CARBON DIOXIDE REQUIREMENT OF GROUP F AND MINUTE COLONY G HEMOLYTIC STREPTOCOCCI

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Received for publication February 24, 1954

The "minute hemolytic streptococci" were discovered first by Long and Bliss (1934) and were established serologically as group F hemolytic streptococci by Lancefield and Hare (1935) and Hare (1935). Somewhat larger colonies among these "minute streptococci" were noted and segregated by Long and Bliss (1934) as group II, and this type of organism later was found by Bliss (1937) to belong serologically to group G, type 1. This organism usually is called the "minute G" hemolytic streptococcus.

The author received a standard strain of group F streptococcus, 36252, from the New York State Department of Health. This is Dr. Lancefield's H60R strain. When this organism was grown on a blood agar plate and aerobically incubated at 37 C, the growth was very poor exhibiting the characteristic "minute" colony. It was noted, however, that when this organism was grown on a blood agar slant in a screw-capped tube, the growth was so luxuriant that the possibility of contamination was suspected. A subsequent plating confirmed the purity of the culture, and the growth was as poor as it was before. The difference in these two types of cultures lies in their gas content, the screw-capped tube being closed to the atmosphere and probably offers less oxygen and more CO_2. When the organism was grown in a CO_2 jar without removal of oxygen, the growth was improved significantly, and after two to three days the colonies were as large as those of ordinary group A streptococci. Several strains of minute hemolytic streptococci isolated locally in Tokyo were identified serologically as group F organisms with antiserum prepared with strain H60R, and all of these organisms exhibited increased growth in a CO_2 jar.

Several standard strains then were obtained directly from Dr. Lancefield, and all of these strains exhibited markedly increased growth in the presence of a high concentration of CO_2. A search of literatures revealed only a short note published by Niven *et al.* (1946) which indicated that an increased concentration of CO_2 was essential for these organisms in a chemically defined medium. The present communication describes the fact that even on a blood agar an increased concentration of CO_2 is necessary for their maximum growth.

EXPERIMENTAL METHODS AND RESULTS

Strains used. Standard strains obtained from Dr. Lancefield were as follows: Group F, type 2, strains H60R, C41F, C468; group F, not type 2, stains 9RP110, 2RP121; group F, type unknown, probably a R variant, strain C628; and group G, type 1, strain F-68-A, "minute". The extent to which these organisms responded to the addition of CO_2 varied, and some strains (C628 and 9RP110) were rather refractory to the effect of CO_2, as pointed out by Dr. Lancefield. However, the growth of these organisms was definitely improved by prolonged incubation.

Strain H60R was used most extensively in these experiments because it was available in two phases, smooth and rough. The data presented here were obtained mostly with this strain. Strain F-68-A, the "minute" G streptococcus, and twenty more strains of locally isolated group F streptococci also were examined with essentially the same results.

Growth on blood agar plate. Blood agar plates containing tryptose-phosphate agar base (Difco) and 7.5 per cent human blood were used in these experiments. The growth of these organisms was studied with various concentrations of CO_2 obtained from CO_2 bomb; pressures being controlled with a manometer. Cultures were incubated with concentrations of CO_2 ranging from 0.5 per cent, 1.0 per cent, 5.0 per cent, and 10 per cent. The extent to which these organisms responded to the addition of CO_2 varied with individual strains but most of these organisms seemed to exhibit maxi-

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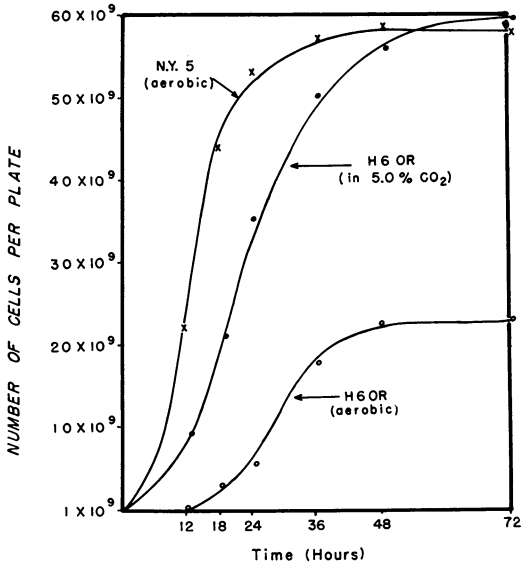

Figure. 1 Growth of strain H6OR (smooth form), a group F streptococcus, compared with that of an ordinary group A streptococcus, stain N.Y. 5. The results are indicated by total numbers of cells harvested from a blood agar plate. The growth of group A streptococcus occurred rapidly within 12 hours and almost reached maximum within 24 hours. The aerobic growth of H6OR was very poor even after 24 hours and reached to certain extent only after 36 hours. When this organism was incubated with 5.0 per cent CO_2, the growth was improved significantly, and after 48 hours, the total cells harvested from a plate were equal to that of the group A streptococcus.

mum growth when the concentration of CO_2 reached 5.0 per cent. All of the subsequent experiments, therefore, were carried out with 5.0 per cent CO_2.

Figure 3 shows the growth of strain H60R on a blood plate incubated aerobically for 48 hour at 37 C. The colonies are very small, and their existence is noted readily only by their hemolysis. Figures 4 and 5 show the growth of smooth and rough forms, respectively, of strain H60R incubated for 48 hours at 37 C in 5.0 per cent CO_2. The colonies were not as large as those of ordinary group A streptococci after 24 hours in the CO_2. They continued to increase in size after this period, while the size of colonies of group A streptococci did not change significantly after 24 hours. At the end of 48 hours, therefore, the size of the colonies of both types of streptococci was essentially the same. Figures 6 and 7 are enlargements of portions of Figures 4 and 5, respectively. These pictures demonstrate the typical colonial types encountered. The smooth colony has an entire edge with a smooth and glistening surface. The cells form a relatively smooth suspension in saline. The rough colony has an irregularly crenated edge which can be almost star-shaped. The surface of this colony is coarse, and it is quite unusual to see the colony of a streptococcus so irregular in shape. The cells do not form a suspension in saline and settle to the bottom of tube.

An ordinary candle jar also was used to grow these cultures with essentially the same results.

An attempt was made to determine quantitatively the growth of these organisms. An ordinary group A streptococcus, strain N.Y.5, was used as control. Liquid media do not provide a suitable environment to study the effect of CO_2 on bacterial growth, and thus it was considered necessary to utilize a solid medium.

Two drops of 18 hour old broth culture (tryptose-phosphate broth containing 0.1 per cent agar) of strains H60R (smooth form) and N.Y.5 were streaked out on blood plates with

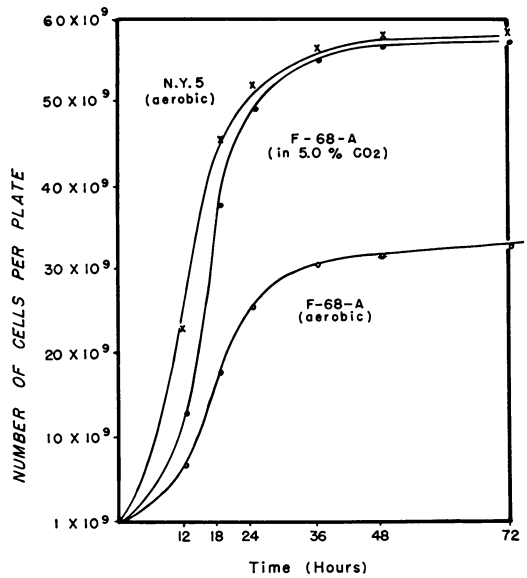

Figure 2. Growth of strain F-68-A, a minute G streptococcus, compared with that of an ordinary group A streptococcus, strain N.Y. 5. When strain F-68-A was incubated with 5.0 per cent CO_2, growth occurred as rapidly as that of strain N.Y. 5 from the beginning, and the growth curve was almost identical with that of this organism.

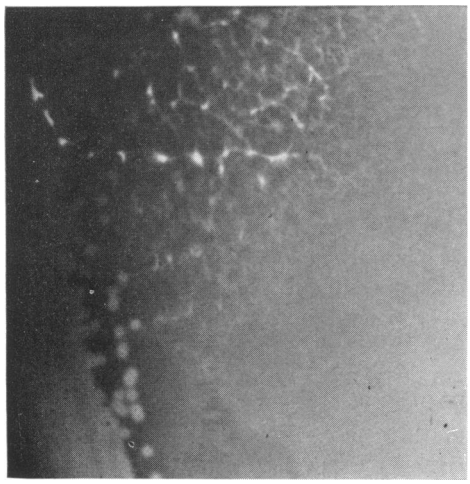

Figure 3. Growth of strain H60R (a group F hemolytic streptococcus) on blood plate incubated aerobically for 48 hours at 37 C. Colonies are very small, and their existence is noted readily only by their hemolysis. Magnification × 1.4.

sterile cotton swabs in such a way as to cover the whole surface of the plates. Twelve plates were inoculated with strain N.Y.5, and 24 were streaked with strain H60R. Twelve plates inoculated with strain H60R were incubated with 5.0 per cent CO_2, and the remaining plates were incubated aerobically at 37 C. This makes 3 groups of plates, i.e., N.Y.5 aerobic, H60R aerobic, and H60R incubated with CO_2. The growth of N.Y.5 made no difference whether grown with or without CO_2. Two plates from each group were taken out after 12, 18, 24, 36, 48, and 72 hours of incubation, and all cells on these plates were scraped off with sterile cotton swabs and suspended in 5.0 ml of saline. The number of cells in these suspensions was estimated by diluting and comparing the dilutions with McFarland's nephelometer. The average values for two plates taken at each time interval are given in figure 1. As will be seen in figure 1, the growth of group A streptococcus occurred rapidly within 12 hours and almost reached maximum within 24 hours. The growth of strain H60R was almost negligible within 12 hours on the aerobically incubated blood plates and still very poor after 24 hours. The growth of this organism, however, was improved significantly when incubated with CO_2, and almost as much as 8 times the number of cells were obtained within 24 hours when compared to those grown on aerobic plates. The growth increased steadily, and at the end of 48 hours the total cells harvested were usually equal to those obtained with strain N.Y.5. The plates incubated aerobically never produced growth comparable to that of group A streptococcus even after 72 hours. Total nitrogen determinations of cell suspensions yielded growth curves which are identical to those shown in figure 1.

A similar experiment was performed with strain F-68-A, the minute G streptococcus. This organism grew considerably better than strain H60R and other group F organisms by aerobic incubation. When it was incubated with CO_2, the growth occurred as rapidly as strain N.Y.5 from the beginning and the growth curve obtained was almost similar to that of strain N.Y.5. The results are given in the figure 2.

Appearance of a variant which grows well in the absence of CO_2. It was noted that when the group F streptococci were subcultured several times in a CO_2 jar and then streaked out to an aerobic blood plate, some colonies appeared which grew extraordinarily better than the normal form. This variant was able to grow well on aerobic plates upon subculture, and a reversion to the original form was not observed within a 10 months period. Figure 8 shows one such culture incubated aerobically at 37 C for 24 hours. The normal

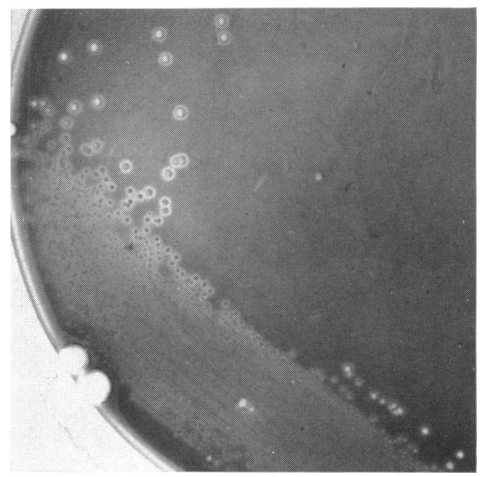

Figure 4. Growth of strain H60R, smooth form, on blood plate incubated in 5.0 per cent CO_2 jar for 48 hours at 37 C. Colonies are many times larger than those on aerobically incubated blood plate, but the zone of hemolysis is very small compared with the size of colonies. The hemolysis is hazy and not a good *beta* type. Double zone hemolysis is noted. Magnification × 1.2.

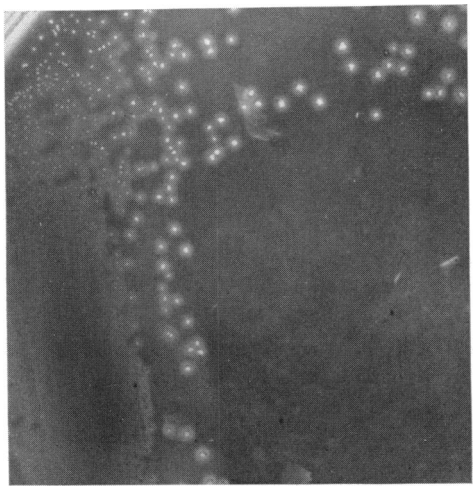

Figure 5. Growth of strain H6OR, rough form, on blood plate incubated in 5.0 per cent CO_2 jar for 48 hours at 37 C. No significant difference was observed in the size of colonies of smooth and rough forms. Double zone hemolysis is shown clearly in this plate. Magnification × 1.2.

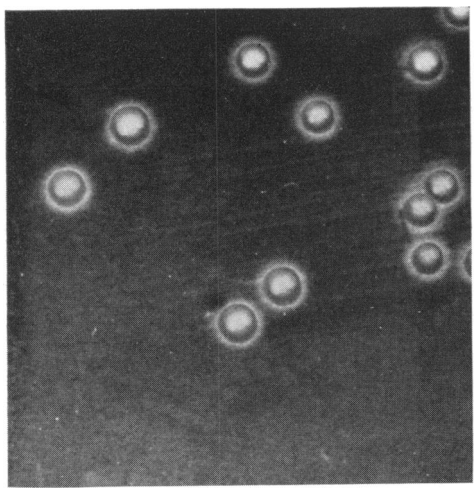

Figure 6. Enlargement of a part of plate II showing the characteristics of smooth colonies of strain H6OR. The colonies have entire edges with smooth and glistening surfaces. Magnification × 5.

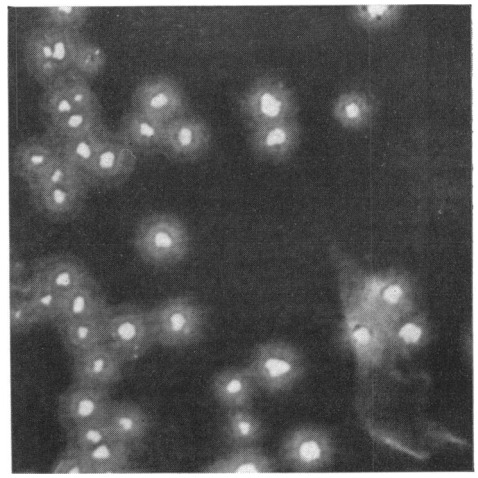

Figure 7. Enlargement of a part of plate III showing the characteristics of rough colonies of strain H6OR. The colonies have very irregular edges and coarse surfaces. Note the double zone hemolysis of colonies on left side of the plate. Magnification × 5.

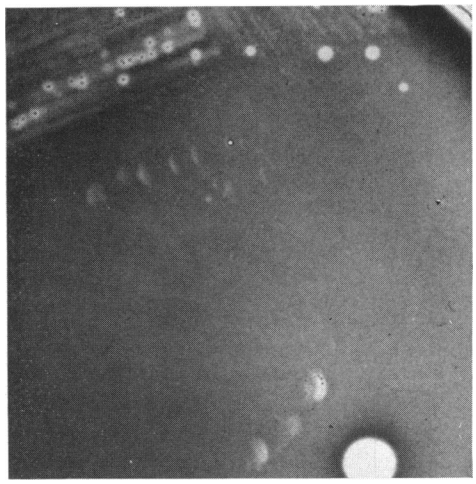

Figure 8. Appearance of variants of strain H6OR which grows well in the absence of an increased tension of CO_2. The plate was incubated aerobically for 24 hours at 37 C. The normal form of this organism is seen as a very faint growth which is indicated by a slight clearing (hemolysis) of blood agar along the line of streak. Several larger colonies, the variants, are seen scattered among these normal forms. Magnification × 1.4.

form of this organism is seen as a very faint growth which is indicated by a slight clearing (hemolysis) of blood agar along the line of streak. Several larger colonies, the variants, are seen scattered among these normal forms.

The effect of CO_2 on hemolysis. Although growths of group F streptococci are improved significantly by CO_2, the hemolysis of these organisms are inhibited. The colonies on aerobic blood plates, although very small, are surrounded by a zone of hemolysis several times as large in diameter as themselves (see figure 3). The colonies on a CO_2

TABLE 1

The ratio of diameter of hemolysis to that of colonies of strain H6OR grown aerobically and in 5.0 per cent CO_2. The numerators indicate the diameters of hemolysis and the denominators express those of colonies in millimeters

ORGANISM \ HOURS	18	24	36	48
Grown aerobic	1.0/0.15 = 6.6	1.2/0.25 = 4.8	1.4/0.4 = 3.5	1.4/0.5 = 2.8
Grown in CO_2	1.2/0.40 = 3.0	1.4/0.60 = 2.3	1.5/0.90 = 1.6	1.6/1.3 = 1.2

plate, however, are surrounded by a very narrow zone of hemolysis which is not always definite resembling the so-called *alpha* prime type (figures 4 and 5). Since the organisms do not readily produce soluble hemolysin in a broth, it was impossible to compare the hemolysin production by titration of soluble hemolysin. Therefore, the ratio of the diameter of hemolysis to that of colony is presented in table 1.

The colonies on aerobic plates were so small that the measurement had to be made with a dissecting microscope. Double zone hemolysis is observed frequently on plates incubated in CO_2 with both smooth and rough forms of streptococci in this group. The hemolysis of strain F-68-A, the minute G streptococcus, was not inhibited, however, by the addition of CO_2. The colonies were larger than those on an aerobic plate, and the hemolysis was also as definite as an ordinary group G streptococcus. The zone of hemolysis was also wider according to the size of colonies.

The effect of CO_2 on the production of group specific substance. It was of interest to determine whether these organisms grown in a CO_2 jar would produce as much C substance (which is responsible for group specificity) as those grown on an aerobic plate. Cells of strain H60R from both types of cultures (48 hours old) were collected to make a suspension in one ml of N/5 HCl containing about 50×10^9 organisms (comparing with the McFarland's nephelometer). These suspensions in ordinary Wassermann tubes were boiled for 10 minutes, neutralized with N/5 NaOH and centrifuged to collect an extract for precipitation test according to a simple modification of Lancefield's technique (1928) described by the author elsewhere (Liu, 1953). Antigen thus obtained was diluted twofold serially, and precipitation tests were performed with the homologous antiserum in capillary tubes. Observations were made after 30 minutes of incubation at 37 C. The results are given in the table 2. As will be seen in table 2, no difference was observed in the concentration of C substance extracted from both smooth and rough forms when they are grown in a CO_2 jar.

It was considered possible, however, that the concentration of the C substance extracted from these suspensions may be limited, not by the total amount of this substance present in the cells, but by their solubility in the HCl. Therefore, the following experiments were conducted. The suspension mentioned above (50×10^9 cells in one ml of N/5 HCl) was diluted twofold serially in one ml N/5 HCl, and extractions of C substance were performed on each dilution of

TABLE 2

The concentration of group specific substance extracted from equal suspensions of cells (about 50×10^9 in one ml of N/5 HCl) of H6OR grown aerobically and in 5.0 per cent CO_2 for 48 hours. The results are indicated by degrees of precipitation with the homologous antiserum

ORGANISM	GROWN	DILUTIONS OF ANTIGENS EXTRACTED							
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Smooth form	Aerobic	+++	+++	+++	++	+	+	±	−
	In CO_2	+++	+++	+++	++	++	+	±	−
Rough form	Aerobic	+++	+++	+++	++	++	+	±	−
	In CO_2	+++	+++	++	++	+	±	−	−

TABLE 3

The concentration of group specific substance extracted from various concentrations of cell suspensions of strain H6OR grown aerobically and in 5.0 per cent CO_2 for 48 hours. The beginning suspension contains about 50 × 10^9 cells in one ml of N/5 HCl and this was serially diluted twofold in one ml of N/5 HCl. Extractions were performed from each dilution of suspension, and results were indicated by degree of precipitation with the homologous antiserum

ORGANISM	GROWN	DILUTIONS OF CELL SUSPENSIONS							
		1/1	1/2	1/4	1/8	1/16	1/32	1/64	1/128
Smooth form	Aerobic	+++	+++	+++	++	+	+	−	−
	In CO_2	+++	+++	+++	++	+	−	−	−
Rough form	Aerobic	+++	+++	++	++	+	−	−	−
	In CO_2	+++	+++	+++	++	+	+	−	−

cell suspension. The results are given in table 3. Practically no difference was noted in the C substance content of this organism when the CO_2 tension was increased for its growth on blood agar. It can be estimated from these data that approximately 6 × 10^9 cells per ml of N/5 HCl were needed to obtain an extract sufficient to give a "++" precipitation.

No difference was noted with strain F-68-A, the minute G streptococcus, in the C substance content of its cell whether it was grown with or without CO_2.

DISCUSSION

Many pathogenic microorganisms are known to require an increased concentration of CO_2 for their maximum growth. *Neisseria gonorrhoeae* and *Brucella abortus* are the examples most familiar to medical bacteriologists. *N. gonorrhoeae* usually does not grow aerobically, and *B. abortus* grows very poorly if it grows any at all. These poor growths, however, are never considered to be "minute" in nature, and the statement is generally made that these organisms require an increased tension of CO_2 for their growth. The situation is exactly identical with group F and minute colony G hemolytic streptococci. It is rather unfortunate that these organisms grow to some extent aerobically because, if they did not, earlier attempts would have been made to determine conditions necessary for their growth. It is suggested, therefore, that in the future these organisms should be described as a group of streptococci which require an increased concentration of CO_2 for their maximum growth, and so-called "minute" colonies are merely a characteristic indicating their inability to grow rapidly in the air.

So far as the isolation of these organisms is concerned, the author still prefers aerobic cultivation. The zone of hemolysis produced by the group F streptococci in the CO_2 jar is very hazy and cannot be described as a *beta* type. It is difficult, therefore, to differentiate them from ordinary *alpha* streptococci. The best procedure for the isolation of this organism from clinical materials is to pick a pin-point colony with *beta* type hemolysis from a blood plate incubated aerobically for 48 hours and subculture on another aerobic plate. After overnight incubation the growth on the second plate, however poor it may be, can be streaked on two blood plates one of which is incubated in a candle jar and the other in an ordinary incubator at 37 C. If the plate incubated in the CO_2 jar exhibits a definitely improved growth over that on the aerobically incubated plate, the organism is very likely to be either group F or a minute colony G streptococcus. If the CO_2 plate exhibits a definite and wide zone of *beta* type hemolysis, the organism is likely to be a minute G streptococcus because, as mentioned above, the hemolysis of this organism is not inhibited by CO_2. If, however, the hemolysis of the isolate becomes hazy with greenish tinge, it is more likely to be a group F streptococcus. The final identification of the organism, of course, has to be made serologically with standard antiserums, but the above mentioned characteristics suggest very strongly the group of the organism isolated.

Colonies of *Haemophilus haemolyticus* may resemble these organisms in their hemolysis and the size of colonies, but they never exhibit in-

creased growth in the CO_2 jar. This organism also can be differentiated readily from the above mentioned streptococci by gram staining.

Decreased hemolysin production by group F streptococci accompanied by increased growth in the CO_2 jar was unexpected, but it is not without precedents in the field of bacterial physiology. For examples, Pappenheimer and Johnson (1936) demonstrated that within certain limits increased concentration of iron in the medium for toxin production of *Corynebacterium diphtheriae* resulted in an increased growth of this organism, but toxin production was inhibited. Totter and Moseley (1953) also demonstrated that the production of fluorescin by *Pseudomonas aeruginosa* was related inversely to the concentration of iron in the media although growth increased with the addition of iron. The role played by CO_2 to the growth and hemolysin production of group F streptococci is similar to that of iron to *C. diphtheriae* and *P. aeruginosa* for their toxin and pigment production, respectively.

It is definitely established that these organisms grown in CO_2 contain as much C substance as when they were cultivated aerobically. Whenever an attempt is made to collect cells of this type of organisms, whether for immunization of animals or for preparation of antigen for grouping procedure, it is advantageous to incubate the culture in a CO_2 jar.

The smooth and rough forms do not vary significantly in their content of C substance.

The technique described here to estimate the content of group specific substance present in a given strain of streptococcus can be used to select strains of streptococci to be used for immunization of animals for production of antiserums. Strains of streptococci within a group vary considerably with regard to their content of group specific substance, and it is better to select strains rich in this substance for immunization of animals.

ACKNOWLEDGMENT

The author wishes to express his gratitude to Dr. Lancefield who confirmed the results and generously supplied the standard strains used in these experiments.

SUMMARY

Growths of group F and minute colony G hemolytic streptococci are markedly improved by an increased concentration of CO_2. Most strains used in these experiments seemed to attain their maximum growth when the concentration of CO_2 reached 5.0 per cent, but few strains were encountered which were rather refractory to the effect of CO_2.

The hemolysin production of group F streptococci decreased with increased growth in a CO_2 jar, but no difference in the content of group specific substance was noted. The hemolysis of so-called "minute" G streptococci was not affected by CO_2.

It is suggested that these organisms should be described as a group of hemolytic streptococci which require an increased tension of CO_2 for their maximum growth. The so-called "minute" colony is merely a characteristic indicating their inability to grow rapidly in the air.

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