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STUDIES ON A CO_2-DEPENDENT STAPHYLOCOCCUS

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A dwarf strain of *Staphylococcus aureus* was isolated in pure culture from styes on the eyelid of a young woman. Incubation of pus cultured on horse blood agar in 5% CO_2 yielded a typical golden staphylococcus, coagulase and catalase positive and penicillin sensitive, but on duplicate plates in air the growth was of tiny white colonies only 0.25 mm. in diameter. These were penicillin-sensitive Gram-positive cocci, but the catalase and coagulase tests were negative. Subcultured with added CO_2, typical 2 mm. colonies of *Staphylococcus aureus* developed, but in air all grew as dwarfs. This coccus was of bacteriophage type 52A.

The patient, who had used penicillin ointment, was now given systemic penicillin and her styes healed, but the dwarf staphylococcus was present in nasal swabs a month later. Swabs from her mother, who had a palmar abscess and then a sore throat, also yielded a dwarf staphylococcus of type 52A which required added CO_2 for normal growth.

Experimental Observations

The CO_2 requirements of these dwarf cocci were studied in parallel with a "normal" staphylococcus of the same bacteriophage type (52A). Some effects of altering the atmosphere and temperature of incubation, and the *p*H and composition of the medium, were observed. In order to standardize conditions all cultures were incubated for 20 hours on single nutrient agar plates in 3.3 litre air-tight jars (unless otherwise stated). Atmospheres are described at the start of incubation because of changes in the gaseous content of the jars during incubation. O_2 and CO_2 gas were obtained from cylinders.

Coagulase tests were done with standard solid inocula, since broth cultures masked variations. A 2 mm. wire loaded from a plate culture was emulsified in 0.5 ml. of 1 in 5 human plasma and incubated at 37° C., the tubes being examined half-hourly for four hours and finally after 24 hours.

In the record which follows intermediate stages are omitted and both strains of dwarf coccus are described together because they gave identical results. Colonies termed "recognizable" gave positive catalase and coagulase reactions. Growth described as "normal" corresponded to the control staphylococcus in air. It was found that the diameter of the cocci was about 0.9 μ whatever the size of the colony. The pleomorphic forms described by Wise and Spink (1954) were not encountered.

Table I shows the relation between the size of the colonies of the dwarf coccus and their reactivity. Small colonies of the control were obtained by short incubation.

Atmospheric Carbon Dioxide and Water Vapour, and Times of Incubation.—In jars of air growth of cultures of the dependent coccus was unrecognizable, but when the air was saturated with water vapour (boiled and distilled) small catalase-positive colonies appeared. Fully normal growth did not occur in saturated atmospheres without 0.75% CO_2 or in dry jars without 1% or more of CO_2 throughout incubation, but six hours in CO_2 during incubation enabled some catalase and coagulase to be produced. Even in CO_2 the first eight hours of growth were abnormally slow. Cultures failed when CO_2 was removed by incubating plates above potassium hydroxide solution, whereas the control staphylococcus grew quite well.

Incubated for five days in air, a colony of 2 mm. diameter was attained after 96 hours, whereas the control reached 4 mm. in 48 hours. In a wet jar of air 4 mm. was attained, but only after 96 hours. With added CO_2 the dwarf behaved like the control. Some sealed cultures stored for months grew well in air on primary subculture but then reverted to the dwarf form.

TABLE I

CHARACTERISTICS OF DIFFERENT-SIZED COLONIES OF THE CO_2-DEPENDENT STAPHYLOCOCCUS COMPARED WITH THOSE OF THE NORMAL CONTROL

Description of Colony and Average Diameter	Dwarf Less than 0·5 mm.	Dwarf 0·5 mm.	Recognizable 0·5–1·5 mm.	Normal 1·5–2 mm.	Normal 2–4 mm.
Pigment	None	None	Poor	+	+++
Haemolysis (horse blood)	,,	None	Slight	+	+
Coagulase (by solid inoculum method)	,,	Partial at 4 to 24 hr.	1½–4 hr.	½–1½ hr.	Less than ½ hr.
Catalase	None	None	Variable	+	+

At comparable colony sizes the control produced more pigment, haemolysin, catalase, and coagulase than did the CO_2-dependent coccus.

Oxygen.—With 1% or more of CO_2 the dwarf coccus grew like the control under atmospheric conditions. Under anaerobic conditions in a MacIntosh-Fildes jar it grew like the control to a diameter of 0.5–1.5 mm., but when KOH or soda lime was added to absorb CO_2 the dwarf coccus failed to reach 0.1 mm. in diameter, while the control reached 0.5–1.5 mm.

Bicarbonate Solutions.—Enough CO_2 for the dwarf staphylococcus to grow normally was released by 100 ml. of 1% aqueous solution of $NaHCO_3$ placed at the bottom of a jar. It was calculated that, at 37° C., 0.75% of free CO_2 would be present in such a jar. Even better growth occurred over a 1.5% bicarbonate solution, corresponding to 0.9% CO_2, but no further improvement resulted from increasing concentration above this level.

H-ion Effects.—Experiments were repeated using media adjusted to *p*H 8.4 and to *p*H 6. The growth of the dwarf organism was not affected and in no case was the final *p*H of the medium outside these limits.

Temperature.—In air the dwarf coccus grew better at 30° C. (diameter 0.5–1.5 mm.) than at 37° C. (diameter less than 0.5 mm.), although in CO_2 it, like the control, grew best at 37° C. (2 mm.).

Some of these details are presented in Table II, in which the results were obtained after 20 hours' incubation on single nutrient agar plates in 3.3 litre sealed jars.

Carbon Dioxide in the Medium

Baryta absorption tests showed that, while nutrient agar (Davies N.Z. powder, autoclaved once) incubated under standard conditions itself released variable amounts of CO_2, greater amounts of CO_2 were released by media to which "analar" sodium bicarbonate was added at 56° C. (Such plates were dried at 37° C. because at higher temperatures much CO_2 was lost.)

TABLE III

GROWTH ON BICARBONATE-CONTAINING MEDIUM

Concentration of $NaHCO_3$ in the Medium (mg. per 100 ml.)	Diameter of Colonies of Dwarf Staphylococcus (mm.)
None	< 0·5
172	0·5–1·5
200	1·5–2 normal
> 350	< 0·5

TABLE II

SUMMARY OF RESULTS OF EXPERIMENTS VARYING THE CONDITIONS OF GROWTH OF THE CO_2-DEPENDENT STAPHYLOCOCCUS

Conditions at the Start of Incubation						Diameter (mm.) of Colonies at End of Incubation
CO_2 from Cylinder			$NaHCO_3$ Strength of Solution (100 ml. inside Jar)	O_2 in Dry Jars	Time (Hr.) in CO_2 before Transfer to Air (1% to 10% CO_2)	
In Dry Jars	In Anaerobic Jars	In Moist Jars				
*Nil to atmospheric	*Nil			100%	2 and 4	0 to 0·5—unreactive dwarf colonies†
0·5% and 100%	Traces, 1% and upwards*	0·03% to 0·5%	Nil to 0·5% (0·4% CO_2)	Nil* (with 5% and up to 100% CO_2)	6 and 8	0·5–1·5—reactive colonies
1% to 10%		0·75%	1% and 1·25% (0·75–0·9% CO_2)	10–20% (with 5% CO_2)	20	1·5 to 2—normal
		1% to 10%	1·5% or more (1·1% CO_2)			2 to 4—large

The control staphylococcus grew full-sized reactive colonies except where an asterisk indicates small colonies between 0·5 and 1·5 mm. in diameter.

Added $NaHCO_3$, 200–300 mg. per 100 ml. of medium, enabled the dwarf coccus to grow to normal size on unsealed plates in the aerobic incubator. Higher concentrations had an inhibitory effect, and more than 150 mg. of $NaHCO_3$ in blood agar plates caused haemolysis.

Sealed plates grew better than unsealed plates in proportion to the number of hours for which the seal was left intact.

Seals were made with a " plasticine " which did not of itself release appreciable CO_2 (see Wilson, 1930).

Increasing the volume of medium improved growth, and, when one-third or more of the volume of any sealed container was occupied by ordinary nutrient agar, growth was normal.

Other Medium Supplements

Growth was no better on blood, chocolate, serum, egg, glucose, lactose, mannitol, or Bordet-gengou media than on nutrient agar. Bacto-difco yeast extract was also ineffective.

Discussion

Dwarf variants of *Staphylococcus aureus* were selected from cultures in antiseptics by Browning and Adamson (1950) and were later isolated from an abscess by Hale (1951), who found them to be CO_2-dependent. Sherris (1952), who described dwarf staphylococci of types 3C and 52A in two patients, thought that the variation might have been induced in one by penicillin, and Wise and Spink (1954) have selected dwarf variants *in vivo* from patients treated with antibiotics. The isolation of an identical organism from an untreated contact of our first case suggests that, whatever their origin, CO_2-dependent organisms are transmissible and may be primary pathogens. The importance of their identification is obvious.

It is unexpected that so definite a need for CO_2 should be satisfied by 1%, for organisms in need of this gas may demand much higher concentrations (Huddleson, 1943). However, Holm (1954) has reported *Actino bacillus* strains which needed CO_2 yet were satisfied by 0.5%, and he too found humidity an advantage.

In the case of a dependent meningococcus CO_2 has been replaced by adding 0.01% of " bacto "-yeast extract to the medium (Tuttle and Scherp, 1952), but this did not satisfy our staphylococcus.

The CO_2 requirement of the dwarf staphylococcus was clearly lower in a water-saturated than in a dry atmosphere. Possibly the entire H_2CO_3 molecule is better absorbed than CO_2, for this is true of other biological cells (Höber, Hitchcock, Bateman, Goddard, and Fenn, 1945).

When metabolism was slowed by anaerobiosis or cool incubation, the dependent coccus grew like the normal control at CO_2 concentrations below 1% ; but if either aerobic or anaerobic cultures were exposed to KOH such further CO_2 deprivation prevented visible growth of the dwarf coccus without much reducing the colony size of controls ; thus the dwarf coccus required abnormal quantities of CO_2 even for anaerobic respiration. After growth in 10% CO_2 the pyruvate dismutation of Hale's strain proceeded normally. His theory that therefore the dependent staphylococci stored enzymes only while growing in CO_2 might explain our findings.

The catalase and coagulase reactions of Hale's strain were not described. When our dwarf staphylococcus was grown without adequate CO_2 both were negative, resembling the G strains of Wise and Spink (1954). Coagulase, catalase, haemolysis and pigment production per unit weight of cells of the dwarf coccus were all increased by increasing the number of hours of growth in 1% or more of CO_2.

Wilson (1931a and b) stated that bicarbonate did not enhance the growth of *Br. abortus*. Andersen (1951), however, found that it did replace CO_2 in sporulating cultures of *Cl. botulinum*, and our medium containing 0.3% sodium bicarbonate provided enough CO_2 for the dependent staphylococcus to grow normally in air. Since there is no evidence that the HCO_3 radicle is utilized directly the success of this medium simply reflects the tolerance of an alkaline *p*H and the low critical threshold for CO_2 of the organism.

Summary

Carbon-dioxide-dependent strains of a haemolytic *Staphylococcus aureus* type 52A were isolated in pure culture from two members of one household.

These organisms were not recognizable as staphylococci on plate cultures incubated for 20 hours in air, for they grew as tiny white G-colonies which were catalase and coagulase negative and might have been streptococci.

With 1% or more of CO_2 growth was like that of a normal control *Staphylococcus aureus*.

Less CO_2 (0.75%) was needed in humid atmospheres.

The coccus grew better at 30° C. than at 37° C. in air, but in CO_2 it grew best at 37° C.

Catalase and coagulase production varied with colony size, which depended upon the amount of CO_2 available in terms of time and concentration.

Sodium bicarbonate was a useful source of CO_2, either in solution within the incubating jar or as 0.3% of the medium.

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