A BIOCHEMICAL AND SEROLOGICAL STUDY OF A GROUP OF IDENTICAL UNIDENTIFIABLE GRAM-NEGATIVE BACILLI FROM HUMAN SOURCES

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In the course of a study of paracolon bacilli a number of cultures were submitted to this laboratory as possible paracolon organisms. Included in this series were 15 identical cultures of gram-negative bacilli that could not be identified. Representative strains were sent to Dr. Kenneth Wheeler and by him to Dr. C. A. Stuart. Dr. Wheeler reported the organisms to be an unidentifiable *Bacterium* species and Dr. Stuart confirmed this report, stating that similar gram-negative bacilli had been encountered in his laboratory, where they were known as "5W." Because of the not infrequent occurrence of these unidentifiable organisms in clinical material from human sources and because the apparent homogeneity of the group suggests the presence of a hitherto undescribed species, the biochemical and serological study to be reported here was undertaken.

The source of the cultures included in the group to be described is given in table I. Figures on their incidence are not available, but their common occurrence in clinical material is evidenced by the fact that in one month 11 strains, 8 of them from urine cultures, were submitted to us for identification by other laboratories of the Johns Hopkins Hospital.

Morphology. Morphologically these organisms are short, fat, coccoid, gram-negative bacilli, showing marked bipolar staining. In newly isolated strains the morphology is regular, and true bacillary forms are seldom seen. In gram-stained preparations the organisms strongly resemble *Neisseria* and morphological differentiation would be difficult if not impossible; in fact, one strain in our series, isolated from the cervix, was received from another laboratory to which it had been submitted as *Neisseria gonorrhoeae*, and several other strains were regarded as possible *Neisseria* species on first isolation. Hanging drop preparations, however, usually demonstrate the true bacillary form of the organisms.

Morphologically stock cultures vary from newly isolated strains in showing definite bacillary forms as well as the coccoid form described above. Rough forms of the organisms are quite pleomorphic, showing coccoid, bacillary, and irregular, curved, filamentous forms, whereas small-colony variants are made up entirely of long, irregular, twisted filaments.

These organisms naturally occur in the "M," encapsulated phase. The capsules are quite small but are easily demonstrated in India-ink preparations. Growth on media known to encourage capsule formation, such as agar plus ascitic fluid with or without milk and glucose, failed to increase the size of the capsule or alter the gross colony morphology. No motility has been demonstrated in repeated studies on each strain.

Growth characteristics. The gram-negative bacilli of this group are strict aerobes, growing well aerobically and at reduced oxygen tension, but failing completely to grow anaerobically. In broth culture they grow heavily with an even turbidity, slight smooth sediment, and a delicate pellicle. The pellicle increases on prolonged incubation, and in 24 to 48 hours a heavy ring is formed. No pigment is produced on solid or liquid media at room or incubator temperatures.

On blood or pancreatic digest agar (Brown, 1948) streak plates, well-isolated colonies are large (2 to 3 mm), white, opaque, raised, convex, smooth, and glistening, with an entire edge. In blood agar pour plates deep colonies are small and biconvex, and there is no hemolysis or discoloration of the blood around either

TABLE 1

Source of cultures

STRAIN NUMBER	SOURCE	CLINICAL IMPRESSION
8	Urine	Urinary tract infection
25	Urine	Urinary tract infection
77	Urine	History not available
80	Left pleural cavity—autopsy	Aspiration pneumonia, atelectasis
81	Urine	Urinary tract infection
83	Pleural and pericardial fluids—autopsy	Postoperative empyema
85	Urine	Urinary tract infection
86	Urine	Urinary tract infection
87	Cervix	Gonorrhea?
90	Urine	History not available
93	Urine	Urinary tract infection
94	Urine	History not available
95	Urine	Urinary tract infection
97	Lung—autopsy	Capillary bronchitis, terminal bronchopneumonia
113	Heart blood—autopsy	Capillary bronchitis

deep or surface colonies. On desoxycholate agar the organisms present a distinctive appearance. In general, the colonies are similar to those described above, but they are unique in that small colonies are colorless or yellowish, whereas larger, well-isolated colonies usually show a definite pink color or pink center in spite of the fact that 1 per cent lactose broth is not fermented. Prolonged incubation of blood agar or desoxycholate agar plates frequently results in the production of small daughter colonies. Transfers from these secondary colonies yield small-colony variants, which will be described later.

Because of the striking morphological resemblance of these organisms to *Neisseria*, they were also studied on chocolate agar plates. Well-isolated colonies on this medium were large and opaque, resembling those on other media. The oxydase test was completely negative on all strains.

Biochemical study. A study of the cultural characteristics of these gram-

negative bacilli has shown them to be biochemically identical. In Durham tubes containing 1 per cent carbohydrates in extract broth, only glucose, galactose, and arabinose were found to be fermented. Fermentation consistently occurs in a characteristic fashion, a slight acid reaction appearing first at the surface of the broth in the outer tube and gradually extending to the bottom of the medium. No growth or fermentation occurs in the inner tube. Glucose is fermented in 24 hours, galactose and arabinose in 2 days. Lactose, sucrose, xylose, mannitol, salicin, maltose, inositol, trehalose, sorbitol, adonitol, raffinose, rhamnose, and dulcitol were not fermented after 1 month's incubation.

When the organisms of this group were first studied, we were investigating the use of 10 per cent lactose agar slants for the identification of paracolon bacilli as recommended by Chilton and Fulton (1946), and these strains were therefore studied on this medium. Although 1 per cent lactose broth or agar slants were consistently negative after prolonged incubation, 10 per cent lactose agar slants were found to be regularly fermented in 24 hours. This apparent discrepancy was later thoroughly investigated and found to be characteristic of these organisms. Ten per cent lactose agar slants show rapid fermentation regardless of the method used for the preparation of the medium, whether the lactose is autoclaved in the complete medium, autoclaved in aqueous solution and added aseptically to the agar, or whether a filtered lactose solution is used. The latter two methods were investigated to eliminate possible breakdown of the lactose during sterilization, and the medium containing 10 per cent lactose so prepared was readily fermented in 24 hours. Varying the pH of the medium from 7.0 to 7.7 also failed to influence the results. Ten per cent lactose broth tubes were fermented more slowly, requiring from 3 to 5 days' incubation. However, if freshly inoculated 10 per cent lactose broth cultures were poured into sterile petri dishes to increase the available oxygen, fermentation occurred in 24 hours as on the 10 per cent lactose agar slants.

A group of control cultures, consisting of a number of strains each of *Escherichia*, *Aerobacter*, *Eberthella*, *Shigella*, *Salmonella*, *Proteus*, *Pseudomonas*, *Pasteurella*, and *Alcaligenes*, were also studied on 10 per cent lactose agar slants. *Escherichia* and *Aerobacter* fermented the medium as would be expected, whereas all strains of *Eberthella*, *Shigella*, *Salmonella*, *Pseudomonas*, *Pasteurella*, and *Alcaligenes* were consistently negative. *Proteus vulgaris*, *Proteus mirabilis*, and *Proteus rettgeri* were also negative, but all strains of *Proteus morganii* gave an acid reaction after 48 hours' incubation on all 10 per cent lactose agar slants regardless of the method of preparation. Therefore, with the exception of a similar but delayed reaction by *Proteus morganii*, fermentation of 10 per cent lactose agar slants correlated with failure to ferment 1 per cent lactose broth or agar appears to be a unique characteristic of these unidentifiable gram-negative bacilli.

As for the other biochemical characteristics of these organisms, all strains gave the following results: indole negative, methyl red positive, Voges-Proskauer negative, citrate positive, nitrates negative, malonate positive, and hydrogen sulfide negative. Urease is produced on Christensen's urea agar (1946), a ++

reaction developing in 24 hours. Gelatin is liquefied slowly, requiring from 6 weeks' to 2 months' incubation. In milk plus bromcresol purple, acid is produced in 3 to 4 days and coagulation in 1 week. There is complete inhibition of growth on Difco SS agar slants inoculated with undiluted cultures. The failure to reduce nitrates is an outstanding characteristic of these organisms and was checked carefully. Cultures were incubated for periods varying from 24 hours to 5 days, and the negative tests were checked with zinc dust (Frobisher, 1944), which in every case showed unreduced nitrates to be present.

It might also be mentioned here that determination of penicillin sensitivity by the agar streak plate, penicillin disk technique of Bondi, Spaulding, Smith, and Dietz (1947) showed these organisms to be highly resistant to penicillin, since there was no inhibition of growth around the penicillin-saturated disk.

Variants. The various strains of these gram-negative bacilli have been carried in stock for a year since their original isolation. During this time variation was noted in several strains. An "S" form has been found that resembles the original "M" form morphologically with the exception of loss of capsules, and varies culturally only in delayed fermentation of glucose and galactose. An "R" form also occurs that shows morphological pleomorphism, no capsules, typical granular growth in broth with loss of pellicle and ring formation, and marked delay in the fermentation of carbohydrates, from 3 to 6 days being required to ferment glucose, arabinose, and galactose. The "R" form also utilizes malonate more slowly than typical strains and fails to produce urease or to liquefy gelatin.

As previously mentioned, prolonged incubation of agar plates results in the production of secondary colonies, isolations from which proved to be small-colony variants. These variants produce very small, streptococcuslike colonies on blood agar and desoxycholate agar plates, and in broth grow poorly with a slight turbidity and sediment and no ring or pellicle. Morphologically they are unencapsulated, long, irregular, extremely pleomorphic filaments with few bacillary forms present. These small-colony variants fail to ferment glucose, galactose, and arabinose, and 10 per cent lactose agar slants, and also vary from the typical organisms in being malonate-, urease-, and gelatin-negative. Only agglutination in high titer with specific antiserum prepared against a typical strain confirms the relation of these small-colony variants to typical organisms of this group.

Serological study. Antiserum was prepared by rabbit inoculation against a typical encapsulated strain of these gram-negative bacilli. Quellung tests set up in the usual manner showed an immediate, marked swelling of the capsule with all strains in the "M," encapsulated phase. The unencapsulated, smooth, rough, and small-colony variants naturally failed to give a positive quellung test. The quellung reaction with encapsulated strains, in which form the organisms naturally occur, offers a rapid method of identification, since quellung tests on a group of control cultures consisting of representative strains of *Escherichia*, *Aerobacter*, *Eberthella*, *Salmonella*, *Shigella*, *Pseudomonas*, and *Pasteurella* were all negative.

Cross-agglutination tests resulted in agglutination to the titer of the serum with

all "M" strains and 80 per cent agglutination of the small-colony variant. The smooth and rough strains failed completely to agglutinate. Agglutinin-absorption experiments were then performed. The antiserum was absorbed with each of the "M" and small-colony variant strains, and the resulting absorbed serum was tested for agglutinins against these same cultures. It was found that each of the typical encapsulated strains completely absorbed the agglutinins against all other such cultures. The small-colony form, however, showed little agglutinin absorption, the titer of the absorbed serum being only slightly reduced with the exception of complete absorption of agglutinins against the homologous absorbing strain.

The serological study clearly demonstrates that in the encapsulated phase these organisms are serologically homogeneous. The smooth, rough, and small-colony forms, however, vary serologically from the "M" strains as might be expected.

Agglutinin tests with the same series of control cultures listed above showed absolutely no cross agglutination even in low dilutions with but one exception. Strains of *Pasteurella multocida* were agglutinated to titer with the antiserum prepared against the organisms under discussion. This apparent serological relationship was investigated and it was found that (1) antisera prepared against strains of *Pasteurella multocida*, which agglutinated *Pasteurella* strains in high titer, failed completely to agglutinate these unidentifiable gram-negative bacilli; (2) absorbed antisera, which were prepared in the agglutinin-absorption experiments described above and which were found to contain no agglutinins against these organisms, agglutinated *Pasteurella multocida* strains to the same titer as the unabsorbed serum; and (3) strains of *Pasteurella multocida* failed completely to absorb the agglutinins against these organisms. Thus it was clearly demonstrated that there is no serological relationship between these gram-negative bacilli and *Pasteurella multocida* and that the agglutination observed in the cross-agglutination experiments was probably due to naturally occurring antibodies against *Pasteurella* in the rabbit serum. Since rabbits are liable to *Pasteurella* infections, the occurrence of such antibodies does not seem improbable.

As for the agglutination of these unidentifiable organisms by antisera prepared against recognized species of gram-negative bacilli, none has been observed to date. This fact was confirmed by Wheeler (1947), who reported that "the serology is unrelated to anything we have on hand." He specifically reported no agglutination with antiserum against the mannitol-negative and mannitol-positive groups of *Shigella* or against the *Shigella dispar* and *Shigella alkalescens* groups, which is of some interest since these organisms resemble *Shigella* in some biochemical characteristics.

Pathogenicity for animals. The pathogenicity of these unidentifiable gram-negative bacilli was tested in mice and guinea pigs. Mice succumb to intraperitoneal injection of 0.01 milliliter of a 24-hour broth culture in less than 24 hours, whereas guinea pigs are killed by a similar injection of 0.25 ml. The organisms were regularly recovered from the heart blood, pleural fluid, and peritoneum of the dead animals and were identified culturally and serologically.

TABLE 2

Differentiation of Bacterium anitratum and culturally similar gram-negative bacilli

ORGANISM	COLONIES ON DESOXYCHOLATE AGAR PLATE	MOTILITY	LACTOSE	GLUCOSE	SUCROSE	XYLOSE	MANNITOL	INDOLE	METHYL RED	VOGES-PROSKAUER	CITRATE	MALONATE	NITRATES	UREASE*	10% LACTOSE AGAR SLANTS	MILK PLUS B.C.P.	SS AGAR	ANAEROBIC GROWTH	QUELLUNG AND AGGLUTINATION TEST WITH B. ANITRATUM ANTISERUM
Bacterium anitratum	Usually pale pink; few strains colorless or yellowish	−	−	A	−	−	−	−	+	−	+	alk	−	+	+	A, coag. 5–10 days	−	−	+
Pseudomonas	Colorless colonies becoming brown in 48–72 hours	+	−	− or sl. A	−	−	−	−	−	−	+	alk	+†	+	−	alk., pep.	+	−	−
Pasteurella	No growth	−	−	A	A	A	A	+	−	−	−	−	+	−	−	sl. A	−	+	−‡
Shigella	Colorless colonies	−	− or late A	A	−	−	A/−	+/−	+	−	−	−	+	−	−	sl. A	+	+	−
Bacterium paropertussis	Colorless colonies	−	−	−	−	−	−	−	−	−	+	−/A	−	+	−	sl. alk.	−	−	−

A = acid, alk. = alkaline; sl. = slight; +/− = may be positive or negative.

* Urease production determined on Christensen's urea agar.

† Nitrite test is negative, but addition of zinc dust shows no unreduced nitrates present; therefore, both nitrates and nitrites are reduced.

‡ Positive agglutination test demonstrated to be due to naturally occurring agglutinins against *Pasteurella* in the rabbit serum.

DISCUSSION

Biochemically these gram-negative bacilli appear to differ from any recognized genera and species described in *Bergey's Manual of Determinative Bacteriology*, sixth edition. A possible relationship to *Pseudomonas* has been suggested, but the organism here described produces no pigment; there are well-defined morphological and biochemical differences and agglutination tests show no serological relationship. In table 2 is shown the differentiation of these organisms from *Pseudomonas*, and also from *Pasteurella*, *Shigella*, and *Bacterium parapertussis*, to which they may bear a superficial resemblance.

As the taxonomic position of these gram-negative bacilli is not clear, it seems advisable at this time to place them in the genus *Bacterium*, which is reserved for organisms not yet assigned to any of the recognized genera. Since it is their failure to reduce nitrates that differentiates them from the great majority of gram-negative bacilli, particularly the *Enterobacteriaceae* to which they appear to be most similar, it seems appropriate to select a species name that will express this characteristic, for example, "*anitratum*." Therefore, the name *Bacterium anitratum* n. sp. is proposed as a name for this group of biochemically and serologically homogeneous gram-negative bacilli.

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SUMMARY

The morphological, cultural, and biochemical characteristics of a group of unidentifiable gram-negative bacilli from human sources have been described.

Cross agglutination and agglutinin-absorption tests have shown these organisms, when in the "M," encapsulated phase, to be serologically homogeneous and to bear no serological relationship to gram-negative bacilli of the *Enterobacteriaceae* and other recognized genera.

Bacterium anitratum n. sp. has been suggested as a fitting name for these organisms.

REFERENCES

BONDI, A., SPAULDING, E. H., SMITH, D. E., AND DIETZ, C. C. 1947 A routine method for the rapid determination of susceptibility to penicillin and other antibiotics. Am. J. Med. Science, **213**, 221–225.

BROWN, J. H. 1948 Media prepared by the pancreatic digestion of meat. J. Bact., **55**, 871–872.

CHILTON, M. L., AND FULTON, McD. 1946 A presumptive medium for differentiating paracolon from *Salmonella* cultures. J. Lab. Clin. Med., **31**, 824–827.

CHRISTENSEN, W. B. 1946 Urea decomposition as a means of differentiating *Proteus* and paracolon cultures from each other and from *Salmonella* and *Shigella* types. J. Bact., **52**, 461–466.

FROBISHER, M. 1944 Fundamentals of bacteriology. 3d ed. W. B. Saunders Company, Philadelphia. *Refer to p.* 231.

WHEELER, K. 1947 *Personal communication.*