THE ANAEROBIC COCCI: GAS FORMATION, FERMENTATION REACTIONS, SENSITIVITY TO ANTIBIOTICS AND SULPHONAMIDES. CLASSIFICATION

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1. INTRODUCTION

Obligate anaerobic cocci have been frequently isolated from pathological conditions in the human respiratory tract, the vagina and more rarely from wounds and abscesses. They have also been found as commensals in the tonsils. There is no doubt that more than one species can be isolated from such material, and although attempts have been made by Harris & Brown (1929), Colebrook & Hare (1933), Stone (1940) and Foubert & Douglas (1948) to evolve schemes for the classification of these organisms, it cannot be said that anything permanent or even useful has emerged. The most comprehensive work has, however, been carried out by Prévot (1925, 1933, 1948) whose scheme of classification has been adopted almost without alteration by Bergey (1948). This comprises eight genera—*Neisseria*, *Veillonella*, *Streptococcus*, *Staphylococcus*, *Diplococcus*, *Gaffkya*, *Sarcina* and *Micrococcus*—to which these organisms are primarily allotted on microscopic appearance alone. Further differentiation into the thirty-two species and several subspecies comprising these genera depends on the ability or inability of the organisms to form gas, the appearance of the colonies in deep agar cultures, fermentation reactions (in which ability to ferment is judged not by pH attained, but by the amount of substrate hydrolysed when incubated with the strain in 2% peptone water (Prévot, 1950) and such biochemical reactions as liquefaction of gelatin, formation of indole, and behaviour in litmus milk. Identification of the organic acids produced from glucose has also been employed (Prévot & Loth,

* Working with grants from the Michael and Sidney Herbert and the Leonard S. Dudgeon Funds, St Thomas's Hospital Medical School, London, S.E. 1.

1941; Prévot & Taffanel, 1942*a*). A number of species are, however, identified by certain peculiarities in shake cultures in agar: *M. niger* (Hall, 1930) because it produces black colonies, *D. constellatus* (Prévot, 1924) because large central colonies are surrounded by a number of small satellites and *Strep. evolutus* (Prévot, 1933) by what are described as 'zones alternantes de fertilité et de stérilité'. Several other species rest on even less secure foundations, being based on an examination of only one strain, many years ago, by entirely inadequate methods and with no record of subsequent isolations.

For these reasons, a study has been made of ninety-nine strains of these organisms collected, for the most part, from the human respiratory tract and vagina, with a view to the evolution of a more satisfactory method of classification, depending less on morphology and more on biochemical reactions. As a result a scheme has been evolved resting partly on ability to form gas from certain carbohydrates or inorganic acids, and partly on certain well-marked fermentation reactions. In this way six groups, I–VI, have been demarcated which show useful correlation with the part of the body from which the organisms are derived, their probable pathogenicity, their microscopic appearances, and their sensitivity to the antibiotics and sulphonamides. The properties of these groups are given in detail in § 7 of this paper.

2. EXPERIMENTAL METHODS

Source of strains. Of the seventy-one strains from the human vagina, twelve were obtained from swabs taken before delivery in the antenatal department of this hospital by our colleague Dr C. S. N. Swan. A further forty-seven were sent by Dr Calman from women in Queen Charlotte's Maternity Hospital, who had had pyrexia following delivery. Other vaginal strains were sent us by Dr A. Beck, Dr A. C. Cunliffe and our colleagues Drs J. Bamforth, W. M. R. Henderson and S. B. Van der Merwe of this hospital. Twenty-three strains from the respiratory tract were isolated from bronchoscopy specimens or purulent sputa by our colleague, Dr G. Weatherley Mien. Dr Martyn of Manchester and Dr R. M. Fry have also contributed strains from various pathological conditions.

Isolation and identification. Because the strains were isolated in different laboratories, it is not possible to give in detail the methods employed. Suffice it to say that great care was taken after their isolation to ensure their purity, and that in our own isolation procedures any coccus, whether Gram-positive or Gram-negative, was collected. All strains were plated in parallel on two blood agar plates, one of which was incubated aerobically and the other anaerobically. If growth occurred on the latter and none on the former, the strain was accepted as an anaerobic coccus. Strains were preserved by growth overnight in Robertson's meat medium under liquid paraffin. Subcultures into fresh tubes of Robertson's meat medium were made every month.

Media. The peptone concentration, presence or absence of glucose or sulphur compounds and the final pH employed varied in the different experiments, but the media were always made in the same way.

To 1000 ml. distilled water, the peptone (Difco or Evans), salts of organic acids,

carbohydrates and sulphur compound were added with 2 g. Difco yeast extract. After the materials had dissolved, the pH was adjusted with N/NaOH. The medium was then brought to the boil, and filtered through hard filter-paper while still hot. It was then tubed in $5 \times \frac{1}{2}$ in. tubes in quantity sufficient to give a column of 40–50 mm. After autoclaving at 15 lb. pressure for 30 min. and while still hot, about 1 ml. of melted vaseline was run into each tube. When cool, the medium was ready for use.

Measurement of volume of gas produced. The following method of measurement was found to be satisfactory and in many duplicate experiments gave similar readings. If gas forms in sufficient quantity, it collects beneath the vaseline seal and forces it upwards. Measurement of the displacement in conjunction with the area of a cross-section of the tube, gives a rough approximation of the volume of gas, but because all the tubes employed were of the same diameter and approximately the same volume of broth was placed in the tubes, the results are recorded merely as length of gas column.

The seal itself should be at least 5–10 mm. thick and the vaseline employed should be sufficiently firm to remain intact and solid at incubator temperature.

Determination of opacities. The opacity tubes of Brown & Kirwan (1914–15) were employed in the majority of the experiments to measure the density of growth in the broth. But in later experiments, a series of tubes containing barium sulphate was constructed of the same diameter as those used in the experiments and carefully calibrated to correspond with those of Brown & Kirwan. These could be compared directly with the cultures.

pH *determinations.* In view of the fact that less than 5 ml. of culture fluid was available and that several hundred determinations might be required in a day, electrical methods could not be employed, with the apparatus at present available. Colorimetric methods were therefore used, employing indicators on porcelain tiles and comparing the colours produced with buffer solutions or the colour plate in Clark's monograph (Clark, 1928).

Duration of experiments. Daily readings of gas volume and opacity of the cultures showed that the maxima were reached between the third and the fourth day. Final readings as recorded in the tables were made on the seventh day.

Gas analysis. Fifty ml. of medium containing 5 % peptone, 0·2 % yeast extract, 1 % glucose and 0·1 % sodium thioglycollate at pH 8·4 was sterilized in a 9×1 in. tube with a right-angled tube let into the butt and sealed with a clip over a rubber tube. A thick vaseline plug prevented escape of gas from the open end. The tubes were inoculated with 5 ml. of an overnight culture of the strain under investigation. In this way, relatively large volumes of gas were collected after incubation for 24–40 hr. The concentrations of the different constituents were, as far as possible, determined by a method based on that described by Sutton (1938) in which the gas is collected over mercury into a bell expanded on the end of a horizontal capillary pipette. The gas is drawn into the capillary and its volume measured on a calibrated scale. It is then returned to the bell and the absorbent introduced into it through the mercury. When equilibrium has been established between the reagent and the gas mixture, any change in volume may be noted by returning the bubble

to the capillary and again measuring the length occupied by the bubble. The percentage of gas absorbed can then be calculated.

A small loop of platinum wire, about 2 mm. in diameter, is used to hold the reagent. If possible, the reagent is used in the solid form. When this cannot be done, a solution of it may be incorporated in a porous bead made by baking a mixture of kaolin and coarsely powdered porcelain. The order in which the reagents are introduced is determined by the composition of the gas. The reagents employed were: for hydrogen, colloidal palladium in a saturated solution of picric acid; for carbon dioxide, solid potassium hydroxide; for oxygen, alkaline pyrogallol; for hydrogen sulphide, saturated lead acetate solution; for ammonia, Nessler's reagent and for sulphur dioxide, acidified potassium dichromate. Methane and carbon monoxide in the residual gases were tested for by sparking in the presence of oxgyen. The experimental error was considered to be not greater than $\pm 5\%$. In all instances the results recorded represent the average of several determinations with each strain, using independently prepared cultures in different batches of medium.

Fermentation reactions. The basal medium consisted of 5% peptone, 1% sodium thiosulphate, together with 1·0% of the substance under investigation. The medium was tubed in 5 ml. amounts, capped with vaseline and steamed on 3 successive days for 30 min. The tubes were then inoculated with 0·1 ml. of an overnight culture in sugar-free broth and incubated for 7 days. The initial pH before steaming was 8·4, and the pH of each tube after incubation was then determined by colorimetric methods on a porcelain tile.

Liquefaction of gelatin. Five per cent peptone broth without glucose, containing 0·1% sodium thioglycollate and 15% Difco gelatin at pH 8·2 and tubed in 5 ml. amounts was sterilized by steaming on 3 successive days. The medium was inoculated by stab, covered with vaseline and incubated for 7 days. After cooling to room temperature, liquefaction was tested after removal of the vaseline.

Formation of nitrite from nitrate. Five per cent peptone broth without glucose containing 0·1% thioglycollate and 0·1% sodium nitrate was tubed and sterilized by autoclaving. It had an initial pH of 8·2 and the tubes were immediately capped with vaseline. After inoculation, the tubes were incubated for 7 days. The presence of nitrites was detected by the sulphanilic acid α naphthylamine method (*Manual*, 1946).

Indole formation. The medium consisted of 5% Difco tryptone, 0·2% yeast extract and 0·1% sodium thioglycollate in distilled water, at pH 8·2 and tubed in 5 ml. amounts. The tubes were sterilized in the autoclave at 10 lb. for 20 min. and capped with vaseline. After incubation for 7 days, the presence of indole was detected by Ehrlich's reagent. A control culture of *Bact. coli*, known to give positive reactions, was always included in any series of tests.

Formation of phenolphthalein phosphatase and phenolphthalein sulphatase. Both tests were carried out in a similar fashion. Medium containing 5% Evans peptone and 0·2% yeast extract (Difco), adjusted to pH 7·0, was dispensed in 3 ml. amounts in 5 in. tubes and sterilized by autoclaving. Immediately after removal from the autoclave, a vaseline seal was added, and then, when cool, 0·3 ml. of

a 0·01 M solution of substrate which had been previously sterilized by gradocol filtration. The potassium salt of phenolphthalein disulphate was given to us by our colleague, Prof. L. Young, to whom we are much indebted for advice in carrying out the tests, and the sodium phenolphthalein diphosphate was obtained from Messrs L. Light Ltd.

The tubes were inoculated from 24 hr. cultures in a similar basal medium, and then incubated for 7 days. At the end of the experiment, N/1-sodium hydroxide was added to the cultures drop by drop. If a pink colour developed, an equal volume of buffer at pH 10·4 was added to stabilize the colour. This indicated that the strain in question was able to form phenolphthalein phosphatase or sulphatase. If no colour change occurred, sodium hydroxide was added until a drop of the medium gave a pink colour when added to phenolphthalein on a porcelain tile.

Formation of fibrinolysin. The method described by Hare & Colebrook (1934) was employed using 48 hr. cultures of the strains in 5% peptone, 1% glucose broth.

Formation of coagulase. To 0·5 ml. of fresh human plasma diluted 1/10 was added 0·1 ml. of a 48 hr. culture in 5% peptone, 1% pyruvate broth. The mixture was incubated and the presence or absence of clots ascertained at 6 and 20 hr.

Sensitivity to sulphonamides and antibiotics. Two-day cultures in 5% Evans peptone, 0·2% Difco yeast extract, 0·1% sodium thioglycollate and 1% pyruvic acid adjusted to pH 7·2 were used for inoculation.

Diffusion methods. Petri dishes containing 2% Bacto agar and 7·5% horse blood in 1% Evans peptone, 1% beef extract (Lemco), 0·5% sodium chloride at pH 7·2 were employed. Where sulphonamides were being tested the blood had been partially lysed by alternate freezing and thawing (Harper & Cawston, 1945). Ditches were cut to one side of the plate, filled with the same medium without blood, and containing the substances in the following concentrations: penicillin, 10 units/ml.; streptomycin, chloromycetin and the sulphonamides, 100 μg./ml.; aureomycin and terramycin, 250 μg./ml.; polymyxin E, 100 units/ml.

In testing the antibiotics, size of inoculum was found to be unimportant, and for this reason a comparatively heavy inoculum with a 2 mm. loop was laid down at right angles to the ditch. With the sulphonamides, however, size of inoculum greatly influenced the result. Only two strains were therefore tested on one plate, and the inoculum was plated out over half the plate at right angles to the ditch so that in part of the area single colonies are obtained.

Because the Oxford strain of *Staph. pyogenes* grew, under anaerobic conditions, at a rate similar to that of the anaerobic cocci, it was employed for purposes of comparison and was inoculated on all plates. The plates were inoculated between 20 and 24 hr. after their preparation. After incubation anaerobically for 48 hr. in a McIntoch and Fildes jar at 37° C. the distance in millimetres between the edge of the ditch and the growth margin was measured with a millimetre scale. In most experiments no difficulty was experienced in judging the point at which growth ceased.

Dilution methods. For the antibiotics, the medium consisted of 5% Evans peptone and 0·2% Difco yeast extract made up in distilled water and adjusted to

pH 7·2 with normal NaOH, dispensed in $5 \times \frac{1}{2}$ in. tubes in 3 ml. amounts and capped with vaseline. No sulphydryl compound was added, since it has been shown that the activity of antibiotics such as streptomycin (Donovick & Rake, 1946) is reduced in the presence of such compounds. Doubling dilutions of the substance under test were made in the same broth and added in 1 ml. amounts after the medium had cooled. The inoculating culture was standardized by adding fresh broth until its opacity was equal to that of Brown and Kirwan's tube no. 1. From this, 0·1 ml. was delivered from a marked Pasteur pipette into each of the dilutions of antibiotic.

The tubes were examined at 24 hr. The end-point was taken as the lowest concentration of antibiotic which completely inhibited growth, as judged by an absence of turbidity.

For the sulphonamides a modification of the method described by Harper & Cawston (1945) was employed in which the strains to be tested were inoculated on to plates consisting of the same lysed horse blood agar as was used with the ditch plates and containing 1000, 100, 10, 1 and 0 μg./ml. of the sulphonamide. The plates were inoculated from 48 hr. old cultures and a small inoculum achieved by plating out. No more than six strains were inoculated on to each plate. The plates were incubated anaerobically for 48 hr. and the bacteriostatic concentration taken as the smallest amount which prevented the growth of discrete colonies.

3. GROWTH IN LIQUID AND SOLID MEDIA

The anaerobic cocci will grow and many strains form gas in tryptic digest broth, infusion broth and simple peptone solutions, provided anaerobic conditions be secured by vaseline or liquid paraffin seal. But since many strains grow very badly and form very little gas in such media, the whole question has been carefully examined and the results are reported in § 4.

On solid media such as tryptic digest broth agar with 5 % horse blood or plain agar made with 5 % peptone broth and 0·2 % yeast extract, they grow readily in the form of smooth, pale grey domes with entire edges reaching a diameter of 0·5–1·5 mm. in 48 hr. But the size of the colonies, their transparency and to some extent their colour, varies according to the degree of dispersion on the plate. It is therefore useless to attempt further description of their surface growth. If, however, two strains known to be biochemically different be plated side by side on the same plate, it is possible to detect differences in the colonies of the two varieties. In this way two or more varieties have on several occasions been isolated from the same pathological material. This, however, is as far as it is possible to go.

The only strain producing any change in blood agar was one sent by Dr R. M. Fry, which produced incomplete haemolysis. None isolated by ourselves has shown such tendencies.

In shake cultures in agar, growth also occurs readily with, in the case of gas-forming strains, fracture and displacement of the fragments. The colonies are usually white, and even when well dispersed there is a tendency to variation in size, some being 0·5–0·75 mm. in diameter, others being merely pin points. Black

colonies of the variety said to be produced by *M. niger* have not been seen, but a slight blackening of the colonies is not unusual. Growth occurs evenly all through the agar up to a sharp line 4–10 mm. from the surface. There were no indications that any particular group was more or less sensitive to oxygen than any of the others. No micro-aerophilic strains have been detected giving a zone of growth a few millimetres below the surface and little or none deeper down.

4. GAS FORMATION

Although Prévot (1948) makes use of gas formation as a basis for classification, only ill-defined media such as glucose broth, peptone water or 'unheated protein' are employed. Foubert & Douglas (1948) have described a semi-synthetic medium, but this, in our hands, was not satisfactory, and it early became apparent that a systematic study of the part played by peptone concentration, carbohydrate and organic acid content on growth and gas formation, was a prime necessity; with, as the work developed, a study of the part played by such auxiliary factors as sulphur compounds. As a result of this, it has been possible to obtain media giving good growth with adequate gas formation.

Peptone concentration. In the first experiments, the medium described by Foubert & Douglas was employed but with concentrations of peptone varying from 1 to 6% at pH 8·4 and containing 0·2% Difco yeast extract, 1·0% glucose and 0·1% sodium thioglycollate. While some gas was formed by strains of groups I, III and V in as little as 1% peptone, larger amounts were formed in 4, 5 or 6% peptone. Those of group II, however, required at least 4%, and not until 5% was reached did all the strains produce gas. For this reason 5% peptone has been employed in many of the subsequent experiments.

Initial pH. Using the same media, with 5% peptone, the effects on growth and gas formation of variations in the original pH are given in Table 1.

On the whole, a high initial pH was better than a low. This certainly applies to strains of groups I, III and VI, while groups II and V were indifferent. No gas was formed at any pH by strains of groups IV and VI, but better growth was obtained at pH 7·8 and 8·4 than in more acid media.

Carbohydrates. The fermentation reactions of the different groups are given in Table 6, but growth and gas formation may be affected by the presence of carbohydrates. This is particularly the case with strains of group I which grow more profusely and produce much more gas in the presence of 1% glucose, laevulose or maltose in a medium containing 5% peptone, 0·2% yeast extract and 0·1% thioglycollate at an initial pH of 8·4. This is well illustrated in Table 2. To some extent, growth and quantity of gas produced by group III is assisted by carbohydrate, and although strains of group VI do not form gas, their growth is also very much better in the presence of carbohydrate. On the other hand, growth and gas formation by strains of groups II and V were unaffected by carbohydrates.

Sulphur compounds. Omission from the medium of sodium thioglycollate, used in all the preceding experiments, led to a suppression of gas formation by strains

of group I in spite of the fact that the amount of growth judged by the opacity of the cultures was only slightly less. Because thioglycollate contains —SH groups,

Table 1. *Gas formation in media of different initial* pH

pH before autoclaving...	5·6		6·6		7·6		8·6		9·6	
pH after autoclaving...	5·6		6·4		6·8		7·8		8·4	
	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas
Group I, 5 strains	0	0	0	0	<1–4	0	6–9	6–29	4–9	11–35
Group II, 5 strains	3, <1 2, 0	0	<1–1	1, 1 1, <1 3, 0	<1–1	1, 0 2, 1 2, 1	<1–1	4, <1	<1	1, 0 4, <1
Group III, 2 strains	0	0	<1 0	3 0	<1 0	2 0	1 <1	13 2	1 1	17 7
Group IV, 8 strains	3, 0 5, <1–1	0	4, 0 4, <1	0	1, 0 7, <1–1	0	1–2	0	<1–4	0
Group V, 4 strains	4, 1	2–10	4, 1	6–10	4, 1	7–9	4, <1	4–8	4, <1	3–6
Group VI, 5 strains	5, <1	0	1, 1 4, <1	0	3, 4 2, 5	0	5, 5	0	1, 1 1, 4 2, 5 1, 6	0

In this and subsequent tables the figures under opacity refer to comparisons made between the culture and the opacity tubes of Brown & Kirwan (1915). The figures given under gas refer to the length of gas column in millimetres in $6 \times \frac{1}{2}$ in. tubes from a column of broth 40–50 mm. in length.

Table 2. *Growth and gas formation in carbohydrate media*

	Control		Glucose		Maltose		Laevulose	
	Opacity	Gas column	Opacity	Gas column	Opacity	Gas column	Opacity	Gas column
Group I, 5 strains	1	3, 0 1, 1 1, 2	1, 8 4, 9	55–64	2, 8 3, 9	50–52	1, 2 4, 9	1, 5 4, 52–65
Group II, 4 strains	3, <1 1, 1	1, 0 3, <1	3, <1 1, 1	3, <1 1, 3	2, <1 2, 1	1, 0 <1	2, <1 2, 1	3, <1 1, 2
Group III, 2 strains	3 1	8 10	5 1	41 40	5 1	38 21	2 1	13 20
Group IV, 5 strains	4, <1 1, 1	0	<1	0	4, <1 1,4	0	3, <1 1, 3 1, 4	0
Group V, 5 strains	<1	1, 0 4, 3–11	<1	4–10	<1	2–10	<1	4–10
Group VI, 4 strains	<1	0	1, 5 2, 6 1, 8	0	1, 1 1, 4 1, 5 1, 6	0	2, 4 2, 6	0

it therefore appeared possible that sulphur might play an unsuspected part in gas production by these strains. Growth and gas production were therefore studied

https://doi.org/10.1017/S0022172400019628 Published online by Cambridge University Press

in a medium containing 5 % peptone (Difco), 1 % glucose, 0·2 % yeast extract at pH 8·4, in which various sulphur compounds were incorporated at a concentration of 1 or 0·1 % (Table 3).

Table 3. *Effect of sulphur compounds on growth and gas formation*

	Control		Sodium thiosulphate 1·0 %		Flowers of sulphur 1·0 %		Sodium sulphate 0·1 %		Sodium sulphite 0·1 %	
	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas
Group I, 5 strains	4, 3 1, 4	0	2, 4 3, 5	7–20	5	7–20	1, 3 4, 4	3, 0 2, 2	2, 3 3, 4	4, 0 1, 1
Group II, 5 strains	2, <1 3, 1	2, 0 3, <1	4, <1 1, 1	1, 0 4, <1	3, 0 2, <1	0	3, <1 2, 1	1, 0 4, <1	1	2, 0 3, <1
Group III, 2 strains	<1	2–11	<1	2–4	0	0	<1	1–4	<1	2–3
Group IV, 5 strains	2, <1 1, 1 2, 2	0	1, 0 3, <1 1, 2	0	0	0	1, 0 3, <1 1	0	4, <1 1, 1	0
Group V, 7 strains	<1	3, 0 3, <1 1, 1	<1	3, 0 3, <1 1, 1	6, 0 1, <1	0	<1	1, 0 5, <1 1, 1	3, 0 4, <1	5, 0 2, <1
Group VI, 4 strains	2, 1 1, 2 1, 3	0	2, 1 2, 2	0	1, <1 3, 2	0	1, <1 2, 1 1, 2	0	1, 0 2, <1 1, 2	0

	Sodium thioglycollate 0·1 %		Methionine 0·1 %		Potassium persulphate 0·1 %		Potassium thiocyanate 1·0 %		l-cystine 0·1 %	
	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas
Group I, 5 strains	5	13–22	1, 3 3, 4 1, 5	3, 0 1, <1 1, 2	1, 3 3, 4 1, 5	3, 0 1, 1 1, 10	<1	0	1, 4 4, 5	6–14
Group II, 5 strains	1, <1 4, 1	1, 0 4, <1	1, <1 4, 1	1, 0 4, <1	1	1, 0 4, <1	3, <1 2, 1	0	2, 1 3, 2	1, 0 3, <1 1, 1
Group III, 2 strains	<1	1–17	1, <1 1, 1	11–16	1, <1 1, 2	4–24	1, <1 1, 0	0	1, <1 1, 1	8–10
Group IV, 5 strains	3, <1 1, 1 1, 2	0	2, <1 2, 1 1, 2	0	2, 1 3, 2	0	2, <1 2, 1 1, 2	0	3, 2 2, 3	0
Group V, 7 strains	3, 0 4, <1	5, 0 2, <1	<1	3, <1 4, 1	1, 0 6, <1	1, 0 1, <1 4, 1 1, 2	3, 0 4, <1	6, 0 1, <1	<1	3, <1 4, 1
Group VI, 4 strains	2, 1 1, 2 1, 3	0	2, <1 1, 2 1, 3	0	1, <1 2, 1 1, 3	0	1, 0 2, <1 1, 2	0	3, 2 1, 3	0

Growth and gas formation by strains of groups II–VI were, on the whole, unaffected by the presence or absence of sulphur compounds, although sulphite apparently inhibited gas formation by group II strains. Group I strains are exceptional in that they either formed no gas at all, or only minute amounts in

https://doi.org/10.1017/S0022172400019628 Published online by Cambridge University Press

the absence of sulphur, but produced much larger quantities of gas in the presence of 1 % thiosulphate, 0·1 % thioglycollate, 1 % flowers of sulphur or 0·1 % L-cystine. The other sulphur compounds were without effect.

When Evans peptone was used instead of the Difco peptone employed in the last experiment, gas in appreciable quantity was sometimes produced by group I strains without added sulphur compounds. This suggests that some peptones may contain sufficient sulphur for gas formation. Tests with eleven strains of group I in Difco 5 % peptone broth containing sodium thiosulphate crystals in concentrations varying from 0·1 to 3 % showed that the most profuse growth and largest gas production occurred at concentrations of 0·5–1·0 %. But at the lowest concentration, 0·1 %, 1–20 mm. (average 6·0 mm.) of gas were produced with none at all in the control tubes. Thus, relatively small amounts of sulphur may suffice and some peptones, such as that of Evans, may contain sufficient for some, if not maximal, gas production.

Salts of organic acids. Foubert & Douglas (1948) have described anaerobic cocci which can split lactate and for this reason the behaviour of our strains towards the sodium salts of organic acids was investigated. At first, broth containing 5 % peptone, but without glucose, at pH 8·4, was employed but better growth and more gas were obtained in media containing less peptone and at a lower pH. The final medium employed for this purpose, therefore, consisted of 2·0 % peptone, 0·2 % yeast extract, 0·1 % thioglycollate and 1 % of the sodium salt of the acid under investigation, adjusted to pH 7·0. Growth and gas formation by representative strains from each group in media of this type were then studied.

The results are given in Table 4, and it is clear that while some salts had no effect, others markedly increased the volume of gas produced. Perhaps the most interesting are the organisms of group II. These strains have consistently proved in almost all media tested to be capable of only very sparse growth and of producing only very small quantities of gas. But in pyruvate, tartrate or citrate broth, much better growth was obtained together with the formation of large amounts of gas, sometimes equalling, if not exceeding, the volume of broth in the tube. To some extent the same applies to strains of group V; while glucose evokes the formation of somewhat larger volumes of gas than in control media, far greater volumes are produced in the presence of citrate, lactate, tartrate, pyruvate, fumarate and malate.

It must also be mentioned that sulphur compounds probably play a part in the formation of gas from pyruvate by group I strains, since in their absence, gas formation is either abolished or markedly reduced.

Gas formation in agar. This occurs as readily as in broth, and depends on the presence of the same substrates.

Composition of the gases. The composition of the gases produced in 1 % glucose broth was ascertained with two typical strains from each of groups I–III and V. The results are given in Table 5. It is at once apparent that not only did the two members of each group behave in very much the same way but that there were wide differences between the groups in the proportions of CO_2 and H_2 formed.

A relatively large proportion of the gas formed by some of the strains could not

be identified, since it gave no reactions with reagents reacting with oxygen, hydrogen sulphide, sulphur dioxide, ammonia, methane or carbon monoxide. Its relatively inert nature, therefore, suggests that it may be nitrogen.

Table 4. *Growth and gas formation in* 1 % *sodium salts of organic acids*

	Control		Citrate		Formate		Acetate		Lactate	
	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas
Group I, 5 strains	3, 1 2, 3	4, 0 1, <1	2, 0 3, <1	0	4, 1 1, 2	0	1, <1 3, 1 1, 2	1, <1 4, 0	4, 1 1, 2	0
Group II, 5 strains	5, <1	<1	1, 1 4, 5	13–39	<1	1, 0 4, <1	4, <1 1, 1	2, 0 3, <1	3, <1 2, 1	<1
Group III, 2 strains	1, <1 1, 1	5–15	<1	<1–4	1	3–5	1	3–17	1, <1 1, 1	4–8
Group IV, 5 strains	5, <1	0	<1	0	<1	0	<1	0	4, <1 1, <1	0
Group V, 7 strains	<1	<1–3	2, 2 1, 3 4, 4	30–39	<1	0–3	<1	2–5	5, 2 1, 3 1, 4	15–30
Group VI, 7 strains	<1	0	<1	0	<1	0	<1	0	<1	0

	Tartrate (racemic)		Pyruvate		Fumarate		L-Malate		Succinate	
	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas	Opacity	Gas
Group I, 5 strains	4, 1 1, 2	0	4, 5 1, 6	10–22	1	0	1	0	1	4, 0 1, <1 1
Group II, 5 strains	1, 1 2, 4 2, 5	15–44	1, 1 1, 4 2, 5 1, 6	15–82	3, <1 2, 1	2, 0 3, <1	<1	3, 0 2, <1	5, <1	2, 0 3, <1
Group III, 2 strains	<1	4–6	1, 2 1, 3	12–65	<1	1–5	<1	3–6	1, 1 1, 3	9–13
Group IV, 5 strains	3, <1 2, 1	0	4, <1 1, 2	0	5, <1	0	5, <1	0	5, <1	0
Group V, 7 strains	2, 1 3, 2 2, 4	40–48	3, 1 1, 2 3, 3	60–80	4, 1 2, 2 1, 4	22–30	6, 1 1, 2	18–32	<1	1–3
Group VI, 7 strains	<1	0	<1	0	<1	0	<1	0	<1	0

Table 5. *Type and percentage concentration of the gases formed in* 5 % *peptone,* 1 % *glucose*

Group	CO_2	H_2	Unknown
I	67	25	8
	94	0	6
II	0	70	30
	0	82	18
III	7	87	6
	0	82	18
	7	65	28
V	9	70	21

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It is of interest that in the case of group I, which forms gas from carbohydrate, relatively large amounts of CO_2 are formed, suggesting that the carbohydrate metabolism is of the same order as that of the *coli-aerogenes* group. In the case of the remaining three groups, II, III and V, it is evident that quite different metabolic processes are at work because large amounts of hydrogen, some inert gas, which is probably nitrogen, and very little or no carbon dioxide are produced.

Thus, so far as can be seen, gas formation by the anaerobic cocci is due to fermentation either of a carbohydrate or of a salt of an organic acid. There is no evidence that gas is formed from peptone or other nitrogenous base, for which reason the term 'gas from peptone' employed by other workers is probably misleading.

It is also probable that a sulphur compound may be necessary for the activation or synthesis of certain enzymes concerned with glycolysis by the organisms of group I. A somewhat similar dependence on sulphur compounds by other species of organisms has been demonstrated by Quastel & Wheatley (1932) and Barron & Singer (1945).

5. FERMENTATION AND OTHER BIOCHEMICAL REACTIONS

In view of the fact that many strains will not grow in simple media such as 1% peptone water or Hiss's serum water, the basal medium adopted for fermentation reactions was the 5% peptone with 1% thiosulphate medium employed in the preceding experiments. It was realized that there are many objections to so high a peptone content, but adequate growth is also very desirable in such reactions. This was considered to outweigh any disadvantages.

Table 6. *Fermentation reactions*

	Glucose	Maltose	Laevulose	Sucrose	Salicin	Galactose
Group I, 12 strains	5·8–6·2	5·8–6·2	5·4–6·4	8·0–8·6	7·2–8·0	7·0–7·8
Group II, 5 strains	7·6–8·0	7·8–8·0	8·0–8·4	8·4–8·8	7·8–8·0	7·6–8·0
Group III, 2 strains	7·4–7·2	6·9–7·4	7·8–7·6	7·6–8·0	6·0–7·6	8·0
Group IV, 8 strains	7·2–8·0	7·8–8·0	7·4–8·4	7·6–8·4	7·6–8·2	7·0–8·0
Group V, 5 strains	6·8–7·4	7·2–7·4	7·0–7·4	7·6–7·8	7·6	6·6–7·0
Group VI, 5 strains	5·0–5·4	5·2–5·6	4·8–5·4	4·8–5·4	1, 5·8 4, 7·8–8·0	5·2–6·2

The upper and lower limits of the final pH determined colorimetrically after incubation for 7 days are given. There was no depression in the pH in media containing the following substances: raffinose, trehalose, sorbitol, lactose, starch, inulin, dextrin, glycerol, aesculin, mannitol.

Representative strains from each of the groups had no apparent action on lactose, raffinose, trehalose, starch, dextrin, glycerol, aesculin, mannitol or sorbitol, but glucose, laevulose, maltose, sucrose, galactose or salicin were acted upon by some of the strains. The results are given in Table 6 and show the final pH obtained after

incubation for 7 days. The initial pH of the tubes varied a little but was always 8·0 or more. The most characteristic picture was obtained with strains of group I which regularly ferment glucose, laevulose and maltose, and with those of group VI which ferment the same sugars together with sucrose and galactose, the results with salicin being variable. The two strains of group III show tendencies to depress the pH in the presence of glucose, maltose, laevulose and one strain in salicin, but not to the same extent as do those of groups I and VI. To some extent this also applies to group V. Groups II and IV, however, showed much less activity.

Thus, fermentation reactions are undoubtedly of assistance in the differentiation of the two groups IV and V, neither of which forms gas.

In the other biochemical tests employed, negative results were, for the most part, obtained. Thus representative strains from each group were unable to liquefy gelatin, produce indole, produce fibrinolysin, coagulase, or give positive Voges Proskauer reactions. On the other hand, all the eleven strains of group V reduced nitrates; the remaining groups being inactive.

Some strains were able to form enzymes capable of splitting phenolphthalein from its compound with phosphoric acid. This test, introduced by Bray & King (1942, 1943) and further studied by Barber, Brooksbank & Kuper (1951), was shown by the latter to be of value in differentiating *Staph. pyogenes* from *Staph. albus*. In our tests no useful information was obtained, since all were able to form the enzyme. A somewhat similar test was suggested to us by our colleague Prof. L. Young, employing the disulphate salt of phenolphthalein. While this substance had been found to be of little value by Barber *et al.* (1951) for the differentiation of staphylococci, it is of interest that the only two strains of anaerobic cocci giving a positive reaction were the two placed in group III.

Although litmus milk has been extensively employed by previous workers, we have found that many of the more delicate strains did not grow in such medium and with those that did, the indicator was merely reduced in the lower part of the tube without coagulation or acid formation. In any event, reproducible results could not always be obtained.

6. SENSITIVITY TO ANTIBIOTICS AND SULPHONAMIDES

Employing ditch plates, it was found that representative strains from all six groups were sensitive to chloromycetin, aureomycin and terramycin (Table 7). They were all sensitive to penicillin, but several strains belonging to group V were less sensitive than the rest. Sensitivity to streptomycin and polymyxin E depends on the group to which the organisms belong; those of groups I–III being relatively insensitive to streptomycin. With polymyxin E, only strains of group V were sensitive.

Sensitivity to the sulphonamides also depends on the group. Thus, groups I and V and, to a lesser extent group II, are sensitive, whereas groups III, IV and VI are quite insensitive.

An attempt was then made to determine the minimal bacteriostatic concentration of these substances. For this purpose, two strains chosen at random from each group were tested by the dilution method, but where marked differences

within a group had been observed in the diffusion test (e.g. group V with penicillin) a number of strains were tested.

Table 7. *Sensitivity to antibiotics and sulphonamides*

	Penicillin	Strepto-mycin	Chloro-mycetin	Aureo-mycin	Terra-mycin	Poly-myxin E	Sulpha-pyridine	Sulpha-thiazole
Oxford staphylococcus	25–30	17–25	18–23	11–15	15–20	0	0	0
Group I, 11 strains	20–30	0	14–25	16–20	22–35	0	29–44	28–36
Group II, 5 strains	28–35	0	17–20	17–20	26–31	0	14–18	10–19
Group III, 2 strains	31–35	0–2	18	8–9	20–22	0	0	0
Group IV*a*, 7 strains	23–34	13–18	10–22	10–13	19–27	0	0	0
Group IV*b* 5 strains	28–36	17–30	18–25	11–22	25–34	0	3, –0 2, 20–27	3, –0 2, 24–30
Group V, 11 strains	18–40	8–20	10–22	5–10	20–31	9–12	30–36	28–39
Group VI*a*, 4 strains	25–31	15–20	22–26	14–16	20–22	0	0	0
Group VI*b*, 1 strain	32	17	21	15	19	0	0	0
Concentration of antibiotic or sulphonamide in the ditch	10 units/ 1 ml.	100μg./ 1 ml.	100μg./ 1 ml.	250μg./ 1 ml.	250μg./ 1 ml.	100 units/ 1 ml.	100μg./ 1 ml.	100μg./ 1 ml.

Figures refer to the maximum and minimum distances in millimetres between the edge of the ditch and the edge of the growth.

Table 8. *Inhibitory concentrations of antibiotics*

Group	No. of strains	Penicillin (units/ml.)	Strepto-mycin (μg./ml.)	Chloro-mycetin (μg./ml.)	Aureo-mycin (μg./ml.)	Terra-mycin (μg./ml.)	Poly-myxin E (units./ml.)
I	2	0·03	> 256	8·0	0·5	0·25	> 256
II	1	0·06	128	2·0	0·5	0·25	256
	1	0·06	128	2·0	0·5	0·25	> 256
III	1	0·5	128	2·0	1·0	0·5	> 256
	1	1·0	> 256	4·0	1·0	0·5	> 256
IV*a*	1	0·5	8	4·0	2·0	1·0	> 256
	1	0·5	16	4·0	2·0	1·0	> 256
IV*b*	1	0·25	2	0·5	0·25	0·06	> 256
	1	0·5	16	8·0	0·25	2·0	> 256
V	1	0·5	256	4·0	2·0	1·0	64
	1	1·0					32
	3	0·25	—	—	—	—	—
	1	1·0	—	—	—	—	—
	1	8·0	—	—	—	—	—
VI*a*	1	0·06	8	2·0	4·0	4·0	> 256
	1	0·13	16	2·0	4·0	4·0	> 256

The results obtained with the antibiotics are given in Table 8. The readings given are those obtained after 24 hr. incubation.

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On the whole, the results obtained are such as might have been expected from the methods employing diffusion. There are, however, certain discrepancies. Group III by the ditch-plate method would appear to be more sensitive than group I to penicillin, whereas the opposite is true with the dilution method. A similar reversal occurs in the case of groups V and II with streptomycin. No explanation can be advanced for these reversals, but it must be pointed out that the same results were obtained on a re-test, and that they cannot very well be due to variations in the rate of growth, since they are not seen with the other antibiotics.

Experiments were also made to determine the bacteriostatic concentrations of sulphapyridine, but because a different method was employed the results have not been incorporated in Table 8. Striking differences between the groups were, however, obtained, which are similar to those obtained using the diffusion method. The strains of groups I and V were all inhibited by only 1μg./ml. sulphapyridine, whereas concentrations as high as 1000μg./ml. were without effect on those from groups III, IV*a*, VI and three strains from group IV*b*. Two other strains from group IV*b* were nearly as insensitive. Group II occupies an intermediate position, one strain being inhibited by 10μg./ml. and the other by 100μg./ml.

Experiments with other sulphonamides showed the same marked differences between the groups, the bacteriostatic concentrations of sulphadiazine, sulphamezathine and sulphathiazole being of the same order as those of sulphapyridine, whereas those of sulphacetamide were somewhat higher.

In view of the work of McLeod, Mayr-Harting & Walker (1944) showing that anaerobiosis decreases the *in vitro* effect of the sulphonamides against certain organisms, it is of interest that groups I and V should be susceptible to such low concentrations.

7. APPLICATION OF THE FOREGOING TO CLASSIFICATION

It is obvious from the experiments reported above that there are very profound differences in the behaviour of strains belonging to the different groups. These differences were most marked in experiments on gas production in different substrates and in fermentation reactions. Thus, by employing three series of tests for (1) ability to form more gas in thioglycollate broth with added glucose than without it, (2) ability to form gas in broth containing different organic acids, and (3) fermentation reactions with certain carbohydrates, it would seem probable that a satisfactory basis for the classification of these organisms might become available. But for any scheme of classification to be of value, it should bear some relationship to the other properties of the organisms, such as normal habitat, pathogenic activity, their morphology, sensitivity to antibiotics and sulphonamides, etc. It is also desirable that it should embrace most of the strains likely to be encountered in nature.

For these reasons all the ninety-nine strains in our possession were tested in this way.

In general, only one strain from each patient was studied, but in six instances it was known that members of two different groups had been isolated from the

same material. One strain of each group was therefore included from each of these patients.

The results are summarized in Table 9 in which the largest and smallest volumes of gas formed and the upper and lower pH levels are recorded, together with data as to the microscopic appearances and the source of the strains.

It is clear that only a minority of the strains submitted to these tests failed to place themselves in one or other of the groups. It is also of interest that there was marked correlation between the group classification and the source of the strains. Thus, except for three of group I, all the strains of groups I–III came from the vagina and all of group V from the respiratory tract. Groups IV and VI were more mixed. Group IV, for instance, characterized chiefly by little measurable biochemical activity, is divisible into two varieties, one (*a*) consisting of relatively large cocci derived from the vagina, and the other (*b*) of smaller cocci from the respiratory tract. Group VI is also divisible into two varieties, one (*a*) forming chains and the other (*b*) growing in masses. There was, however, no very definite correlation with source.

It is thus clear that, on the whole, the strains behaved with reasonable regularity in our tests and that there is useful correlation between these biochemical activities, morphology, staining reactions and source. And although groups IV and VI may require subdivision on a firmer basis when further study can be given to them and more strains become available, this does not invalidate the scheme as a whole.

The method of classification proposed is probably the best yet suggested and is reasonably certain with the strains so far tested. We shall therefore summarize what is known of the properties of each of the groups, and attempt to correlate them with species described by previous workers.

Group I. This is a very distinctive and easily recognized group. Glucose, laevulose or maltose are fermented, with the production of large volumes of gas, a high proportion of which is CO_2. Sulphur compounds are necessary for gas production but not for fermentation. Only pyruvate amongst the organic acids tested is attacked. Growth in glucose broth is profuse in the form of chains of Gram-positive cocci 0·6–0·7 μ in diameter, which show a tendency to pleomorphism and the formation of clubs or bacillary forms. With the exception of ability to form phosphatase the auxiliary tests referred to in § 5 are not very helpful since negative results are obtained. These strains are sensitive to the sulphonamides and all the antibiotics except streptomycin and polymyxin E.

No less than forty-five strains behave in this way, although two strains similar in all other respects to the remainder are able to ferment sucrose as well. With the exception of one strain from an abscess of the brain, one from an infected foot and a third from an appendix, all were derived from the vagina.

There can be little doubt that *Strep. putridus* or *putrificus* first isolated from a case of bartholinitis by Veillon (1893), but first adequately described by Schottmüller (1910) and again by Prévot (1925, 1933) is the same organism. Confirmatory evidence is afforded by the fact that according to these authors, gas is not formed in 'heated media' but does appear in quantity in media containing 'fresh protein (blood)'. Although they were unaware of the reasons for this, it is

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Table 9

Group	No. of strains	Gas column in thiosulphate broth: + glucose	Gas column in thiosulphate broth: No glucose	Final pH in broth + 1 % thioglycollate + 1 % of: Glucose	Laevulose	Maltose	Sucrose	Galactose	Salicin
I	45	7–64	0–2	5·6–6·6	5·6–6·4	5·6–6·4	6·8–8·2	6·8–8·4	6·8–8·0
	2	45	< 1	5·8–6·0	5·6–6·0	5·8–6·0	6·0–6·2	6·8–7·2	7·0–7·8
II	7	< 1	0–< 1	7·8–8·6	7·6–8·4	7·4–8·4	7·6–8·6	7·2–8·6	7·6–8·0
III	2	5–2	7–10	7·4	7·4	7·4	6·0	7·4	7·8–8·0
IV (*a*)	9	0	0	7·6–8·6	7·6–8·6	7·6–8·6	7·4–8·6	7·4–8·6	7·4–8·0
IV (*b*)	6	0	0	7·2–7·6	7·4	7·8–8·0	7·6–8·0	7·0–7·4	8·0–8·2
V	11	3–10	< 1–6	6·8–7·4	7·0–7·6	7·0–7·8	7·4–8·0	6·6–7·4	7·6–7·8
VI (*a*)	6	0	0	4·8–5·2	4·8–8·0	5·2–8·0	4·8–5·6	5·0–6·0	5·2–8·0
VI (*b*)	4	0	0	4·8–6·2	4·8–6·0	5·0–7·0	5·0–5·4	5·2–6·6	5·0–8·0
Unassigned	4	< 1–4	1–3	7·0–7·8	7·2–7·6	7·4–7·8	7·2–8·0	7·0–7·8	7·8–8·0
	1	0	0	7·4	5·0	7·4	5·0	5·6	8·0
	1	< 1	0	8·2	8·4	8·6	8·4	8·2	7·8
	1	2	0	6·4	6·2	6·2	6·8	6·4	7·2

Group	No. of strains	Gas column in broth + thioglycollate + 1 % of: Pyruvate	Lactate	Malate	Fumarate	Tartrate	Citrate	Morphology of cultures after 24 hr. in broth: Morphology	Gram reaction	Diameter of cocci (μ)	Place of origin
I	45	9–30	0–< 1	0–2	0–3	0–< 1	0–< 1	Chain	+	0·6–0·7	Vagina 42, brain 1, foot 1, appendix 1
	2	16–20	0	0–< 1	0	0	0	Chain	+		Vagina
II	7	65–90	0–2	0–< 1	0–< 1	20–47	7–85	Masses	+	0·7–0·8	Vagina
III	2	25–17	1–2	0–2	5	1–< 1	5–7	Diplococci	+	0·9–1·7	Vagina
IV (*a*)	9	0	0	0	0	0	0	Masses	+	0·7–0·8	Vagina
IV (*b*)	6	0	0	0	0	0	0	Masses	+	0·4–0·5	Lung
V	11	45–80	15–50	9–35	22–34	8–48	15–36	Masses	±	0·5–0·8	Lung
VI (*a*)	6	0	0	0	0	0	0	Chain	+	0·5–0·7	Lung 4, frontal sinus 1, vagina 1
VI (*b*)	4	0	0	0	0	0	0	Masses	+	0·8	Lung 2, Vagina 2
	4	0	0–2	0–3	0–< 1	0–2	0	Masses	+	0·6–0·8	Vagina
	1	0	0	0	0	0	0	Chain	+	0·5	Blood
	1	18	0	0	0	0	0	Masses	+	0·8	Vagina
	1	0	0	0	0	0	0	Tetrads	+	1·0	Vagina

probable that the sulphur radicle in the cystine of the blood or serum added to the medium was responsible for activating the glycolytic enzymes.

It is therefore highly probable that the organisms of this group were responsible for many of the severe post-partum infections of the uterus described by Schottmüller (1928), Colebrook (1930), Schwarz & Dieckman (1927) and many others, since all agree that chain-forming organisms were responsible and that many of them were able to form gas. For the same reasons it is also probable that these organisms may have been responsible for infection of war wounds (Fleming, 1915; MacLennan, 1943).

The remaining streptococci which form gas bear little resemblance to our strains. Thus *Strep. anaerobius* (Krönig, 1895; Natvig, 1905; Prévot, 1925, 1933) forms gas in all media and grows in the form of clumps in broth. *Strep. foetidus* (Veillon, 1893; Prévot, 1925, 1933), according to the illustrations given by Prévot, bears only a superficial resemblance to a streptococcus, since it forms masses, tetrads, pairs, short chains and parallel chains. It also ferments galactose, sucrose, glucose and laevulose but not maltose. *Strep. productus* (not referred to by Bergey) was isolated from a case of pulmonary gangrene by Prévot (1941) and besides clotting milk, ferments glucose, laevulose, lactose, arabinose, xylose and sorbose. Lastly, there is *Strep. lanceolatus* which does not appear to have been seen again since Tissier (1926) isolated one strain from the stool in a case of diarrhoea and so named it because of its resemblance to the pneumococcus.

Group II. This group is metabolically quite unlike group I. Only small bubbles of gas, consisting largely of H_2, are usually produced in plain broth and glucose broth. There is no fermentation of any of the carbohydrates. On the other hand, very large volumes of gas may be produced from pyruvate, citrate and tartrate. Sulphur compounds are apparently unnecessary for gas production. In the auxiliary tests, apart from splitting phenolphthalein phosphate, the strains are quite inactive. Sensitivity to antibiotics and sulphonamides is of the same order as that of strains of group I.

Growth in glucose broth is generally very poor and in the form of a sticky mass at the bottom of the tube. Microscopic examination shows that it consists of thick masses of Gram-positive cocci, apparently held together by Gram-negative material. The individual cocci are 0·7–0·8μ in diameter. All the strains were derived from the human vagina, and although some cases were pyrexial, there is no evidence that it was due to the presence of these organisms.

We have so far been completely unable to identify these strains with those described by other authors. While their general appearance under the microscope is suggestive of staphylococci, diplococcal forms, tetrads and short chains are sometimes seen, and some authors might even look on them as micrococci. If therefore they have in the past been considered to be anaerobic gas-forming staphylococci, Prévot lists three species. *Staph. aerogenes* (*M. aerogenes* in Bergey) was first isolated from cases of puerperal infection by Schottmüller (1912) and from the tonsils by Prévot (1933). Unlike our strains, glucose and laevulose are fermented. *Staph. activus* (not referred to by Bergey) was also isolated from a puerperal infection by Prévot & Taffanel (1942*b*) and is even more active,

liquefying gelatin, and fermenting glucose, laevulose, maltose, galactose and sucrose. *Staph. asaccharolyticus* (*M. asaccharolyticus* in Bergey) (Distaso, 1912) is of doubtful authenticity, but the original strain was stated to be twice the size of a staphylococcus, and although forming indole was unable to ferment.

The only gas-forming *Diplococcus* previously described is *D. glycinophilus* (Cardon & Barker, 1947) isolated from black marine mud in California. Not only does this organism vary greatly in size but it only forms gas in media containing glycine and the gas produced is CO_2. *Gaffkya anaerobia*, isolated from the intestine of a horse by Choukevitch (1911), produces gas and is somewhat larger than our strains (1·0–1·5μ), but its description is too inadequate to permit of identification.

One species of *Micrococcus*, *M. niger*, is also stated to form gas. One strain only was isolated from human urine by Hall (1930) and another by Prévot & Senez (1944). Carbohydrates are not fermented. Its chief characteristic is the formation of black colonies in deep cultures in agar. No attempt seems to have been made to elucidate the reasons for this, and it is doubtful whether a single characteristic of this nature is sufficient to warrant the description of the organism as a distinct species. Lastly, the gas-forming streptococci must also be considered because short chains may sometimes be seen. While these organisms bear little resemblance to *Strep. putridus*, *anaerobius*, *productus* or *lanceolatus*, they do to some extent resemble *Strep. foetidus* as figured by Prévot (1925). But this organism apparently ferments glucose, laevulose and galactose.

It is therefore clear that no correlation between our strains and any previously described can be made out.

Group III consists of only two strains, both derived from the vagina, but which behave in very similar fashion. Growth is poor in broth or glucose broth, consisting of little more than a cloud, but in spite of this adequate amounts of gas are formed. The amount of gas formed is usually, but not always, increased by the presence of glucose and considerably more so by pyruvate. Added sulphur compounds are not, however, necessary. The gas consists of 82–87 % H_2 with 0–7 % CO_2.

There is a tendency for a fall in the pH to occur in the presence of carbohydrates, but this is not sufficiently regular or of sufficient degree to suggest that these strains are very active fermenters.

In the other tests these strains are almost completely inert, failing to liquefy gelatin, form coagulase, fibrinolysin or indole. Nor do they reduce nitrates. While forming phenolphthalein phosphatase, they stand apart from all other strains in forming phenolphthalein sulphatase.

The colonies on solid media tend to be rather larger than those of the other groups, but this is not a constant occurrence.

Microscopically, these organisms are much larger than those of any of the other groups, being 1·0–1·7μ in diameter. They generally appear as rather loose masses, but diplococci or tetrads are by no means unusual. They are Gram positive. Both strains came from the vagina of patients with post-partum pyrexia. Both strains are sensitive to penicillin, chloromycetin, aureomycin and terramycin but not to streptomycin, polymyxin E or the sulphonamides.

We have also failed to place these organisms. They are too small to be placed amongst the Sarcinae and too large for the Staphylococci or Micrococci but do resemble *D. magnus* (1·5–1·8μ), *D. glycinophilus* (0·7–2·5μ) and *G. anaerobia* (1·0–1·5μ) in this respect. The first may be neglected since it does not form gas (Prévot, 1948), and the second and third are referred to more specifically under group II, but they bear little resemblance to our organisms.

Group IV. The chief characteristic of this group is inability of its members to achieve any recognizable biochemical activity, no gas formation or fermentation reactions being detectable. In the auxiliary tests employed, these strains are equally inert; phosphatase formation being the only sign of activity.

The colonies on solid media are not distinctive. In broth, a thin cloud with a tendency to settle after 2 or 3 days' growth is the best that can be achieved.

Morphologically, however, two distinct varieties can be distinguished. One, denoted as group IV*a*, derived from the vagina, grows in the form of Gram-positive masses with occasional diplococci or tetrads as outliers and with diameters of 0·8μ, whereas the other (group IV*b*) from the respiratory tract grows in much the same way, but the individual cocci are 0·4–0·5μ in diameter.

All the strains are sensitive to penicillin, and while their sensitivity to streptomycin, chloromycetin and aureomycin is variable, they are, on the whole, much less sensitive than to penicillin. They are completely insensitive to polymyxin E and, with the exception of one strain from the upper respiratory tract, they are also insensitive to the sulphonamides.

There is no very definite evidence that these strains are highly pathogenic.

Staph. anaerobius (Jungano, 1907; Prévot, 1933) resembles our vaginal strains because of its biochemical inactivity and the size of the cocci. The rather smaller organisms (0·4–0·5μ) isolated from the respiratory tract are more difficult to place, since no other species of staphylococci unable to form gas has been described. Although it is improbable, they might have been described as diplococci, in which case, five species unable to form gas must be considered. *D. paleopneumoniae* (Bolognesi, 1907), *plagarum belli* (Adamson, 1918–19), and *morbillorum* (Tunnicliff, 1917) are of very doubtful authenticity since no subsequent worker, including Prévot (1933), seems to have encountered them. *D. magnus*, like our strains, is unable to ferment but it is considerably larger (1·5–1·8μ). *D. constellatus* (Prévot, 1924, 1933) characterized on the somewhat shaky basis of ability to produce large numbers of small colonies surrounding a central large one when grown in agar and able to ferment glucose, laevulose, galactose, maltose, sucrose and arabinose, is also quite unlike our strains. Finally, there is *M. grigoroffi* (Grigoroff, 1905), first isolated from appendicitis, and by Prévot & Senez (1944) from the mastoid region, with a diameter of 0·7μ and able to ferment sucrose, glucose, galactose, xylose, sorbite, mannite and inulin, in which respects it differs from our organisms.

Group V is readily differentiated from all the other groups because carbohydrates are not fermented, and while glucose increases gas formation to a slight extent, pyruvate, lactate, malate, fumarate, tartrate and citrate are all extremely active.

To some extent, therefore, the metabolism of these strains resembles that of group II. As might be expected only 7–9 % CO_2 is formed, but 65–70 % H_2.

In other respects, these organisms are also distinctive because nitrates are reduced, and morphologically they appear as Gram-negative masses of cocci in overnight culture, although Gram-positive elements appear in young cultures. Colony appearance and growth in broth are of no diagnostic value.

All eleven strains of this group are sensitive to penicillin, streptomycin, chloromycetin, aureomycin and terramycin, and, provided small inocula be used, to sulphapyridine and sulphathiazole as well. These organisms are the only strains sensitive to polymyxin E.

All the strains came from the respiratory tract. Their pathogenicity is doubtful, but two were directly isolated (along with several other species of organisms) from suppurative processes.

There can be little doubt that these organisms belong to the Veillonellae. *V. parvula* was isolated by Veillon & Zuber (1898) from appendicitis, and according to Prévot (1948) is a common organism, producing gas, indole and reducing nitrates. It also ferments glucose and laevulose. *V. variabilis* was isolated by Magrassi (1946) from a mastoid infection. It also forms gas and ferments glucose, laevulose and sucrose. Because our strains are not glycolytic, they cannot very well belong to these two species, but they do bear a strong resemblance to *V. alcalescens*. This organism was first isolated by Lewkowicz (1901) from the mouths of breast-fed infants, and Choukevitch (1911) found similar organisms in the intestines of horses. Other strains have been isolated by Hall & Howitt (1925) from saliva, by Foubert & Douglas (1948) and Douglas (1950) from tonsils and renamed by them *M. lactilyticus*, by Benstead (1950) from bronchoscopy specimens and by Johns (1951) from the rumen of sheep. Much has also been done to elucidate the metabolism of these organisms by Whiteley & Douglas (1951) and by Johns (1951), the former showing that purines can be decomposed by them while the latter demonstrated that lactate, fumarate, pyruvate, malate, succinate and oxal-acetate are attacked by them.

Group VI consists of organisms unable to form gas but capable of fermenting carbohydrates and, in particular, glucose, sucrose and galactose. All except one strain ferment laevulose and all except two, maltose as well.

In other respects these strains are relatively inactive, having no action on gelatin or nitrates, being unable to form coagulase, fibrinolysin or indole, and although some form phosphatase none forms sulphatase.

The colonies on agar tend to be rather smaller than those of other groups, while growth in broth is generally very sparse.

Morphologically, seven of the strains are definite Gram-positive streptococci, growing in long chains of cocci 0·6–0·7 μ in diameter. These have been placed in Group VI*a*. Another four, having exactly similar biochemical reactions, grow as small, rather loose masses of Gram-positive cocci about the same size and have been designated as group VI*b*. It is, therefore, possible that two different species are involved, but none of the auxiliary tests we have employed serves to distinguish between these two varieties.

Four of the chain-forming and two of the mass-forming organisms are sensitive to penicillin, streptomycin and less so to chloromycetin, aureomycin and terramycin. None is sensitive to polymyxin E or the sulphonamides.

Little can be said about the pathogenic activities of these organisms.

Four species of chain-forming cocci unable to form gas have been described. *Strep. evolutus* (Gräf & Witneben, 1907; Prévot, 1925, 1933; Weiss & Mercado, 1938) is characterized chiefly by the fact that it liquefies gelatin, and although a strict anaerobe when first isolated, tends to become aerobic, passing in the process through a phase when alternate zones of growth or no growth are seen in deep cultures in agar. *Strep. micros* (Lewkowicz, 1901; Prévot, 1925, 1933), *parvulus* (Repaci, 1910; Weinberg, Nativelle & Prévot, 1937) and *intermedius* (Prévot, 1925, 1933) are evidently looked upon by Prévot as a series with progressive increase in size and biochemical activity. Thus *Strep. micros* is 0·25–0·4μ in diameter and unable to ferment carbohydrates, *Strep. parvulus* is slightly larger, 0·3–0·4μ, able to clot milk and ferment glucose and lactose, whereas *Strep. intermedius* is 0·5–0·7μ in diameter, also clots milk and ferments glucose, laevulose, maltose, galactose and lactose. Of these, the last more closely resembles our strains. Somewhat similar organisms were also isolated from the respiratory tract by Benstead (1950) but, in his hands, did not ferment glucose or laevulose. This may have been due to unsuitable media, since these organisms do not grow at all readily.

In regard to the strains having the same biochemical reactions but which are unable to form chains, they may have been described as non-gas-forming staphylococci, diplococci or micrococci.

Only one non-gas-forming staphylococcus has been described, *Staph. anaerobius*; but this is a poor fermenter and more closely resembles some of our group IV strains. The non-gas-forming species of diplococci—*D. magnus*, *paleopneumoniae*, *morbillorum*, *plagarum belli* and *constellatus*—have already been referred to under group IV. The first is larger than our strains (1·5μ). The sizes of the remainder approximate more closely to ours. All are capable of glycolysis, in which respect *D. constellatus*, which ferments glucose, laevulose, galactose, maltose, sucrose and arabinose, resembles our strains, but the characteristic colonies in agar have not been observed. Unfortunately, the size of *M. grigoroffi*, also referred to under group IV, resembles that of our organisms and its fermentation reactions are of a similar order. For this reason, it is not possible to identify our strains completely.

Unassigned strains. Of the seven strains not assigned, four form small quantities of gas and resemble group II in most respects, but citrate, pyruvate and malate do not increase gas production. One of these strains was also tested in formate, acetate, lactate, fumarate, malate and succinate, but without increasing gas production.

The remaining strains differ in some respects from the remainder of the groups and will need further study.

Finally, it should be mentioned that we have never encountered strains able to ferment lactose, liquefy gelatin or produce indole, although strains capable of these activities have been previously described. No very good reasons are apparent for this discrepancy.

8. DISCUSSION

Only limited acquaintance with the anaerobic cocci isolated from human beings shows that more than one species are involved. But there is little justification for the large number of species which have been described in the past. The description of some of them is certainly very inadequate and the criteria adopted for species differentiation of others are likewise of very doubtful authenticity.

The system of classification suggested makes little or no use of the colony or microscopic morphology extensively employed by previous workers, and depends on such easily controlled reactions as gas formation and fermentation reactions in different types of substrate using semi-synthetic media. The marked degree of correlation between the groups demarcated in this way, their habitat, probable pathogenicity, their microscopic morphology and their sensitivity to antibiotics and sulphonamides, suggest that the classification may be of value.

While many of the less well authenticated species described by previous authors cannot be correlated with this scheme, it is perhaps significant that the better differentiated species such as *Strep. putridus* of Schottmüller (1910), *Staph. anaerobius* of Jungano (1907) and *V. alcalescens* of Lewkowicz (1901) do find places within it.

9. SUMMARY

1. A total of ninety-nine strains of anaerobic cocci from human beings have been studied and six groups demarcated on the basis of gas formation and fermentation reactions.

2. Gas formation by strains of group I is due to fermentation of glucose, laevulose or maltose, with a sulphur compound as an activator and with the production of gas rich in CO_2.

3. Gas formation by strains of groups II, III and V is due to fermentation of different organic acids, sulphur not being required, and accompanied by the production of gases containing a high proportion of H_2.

4. Two groups, IV and VI, do not form gas, but the latter has marked fermentation abilities.

5. There is correlation between microscopic appearances, probable pathogenicity, sensitivity to antibiotics and sulphonamides and the groups demarcated.

6. Ninety-two out of ninety-nine strains isolated from human beings could be placed in one or other group.

We are greatly indebted to Dr H. J. Parish of the Wellcome Research Laboratories for a sample of Polymyxin E, to Prof. L. Young for much assistance and advice and to those colleagues, particularly Dr R. M. Calman of Queen Charlotte's Hospital, who sent us strains.

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(*MS. received for publication* 2. XI. 51.)