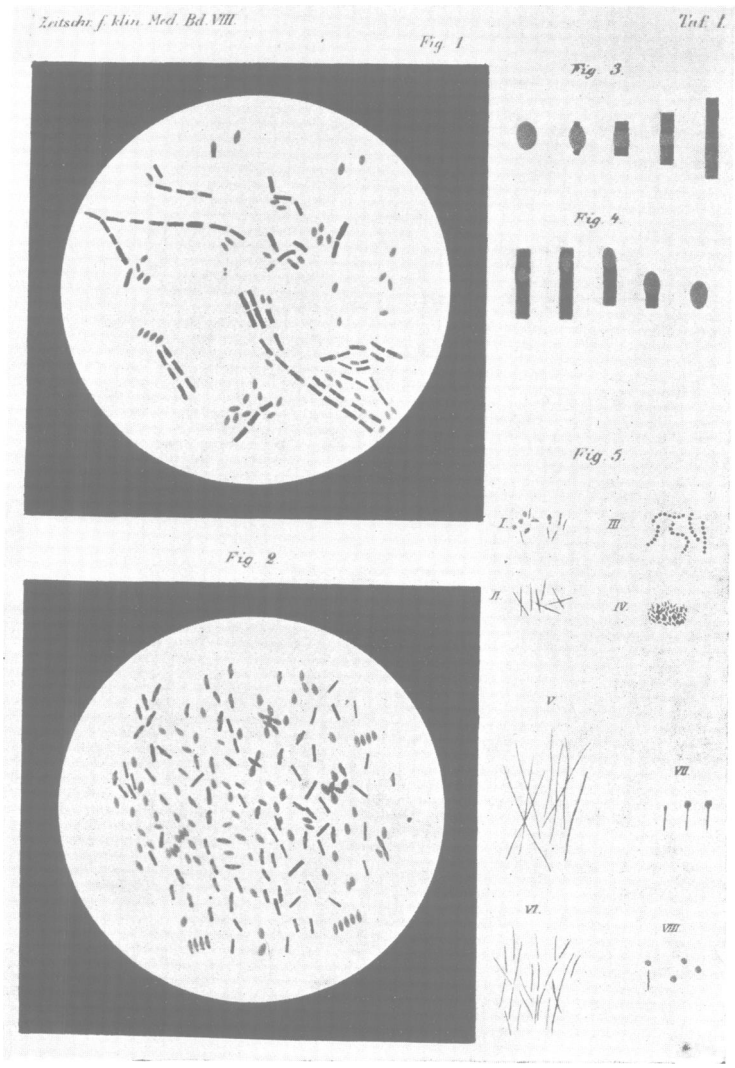

FIG. 1. PHOTOGRAPHIC REPRODUCTION OF BIENSTOCK'S PLATE I, SHOWING VARIOUS BACTERIA SEEN IN FECAL CULTURES

These were apparently drawn in colors diagrammatically, and probably on different scales, from slides stained with warm aniline fuchsin, decolorized in $33\frac{1}{3}$ per cent nitric acid and counter-stained with methylene blue according to the methods of Neisser and Ehrlich. Figure 7 represents the supposed "Bazillus der Eiweissfaulniss," whose actual identity is now only conjectural.

robic bacilli described by Liborius (1886), Gruber (1887), Lüderitz (1889), Sanfelice (1893), or Tavel (1898) owing to their

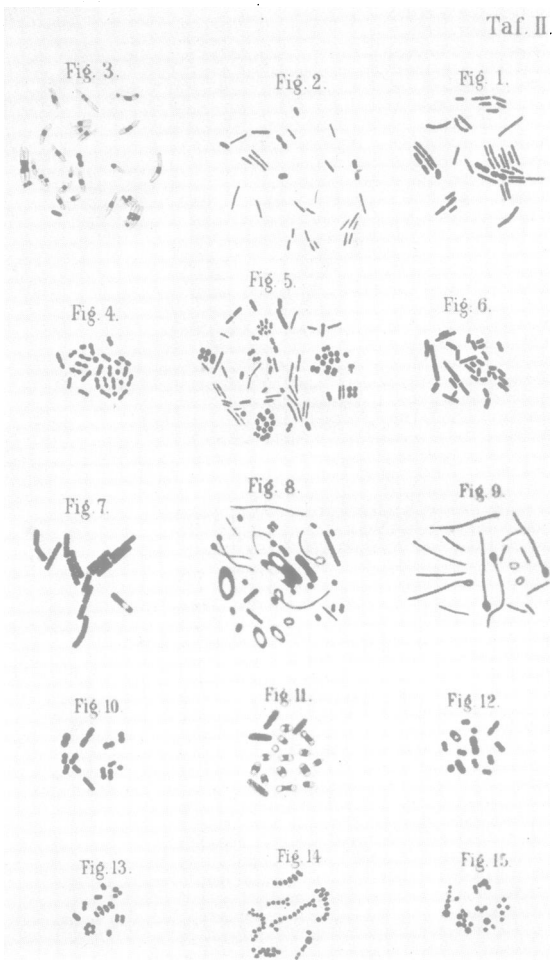

FIG. 2. PHOTOGRAPHIC REPRODUCTION OF ESCHERICH'S PLATE II, SHOWING VARIOUS BACTERIA SEEN IN MECONIUM AND IN FECAL CULTURES

The "Köpfchenbakterien" ("Bienstock's Eiweissbazillus") are shown in figures 8 and 9.

inadequate description for modern identification. On the other hand "*Köpfchenbakterien*" have been isolated since 1898 by various investigators.

In 1899, Klein recovered obligately anaerobic non-pathogenic "*Trommelschlägerformen Köpfchenbacillen*" from buried bodies which he sharply distinguished from Bienstock's aerobic "*Bacillus putrificus coli*" under the name "*Bacillus cadaveris sporogenes.*"

In the same year, Bienstock (1899) attempted unsuccessfully again to recover from feces a proteolytic aerobe similar to his original "*fäulnisserregender Bazillus.*" Failing this, he succeeded in isolating from street manure obligately anaerobic "*Köpfchensporen*" which he regarded as identical with the "*Eiweissfäulniss Bazillus,*" taking the view that his earlier culture was a contaminated symbiotic mixture of this organism with aerobic forms. In the following year (1900) Bienstock admitted that his original fibrin medium in 1884 was insufficiently sterilized, that *B. putrificus* rarely if ever occurred in feces, and that it was probably identical with Klein's *Bacillus cadaveris sporogenes.* This viewpoint was accepted by von Hibler (1908).

In these papers, Bienstock emphasized the peculiar morphology of *B. putrificus*, its liquefying action upon proteins, the failure of various carbohydrates to protect the proteins against decomposition by pure cultures, and the inhibitive action of *B. coli* upon proteolytic changes in milk when grown with *B. putrificus.*

Subsequently, Achalme (1902) recorded the fermentation of several carbohydrates by cultures of "*B. putrificus*" secured from the Pasteur Institute in Paris, but Tissier and Martelly (1902), and later Rodella (1905), pointed out that the Paris cultures were contaminated by *Bacillus bifermentans sporogenes* and that pure cultures of *B. putrificus* did not ferment the sugars. Their failure to do so was re-iterated by Bienstock in 1906.

This point is important because it lays the basis for the later identification of *B. putrificus* by Reddish and Rettger (1922) (1923) (1924), Hall (1922), Kahn (1922), Cunningham (1932), and others, and our recognition of the "*Köpfchenbakterien*" as belonging to a single separate species which ferments several sugars and has neither gelatinolytic nor proteolytic action. For the same reason we are unable to accept Sittler's (1909) and Cruickshank's (1931) identification of "*Köpfchenbakterien*" with *Bacillus putrificus*, or to understand the inclusion in the species

B putrificus, of various non-pathogenic anaerobes with oval terminal spores, by Weinburg, Aznar, and Duthie (1924) irrespective of fermentation reactions. Niszle's (1929) recent summary clearly shows the generally confused state of present knowledge concerning these microörganisms.

The first confirmation of "*Köpfchenbakterien*" in the feces of newborn infants after Escherich appears to have been made by Tissier (1900), who, however, made the common mistake of regarding them as "attenuated" strains of *B. putrificus* until Rodella (1902) pointed out their lack of proteolytic properties, a criticism which Tissier graciously acknowledged in 1905 and again in 1908. Rodella's work with infants' stools began in 1901 in a study of the acidophilic bacteria which had just been so carefully described by Tissier (1900) and Moro (1900). In the following year Rodella (1902) described three numbered but unnamed species of obligately anaerobic gas-forming bacilli from the normal stools of nursing infants. Rodella regarded No. III as identical with Escherich's "*Köpfchenbakterien*," and described it in pure culture as forming slender Gram-positive rods with round terminal spores, not liquefying gelatin, or changing milk, producing abundant gas in broth, and being non-pathogenic for laboratory animals.

Rodella's Bacillus III was again found by Tissier (1905) in the lower bowels of newborn infants during the early stage of intestinal invasion by bacteria, in the stomachs and both upper and lower bowels of puppies, and later (1908) in the feces of infants one to five years old.

Rodella's observations on Bacillus III and its identity with Escherich's "*Köpfchenbakterien*" were also confirmed by Moro (1905). This writer recorded the occasional coagulation of milk and the motility of the vegetative rods which Rodella had failed to see. Moro erred, however, in regarding the "*Köpfchenbakterien*" as sporulating forms of *Bacillus* (*Lactobacillus*) *bifidus*.

Passini (1905) likewise studied several strains of anaerobic bacilli among which were some identified as Escherich's "*Köpfchen-Putrificuskeim.*" Bienstock (1906) regarded these as corresponding to his "*Bacillus paraputrificus*" which, unlike his *B.*

putrificus, often coagulated milk, forming an acid whey without subsequent liquefaction of the casein, and fermented both glucose and lactose. It seems entirely probable that Bienstock's *Bacillus paraputrificus* was identical both with the *Köpfchenbakterien* of Escherich and with Rodella's Bacillus No. III. Although the name *Bacillus paraputrificus* has apparently not since been connected in the literature with either of these microörganisms, we are quite convinced of its validity and shall henceforth use it for the species under discussion.

In 1908 von Hibler described as Bazillus IX, a non-pathogenic obligately anaerobic rod isolated ten years before from the emphysematous tissues of a boy having a compound fracture of the humerus. Von Hibler considered this culture similar to *Bacillus solidus* of Lüderitz, but its properties clearly identify it with Rodella's Bacillus III, as suggested by Robertson (1916).

Fleming (1915) and Goadby (1917) had also observed terminally spored bacilli in war wound infections, and Robertson regarded these and certain morphologically similar cultures, which she isolated, as also identical with Rodella's Bacillus III and von Hibler's Bacillus IX. These cultures were subsequently named *Bacillus tertius* by Henry (1917), thus starting a new train of confusion which has been perpetuated by Weinberg and Sequin (1917), McIntosh (1917), Bullock (1919), Douglas, Fleming and Colebrook (1920) and Robertson (1929). For, in spite of their superficial resemblance, *Bacillus paraputrificus* (Bienstock) is clearly distinct from *Bacillus tertius* (Henry), as the following comparison shows.

	Bacillus paraputrificus	*Bacillus tertius*
Aerobic growth	−	+*
Morphology of spores	Oval	Elongate
Fermentation of xylose	−	+
Fermentation of mannitol	−	+

* See Hall and Matsumura (1924).

In short, von Hibler's Bazillus IX was probably identical with Bienstock's *Bacillus paraputrificus* and Rodella's Bacillus III, but *Bacillus tertius* is a distinct species. This conclusion suggests

that the English observations cannot be accepted as evidence of the occurrence of Rodella's Bacillus III or von Hibler's Bacillus IX in wound infections; on the other hand, the only valid evidence we have of its occurrence in wound infections rests upon von Hibler's limited observations and our own.

We believe that the only significance attached to the occurrence of *B. paraputrificus* in wound infections is the implication which it carries of fecal contamination. On the other hand, while *B. paraputrificus* frequently attains a moderate development in the intestinal tracts of newborn infants during the first week of life, the acidity of the normal meconium (pH 6.0 to 6.6) is by no means optimal; Adam (1921, 1922) has shown that the "*Köpfchenbakterien*" grow best in alkaline media (pH 6.9 to 8.2), and they disappear more or less completely upon the establishment of the normal milk stool because of the higher acidities which definitely favor *Lactobacillus bifidus* (optimum pH 5.0 to 5.8). However, *B. paraputrificus* may frequently be recovered from older children and from adults.

In view of the preceding studies in Germany, it is remarkable that Zeissler and Käckell (1922), in a study of 32 specimens of feces from 14 healthy and 11 ill babies, failed either to discuss or to isolate the "*Köpfchenbakterien*," and still more so that Schüssler (1924) in a special study recovered them only 4 times from 112 specimens of meconium from 48 newborn infants, and that he interpreted them as "degenerationsformen des *Bacillus amylobacter*." In this country Upton (1929) claims to have seen the "*Köpfchenbacillus*" only once during a study of the anaerobic intestinal flora of 89 specimens of feces from newborn infants (one to six days), but there is no indication that she succeeded in isolating it in pure culture.

SOURCES OF BACILLUS PARAPUTRIFICUS

All of our strains of *Bacillus paraputrificus* were isolated in our own laboratories during the past ten years. The first was recovered in 1924 from the feces of a normal child ten years old, and others were isolated at irregular intervals from materials secured from human autopsies and from various infections, but

it was not until we began our systematic study of the feces of newborn infants in 1930 that we connected these organisms with Escherich's "*Köpfchenbakterien.*" The twenty strains studied in detail were derived as follows:

	Number of strains
Feces of newborn infants	7
Feces of ten-year-old child	1
Fecally contaminated surgical wound (colostomy)	1
Decubitus ulcer on buttock	1
Gaseous gangrene (amputation of leg)	1
Postmortem heart blood cultures	5
Postmortem peritoneal fluid cultures	3
Postmortem pleural fluid cultures	1
Total	20

Analysis of our data shows that in all cases other intestinal bacteria were associated, as follows:

	Number of strains
Bacterium coli	14
Streptococcus species	9
Micrococcus species	6
Bacterium aerogenes	6
Bacillus Welchii	5
Bacillus tertius	3
*Bacillus difficilis**	2
Bacterium alkaligenes	2
Bacillus centrosporogenes	2
Lactobacillus species	2
Bacillus Sordellii	1
Bacillus botulinus	1
Bacterium proteus	1
Bacterium pyocyaneum	1
Bacterium flavescens	1

* *Bacillus difficilis* was recently described as a new species of pathogenic anaerobe by Hall and O'Toole (1934).

ISOLATION OF PURE CULTURES

It will readily be understood that isolation of pure cultures of *B. paraputrificus* was by no means an easy task, although less difficulty was encountered than in the case of *B. difficilis*, and when these two anaerobes occurred together in the feces of infants,

B. paraputrificus was always recovered first in pure culture. On the other hand, when other bacilli were associated they were generally isolated before *B. paraputrificus.*

In general, the methods used were those advocated by Hall (1932). Primary mixed cultures in deep brain medium were transferred to blood agar slants, made anaerobic by means of alkaline pyrogallol and incubated at 37°C. for forty-eight hours. These cultures were placed in a bath of boiling water for two minutes and the melted agar transferred by pipette to tubes of deep brain medium similarly heated to drive out dissolved oxygen. When anaerobic bacilli were present, incubation of these resulted in gas production. Some of these cultures proved to be pure *B. paraputrificus;* several contained other sporulating anaerobes. In any event, dilutions were streaked out upon blood agar slants which were then made anaerobic with alkaline pyrogallol and incubated, or dilutions were made in deep agar tubes; from either of these, well separated colonies were picked into deep brain medium. These procedures often had to be repeated or alternated several times before pure cultures were secured.

MORPHOLOGY

One occasionally sees in smears of feces of infants, or of other sources, sporulating rods resembling spermatozoa. These may or may not prove to be *Bacillus paraputrificus.* In cultures vegetative cells appeared as Gram-variable slender rods with rounded ends and measuring 2 to 6μ in length and 0.3 to 0.5μ in width. The rods were straight or slightly curved and occurred singly, in pairs, and sometimes in short chains.

Spores developed readily in brain medium and blood agar slants under alkaline pyrogallol, and were seen in all stages. Young spores (orgonts) stained solidly by Gram's method, but the mature oval spores were refractory to staining; they measured 1.2μ in length, 1.0μ in width. Figure 3 shows orgonts, spores, and vegetative rods as they appeared in a three-day-old blood agar slant.

MOTILITY

All of the strains were found to be motile in glucose broth and brain media, but the majority of the rods were non-motile. This observation is perhaps significant because Rodella failed to observe motility, but this is the only discrepancy between his observations and ours.

FIG. 3. B. PARAPUTRIFICUS (BIENSTOCK), FROM THREE-DAY-OLD BLOOD AGAR SLANT, STAINED BY GRAM'S METHOD TO SHOW VEGETATIVE RODS, ORGONTS, AND SPORES. × 1000

Flagella were stained by Bailey's method (1930); they were peritrichic and numbered 6 to 15, as shown in figure 4.

Sporulation generally resulted in loss of motility, but in one instance an orgont was stained with three flagella still attached, as shown in figure 5.

CULTURAL PROPERTIES

Deep 1 per cent agar. Colonies appeared in twenty-four to forty-eight hours as small irregular, opaque, dense, cottony

masses rarely exceeding 1 mm. in diameter. They are illustrated in figures 6 and 7. Even with moderate seeding, considerable gas was generally produced.

Blood agar slants under alkaline pyrogallol. All strains produced delicate, irregular, non-hemolytic, round-topped colonies resembling dewdrops in young culture; older colonies were flat-topped.

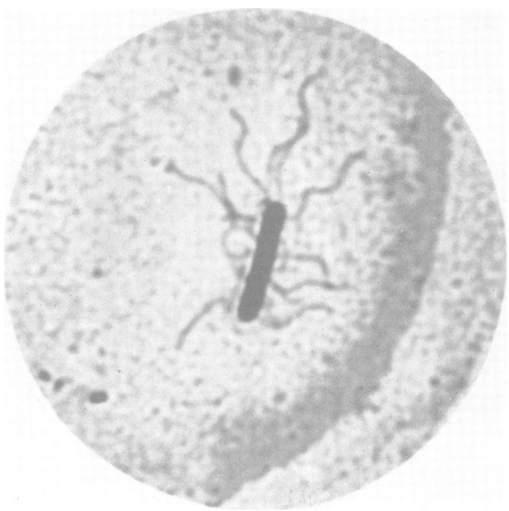

FIG. 4. BACILLUS PARAPUTRIFICUS (BIENSTOCK) FROM THREE-DAY-OLD BLOOD AGAR SLANT SHOWING PERITRICHAL FLAGELLA
Stained by Bailey's method. About 15 flagella can be counted. × 3300

Deep brain. Abundant gas was produced overnight. No blackening occurred on prolonged incubation.

Deep brain with iron wire. The results were the same as without iron during the first ten days. After that a moderate gray discoloration appeared at the top of the medium and along the upper part of the wire. Cultures capped with tin foil under cellophane showed after five months a brown deposit where the iron touched the glass, probably iron oxide; the surface of brain substance was covered with a gray deposit, but there was no blackening in the depths of this medium such as one sees in the case of

putrefactive anaerobes where abundant H_2S is produced. Special tests of brain medium with 0.1 per cent lead acetate failed to show any blackening whatever, but we suspect nevertheless from the appearance of old iron brain cultures that a small amount of H_2S is formed.

There was little or no liquefaction of the brain substance; the microörganism is essentially non-proteolytic.

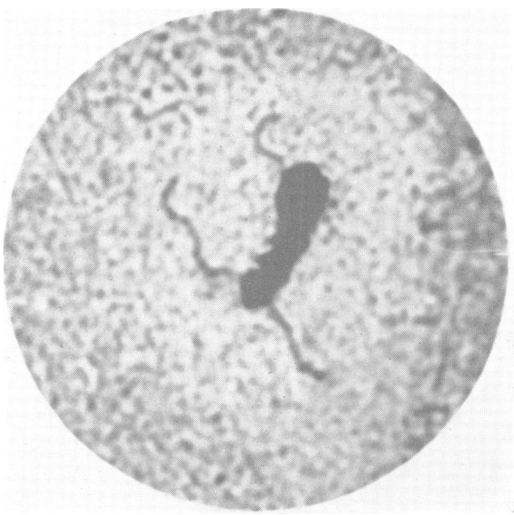

FIG. 5. BACILLUS PARAPUTRIFICUS (BIENSTOCK) WITH THREE FLAGELLA STILL ATTACHED TO A ROD UNDERGOING SPOROGENESIS
Stained by Bailey's method. × 3300

Löffler's coagulated blood serum. No liquefaction or discoloration occurred in thirty days.

Gelatin. Turbidity and a small amount of gas appeared under both vaspar and marble seals in constricted tubes in twenty-four hours. The turbidity disappeared in a few days, due to settling. No liquefaction occurred during twenty days observation.

Ten per cent powdered milk solution. Gas was produced both under marble seals in constricted tubes and under vaspar seals. One strain slowly coagulated the casein, but the remaining strains showed neither coagulation nor liquefaction in ten days.

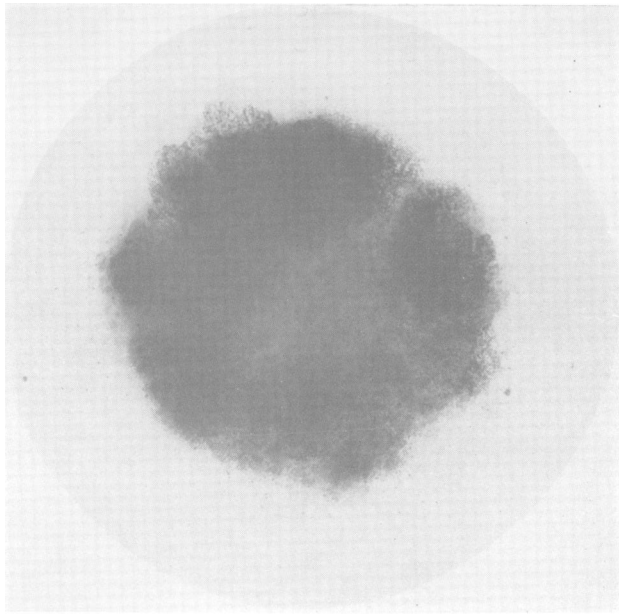

FIG. 6

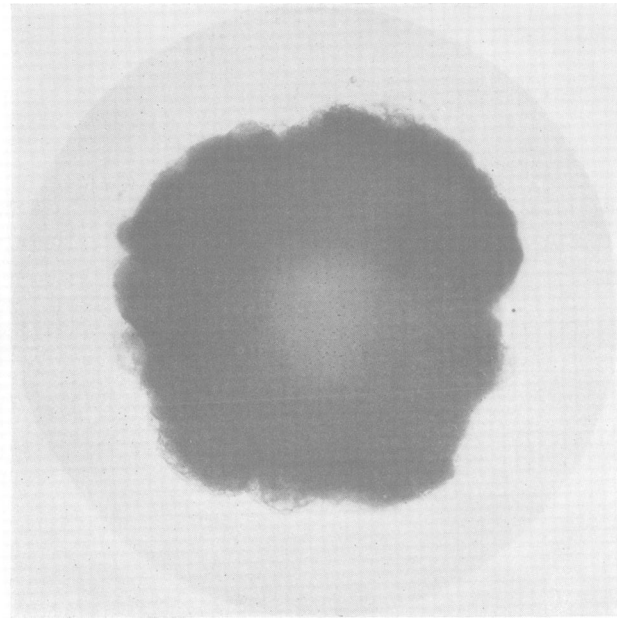

FIG. 7

FIGS. 6 AND 7. THREE-DAY-OLD COLONIES OF TWO DIFFERENT STRAINS OF BACILLUS PARAPUTRIFICUS IN DEEP 1 PER CENT AGAR (× 100), INDICATING THE EXTREMES OF VARIATION IN DENSITY AND TEXTURES OF SUCH COLONIES

Sterile cow's milk. Abundant gas production was followed by slow coagulation in all but two strains in six to ten days. There was no liquefaction of the coagulated casein.

Difco proteose-peptone broth for indol. Turbid forty-eight hour cultures in this medium under marble seals in constricted tubes were shaken with ether and tested for indol by adding Ehrlich's reagent (paradimethylaminobenzaldehyde) to the ether layer. This test gave negative results with all of the strains. Control tests with *Bacillus tetani* gave positive results.

Fermentation reactions. These reactions were tested by two methods, as follows:

(*a*) Hiss serum water media containing 1 per cent of the various carbohydrates were prepared by filling slender tubes (150 mm. by 10 mm.) half full. Litmus was used as an indicator. The tubes were boiled for a few minutes to expel air, cooled, and inoculated with a few drops of fresh glucose broth cultures of the various strains. Glucose, levulose, galactose, maltose, lactose, sucrose, raffinose, dextrin, soluble starch, amygdalin, and salicin were fermented, as was shown by coagulation of the serum and by reddening and reduction of the litmus. No liquefaction of the coagulated serum occurred. Xylose, inulin, mannitol, and glycerol were not fermented.

We have used this method successfully for several actively fermentative anaerobes other than *B. paraputrificus*, but it is not recommended for general use; it failed entirely with the putrefactive anaerobes.

(*b*) The tests were repeated in sugar-free broth in constricted tubes with marble seals to which, after boiling to expel oxygen, sufficient 10 per cent sterile carbohydrate solutions were added to yield 1 per cent concentrations. The same sugars were fermented as before, as indicated by abundant gas production, and acid reaction to brom-thymol-blue upon removal to a spot plate.

Pathogenicity. Each strain was tested in guinea pigs by subcutaneous injection of 2 cc. of forty-eight-hour glucose broth cultures. No symptoms or lesions were observed.

Several of the strains were likewise repeatedly injected intra-

venously into rabbits in the preparation of agglutinating serums, without any evidence of pathogenicity.

SUMMARY

We have recovered from the feces of seven newborn infants, from the feces of a ten-year-old child, from a fecally contaminated surgical (colostomy) wound, from a decubitus ulcer, from five postmortem heart blood cultures, from three postmortem peritoneal fluids, from one post-mortem pleural fluid, and from the amputated stump of a gangrenous leg, an obligately anaerobic bacillus which appears to be identical with the *Bacillus paraputrificus* of Bienstock.

A study of the literature indicates the identity of this organism with Rodella's Bazillus III, von Hibler's Bazillus IX, and Escherich's *"Köpfchenbakterien."*

The improper confusion of Escherich's "Köpfchenbakterien" with *Bacillus putrificus* (Bienstock), and of Rodella's Bacillus III and von Hibler's Bazillus IX with *Bacillus tertius* (Henry) has been traced in the literature, and the true distinctions between *Bacillus putrificus* (Bienstock), *B. paraputrificus* (Bienstock), and *Bacillus tertius* (Henry) have been pointed out.

Bacillus paraputrificus (Bienstock) is a motile, Gram-variable, slender, obligately anaerobic bacillus, with oval terminal spores, which forms small opaque cottony colonies in deep agar, delicate irregular non-hemolytic surface colonies on blood agar, abundant gas in brain and other liquid or semisolid media, without evidence of H_2S or indol production. It is non-proteolytic, non-gelatinolytic, and produces gas and often coagulum, but no liquefaction of casein in milk. It ferments glucose, levulose, galactose, maltose, lactose, sucrose, raffinose, dextrin, soluble starch, amygdalin, and salicin, with production of acid and gas; it fails to ferment xylose, inulin, mannitol, and glycerol, and is non-pathogenic for guinea pigs and rabbits.

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