

A MICROORGANISM DECOMPOSING GROUP-SPECIFIC A SUBSTANCES

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Received for publication March 29, 1938

The use of bacteria to effect the degradation of serologically active carbohydrates was initiated by Avery and Dubos (1930; see also Dubos and Avery 1931) in studies on the capsular carbohydrate of *Pneumococcus* Type III; and other microorganisms capable of splitting polysaccharides of bacterial origin have since been found. The present report concerns the isolation and cultural characters of an apparently hitherto undescribed bacterium, having specialized food requirements and not growing on most common culture media, which is able to decompose actively the group-specific substance found in the saliva of human beings of blood group A and in various group A preparations obtained from animal materials, namely horse saliva (Landsteiner 1936) commercial pepsin and gastric mucin (Landsteiner and Chase 1936). From chemical investigations it has become probable that the substances underlying the serological differentiation of human blood groups are of polysaccharide nature (for literature, see Landsteiner 1936, Landsteiner and Chase 1936). Apart from adding to the relatively small number of organisms known to attack "specific" polysaccharides—a property not as common as might have been supposed—the description of a new species with unusual characters may be of interest.

Of bacteria which decompose serologically active complex polysaccharides, mention may be made of the following: the *Myxococcus* of Morgan and Thaysen which acts upon several specific polysaccharides of bacterial origin (Morgan and Thaysen 1933; Meyer and Morgan 1935) and also "group specific" A substances (Landsteiner and Chase 1935a); strains of bacteria,

isolated by Sickles and Shaw, which can degrade different pneumococcal carbohydrates (Sickles and Shaw 1934, 1935) and of which one, *Saccharobacterium ovale*, acts on group A substances as well (Landsteiner and Chase 1935b); and *Clostridium welchii* found by Schiff (1935) to attack group A substances. With regard to the decomposition of group A substances by other microorganisms, it may be mentioned that we found these preparations to be unaffected when incubated with a number of samples of decomposing vegetable matter; they were not altered by many common bacterial species, as Schiff (1935) has reported also, or by a chitinovorous bacterium¹ and several cellulose-decomposing organisms. In two cases, some degree of destruction of the A substance was detected after inoculation with rotting vegetables. Rather active decomposition was noted with a sample of aged, dry leaf mold and from this there was isolated the bacterium to be described.

Leaf mold was introduced into a medium comprising the A substance from commercial pepsin dissolved in the mineral base of Dubos and Avery (1931) (1 gram ammonium sulfate and 2 grams dipotassium phosphate per liter of water); after some days the culture medium had lost its specific property of inhibiting the hemolytic action (for sheep cells, in the presence of complement) of rabbit antisera to human group A erythrocytes. In isolating the organism, serial passages in 3 cc. portions of medium containing 0.02 per cent of the A preparation were employed in the beginning, but the persistence of extraneous bacteria in much greater numbers than the active organism necessitated plating on a medium containing A substance, mineral base and 1 per cent agar, the fishing of small areas of confluent growth (rather than single colonies) into the fluid A medium, and continued plating of such mixtures as were found after incubation to effect destruction of the specific substrate. After a few repetitions of this procedure, colonial fishings were practicable, and yielded a pure culture of the organism. As a precaution, eight consecutive platings from single colonies were made.

¹ A culture belonging to group I, supplied by the kindness of Dr. Anne G. Benton (1935).

For maintenance and study of the strain, two types of culture media were used. The first was a mineral medium containing 0.1 per cent of preparations rich in A substance, made from commercial pepsin and gastric mucin² (hereafter designated as "P" or "M" respectively), or slants of 0.5–1.0 per cent A substance and 1 per cent agar in mineral medium, employed usually at pH 7.0 to 7.2 (the optimum range is pH 6.0–6.5) because of moderate acid production during the growth of the bacteria.³ After a preliminary study of the organism a second type of medium, termed "SP", was developed, based upon a utilizable carbohydrate and proteose peptone (Difco), which supports growth, probably chiefly because it has a relatively high content of group-specific A substance. The medium consists of 1 per cent Difco proteose peptone, 1 per cent sucrose, 0.1 per cent $(\text{NH}_4)_2\text{SO}_4$, 0.2 per cent K_2HPO_4 , with 1 per cent agar as desired, in tap water, adjusted preferably to pH 6.5: the sucrose was added as a sterile solution to the autoclaved base. It should be mentioned that after cultivation for over a year on this "SP" medium the organism showed some decided alteration in cultural characters, as contrasted with a line maintained on medium "M".

When first isolated in pure culture, the bacterium was transplanted to a variety of common substrates, as well as synthetic media containing ammonium lactate or asparagin and tryptophane. There was no evidence of growth except in the cases of rabbit-blood beef-infusion Witte peptone agar, and litmus milk. On the former, a few small colonies slowly developed; with continued cultivation, the organism could be adapted to a scanty, unsatisfactory growth on beef infusion Witte peptone agar, again probably because of A substance in the peptone.

² The latter material, as used in the media, was prepared from crude gastric mucin (Wilson and Co., No. 1701-W) by heating a slightly acidified 2 per cent aqueous solution at 100°C., discarding material sedimenting upon centrifugation, and selecting the alcohol-insoluble fraction precipitating between $\frac{3}{4}$ and 2 volumes of alcohol, substances insoluble at the lower alcohol concentration first being removed by Berkefeld filtration; the material was reprecipitated once by addition of 2 volumes of alcohol to an aqueous solution.

³ Whenever growth was evident in fluid media "M" or "P" (within 20 to 48 hours), degradation of the A substance could be confirmed serologically.

PURE CULTURE STUDY

General

For the determination of cultural characters, the organism was examined, when appropriate, on both "SP" and "M" media. For other substrates, 1 per cent proteose peptone in mineral medium was employed as a basal fluid, and growths on proteose peptone were used as seeding cultures. For nitrate reduction, 0.1 per cent potassium nitrate was added to this solution, while indol production by the organism was examined on the proteose peptone solution alone and with added "tryptone" or with 0.03 per cent tryptophane. Cellulose digestion was tested on fluid and solid media containing amorphous cellulose, prepared according to the method of McBeth (1916), in the peptone base or in medium "M". In making the biochemical tests, procedures recommended in the Manual of Methods for Pure Culture Study of Bacteria were followed.

Morphology

The best preparations were made from growths on fluid medium "M" at pH 5.5 to 6.5, incubated at temperatures between 25° and 32°C. for one to four days; the cells were then fairly uniform and stained clearly, the organism being a coccoid rod, occurring singly and in pairs, the somewhat oval forms measuring about 0.3–0.6 μ by 0.5–0.7 μ . On agar medium "SP" at pH 6.5, the individuals vary in size to a greater degree than noted above, averaging about 0.5 by 0.6 μ , but large cells 0.9 by 1.2 μ are also seen. On fluid medium "SP" at pH 6.5, the cell is not sharply outlined, and there is some variability in the intensity of the stain. Organisms grown in fluid media at reactions more alkaline than pH 6.5 stain increasingly poorly the higher the pH value, and rapid and complete autolysis occurs in but weakly alkaline fluids, e.g. upon treatment with dilute ammonium hydroxide.

Carbohydrate utilization

The organism did not grow on solutions of the test carbohydrates in the absence of proteose peptone. In media at pH 7.0 having 1 per cent carbohydrate and 1 per cent proteose peptone in the mineral base, a greater final amount of growth than

in the peptone control occurred in the presence of glucose, lactose, sucrose, galactose, mannose, xylose, maltose, inulin and dextrin, whereas mannitol, salicin and glycerol appeared not to be utilized.⁴ The rate of development of the organism was markedly retarded over that in the peptone control by the presence of mannose, xylose, or salicin. There were only inconsiderable increases in acidity, about 0.2 pH unit, with the most vigorous growths; further studies with slants (1 per cent agar) made from sugar-peptone solutions and indicators revealed the development of acidity during growth on lactose (pH 6.6) and glucose (pH 6.8), but no or only insignificant changes in reaction with sucrose, galactose and maltose. No gas could be detected in Smith tubes (or in agar stabs) during active utilization of added carbohydrates; the growth was however limited to the surface of the open arm.

CULTURAL CHARACTERS

Coccoid rods, 0.3 to 0.6 by 0.4 to 0.9 microns, occurring singly and in pairs. Non-motile. Gram-negative. No spores demonstrated.

Gelatin "M" stab: no liquefaction.

Agar colonies: On beef-extract agar, no growth. On 0.75 per cent "M", 1 per cent agar: after two days at 32°, visible colonies 0.75 mm. in diameter, increasing in size to 5-7 mm. in 12 days. Circular, smooth, slightly raised, with an opaque raised dot set in a central depression. The internal structure appears coarsely granular. By transmitted light, bluish with thin light margins, by reflected light faintly yellow.⁵ The consistency is butyrous to viscid. On this medium, only, daughter colonies often arise near the periphery of the parent colony after the fifth day. On "SP" 1 per cent agar: as above, except that the colonies are opaque and somewhat smaller. On blood agar (usual beef infusion peptone base): after 17 days at 32°, 0.75 mm. diameter, circular, raised, glistening, rather white.

⁴ The utilization of the carbohydrates was confirmed by means of the Hanes' modification (1929) of the Hagedorn Jensen method for the microestimation of reducing sugars; media containing sucrose, inulin, dextrin, salicin, or lactose were first subjected to acid hydrolysis. The author wishes to thank Mr. Robert A. Harte for these determinations.

⁵ When the agar concentration is higher, the colonies are smaller and quite transparent at first, but a decided brownish yellow color eventually develops.

Subsurface agar colonies: On 0.75 per cent "M", 1 per cent agar: distinctly brownish, often branched or lanceolate, opaque.

Agar stroke: On 1 per cent "M", 1 per cent agar: after 5 days at 32°–35°, moderate growth, glistening, contoured, flat, with margins rugose; translucent, slightly iridescent along margins; secondary colonial growths commonly observed along line of original streak; some irregular, cleared areas on old slant growths.

Infusion broth: no growth.

"M" or "SP" fluid media: growth at surface, settling to give an even turbidity.

Litmus milk: becomes slimy, reaction unchanged.

Lead acetate: slight browning?

No growth on potato.

Indol not produced.

Nitrates reduced.

No gas or pronounced acid production in carbohydrate media, but glucose, galactose, mannose, lactose, sucrose, maltose, xylose, inulin and dextrin are utilized. Very slight acid reaction detected with lactose and glucose.

Starch not attacked.

No characteristic odor.

Obligate aerobe; in agar shake cultures, growth does not occur more than 2 mm. below the surface.

Optimum temperature: about 32°; no growth at 40°C.

Thermal death, 2 day growth in "M" or "SP" fluids, after neutralization: 10 minutes at 51°C. not at 48°C.

Reaction of medium: pH limits of growth from 5.5 to 7.7, optimal range 6.0 to 6.5.

Cellulose agar not attacked.

Habitat: isolated from leaf mold.

CLASSIFICATION

The systematic position of a Gram-negative, non-motile, obligately aerobic, coccoid bacterium with the cultural peculiarities, as regards common sources of energy, of using certain peptones only, of growing either not at all or sparsely on common media, and—in the presence of peptone—of utilizing a number of sugars without frank production of acid or gas is difficult of decision.

A kinship with the two organisms in the genus *Saccharobacterium* erected by Sickles and Shaw (1934) in the family Mycobacteriaceae seems excluded on morphological grounds, also because of the failure of certain substances to inhibit growth when added to a favorable medium, namely the presence of 0.7 per cent sodium chloride, or 0.3 per cent beef extract, or peptone (a brand which was less satisfactory for supporting growth was tested for inhibition). The description given in Bergey's *Manual of Protaminobacter alboflavum* would appear to permit a relationship, but a comparative study of bacteria of this type was not undertaken. It seems preferable, therefore, not to suggest a generic or species name for the A-splitting organism at the present time.

ACTION ON GROUP A SUBSTANCES

As already stated, the organism decomposes the group specific A substances from different sources, as human A saliva, horse saliva, and pig stomach (a material available in commercial pepsin and crude gastric mucin). The A preparations from pepsin and mucin have been used chiefly. During decomposition of the substances acid is formed (no gas is detectable by the common methods), and if the reaction is suitably readjusted to pH 6.5-7.0 at intervals during the period of incubation the organism will destroy the activity of 2 per cent solutions of the A preparations in the mineral base; this capacity for attacking the responsible serological structure is greater than that of *Saccharobacterium ovale* and exceeds by far the destructive activity of the *Myxococcus*. The action of the bacterium appears to lead to a thorough destruction, not for instance a mere splitting off of acetyl groups, since only traces of substances of polysaccharide nature were recovered following the action of the organism. No exoenzyme has been demonstrated. The bacterium did not attack the specific polysaccharides of *Pneumococcus* types I⁶ or II⁷, whereas *Pneumococcus* II polysaccharide is utilized by some other bacteria known to break down A substance, the *Myxococcus* of Morgan and *Saccharobacterium ovale* of Sickles and Shaw. In conclusion,

⁶ The preparation (SSS, prepared by the older methods) was kindly furnished by Dr. Harry Sobotka.

⁷ This material was supplied through the courtesy of Dr. W. F. Goebel.

the hope may be voiced that by means of such biological degradations of serologically active complex materials, information on the structure of these substances eventually may be yielded.

SUMMARY

The description is given of a bacterium having unusual characters and capable of destroying group A substances, that is, substances, presumably of polysaccharide nature, reacting with immune sera obtained by injection of human blood of group A. The organism does not grow on most common media, but can be cultivated in the presence of A substance.

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