STUDIES ON STAPHYLOCOCCUS MUTATION: A NATURALLY OCCURRING "G" GONIDIAL VARIANT AND ITS CARBON DIOXIDE REQUIREMENTS.

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SMALL colony variants have been described by many workers. They have been selected from cultures with the aid of inhibitory media, and a few have sometimes been seen among normal colonies on plates obtained from lesions. In the case reported a pure culture of a small colony variant staphylococcus was obtained. A patient of this hospital developed an abscess and culture of the pus on blood agar plates resulted in a pure growth of minute colonies, smears of which showed typical staphylococcal morphology. Plates from the same material incubated in the presence of CO_2 gave a growth of typical large staphylococcal colonies. This variant was not only isolated from the abscess, but was also shown to be carried in the patient's nose.

The effect of CO_2 in enhancing the growth of many bacterial species is well known, but its action as an essential factor for normal growth of a staphylococcus appears to be a new phenomenon. Furthermore, the isolation of a small colony variant in pure culture from a well-developed lesion raises certain points of both theoretical and practical importance. The organism was therefore investigated with a view to elucidating the role of CO_2 in the metabolism.

METHODS.

Preparation of bacterial suspensions.—The variant and "wild type" were grown on blood agar, both in air and in the presence of added CO_2. The growth was washed off the plates with physiological saline, centrifuged, washed, re-centrifuged and finally re-suspended in saline to give thick suspensions.

Estimation of dried weight of organisms.—Three aliquots of each thick suspension were pipetted into weighted tubes and an identical quantity of physiological saline (as used in making suspensions) was pipetted into another weighed tube. Tubes were dried at 100° to a constant weight, and the difference in weight between dried suspension and saline enabled the dried weight of the organisms used in the experiment to be calculated.

Measurement of oxygen uptake.—This was measured by the Warburg technique at 37°: 0·5 ml. of cell suspension, 0·5 ml. of substrate and 2 ml. of 0·158 M phosphate buffer, pH 7·2 were placed in the flasks. The final substrate concentration is given in the appropriate tables. Results are expressed as the QO_2 µl. of O_2 per mg. dried cells per hour.

Anaerobic glycolysis.—These measurements were performed at 37° using an atmosphere of N_2 containing 5 per cent CO_2, yellow phosphorus being placed in

the centre well of the Warburg flask to ensure complete anaerobiosis. For these experiments cells were suspended in the physiological salt solution described by Krebs and Henseleit (1932) and the method described by Dixon (1943) was followed. Results were expressed as the QN_2/G µl. CO_2 per mg. dried cells per hour.

Anaerobic dismutation of pyruvate and decarboxylations.—These were carried out by the method described by Smyth (1940). The gaseous phase was nitrogen, 5 per cent CO_2, anaerobiosis being ensured as in glycolysis experiments.

RESULTS,

Effect of CO_2.

Nutrient agar plates seeded with the variant and incubated aerobically for 24 hours yielded small colonies about 0·15 mm. in diamater. The use of blood agar resulted in an even smaller colony. Typical large staphylococcal colonies were obtained on both media after growth in the presence of CO_2, and they retained this large colony form through repeated subculture in these conditions. Reversion to small colonies followed immediately upon aerobic incubation. After 3–4 successive aerobic subcultures of the small variant a few large colonies appeared. These large colonies retained this form and were taken to represent what may be termed the " wild type " staphylococcus, that is, the type of organism from which the small variant was probably derived. Colony sizes of this " wild type " and other strains of staphylococcus were little affected by growth in CO_2; some showed very slight increases, others slight decreases. Incubation of the small colony variant in closed jars containing soda lime for the absorption of all traces of CO_2 resulted in even smaller colonies, whereas the wild and other strains of staphylococci were unaffected. In closed jars without soda lime colonies of the variant were slightly larger than colonies of ordinary aerobic cultures, owing to the accumulation of CO_2 from the organism's metabolism.

The experimental results are summarized in Table I.

TABLE I.—*Effect of CO_2 on Growth of the Variant and Other Staphylococci.*

Strain.	Mean diam. of colonies (mm.) when incubated under following conditions.			
	Air.	10% CO_2.	Closed jar.	Closed jar + soda lime.
Variant (Nutrient agar)	0·15	1·0	0·25	0·01
Variant (Blood agar)	0·05	1·0	—	—
Wild type	1·0	1·0	1·0	1·0
Albus	1·0	1·0	1·0	1·0
Aureus 18L	1·0	1·0	—	—
Aureus 35L	0·7	1·0	—	—
S. Newman	1·0	1·0	—	—
Aureus E	1·1	1·5	—	—
Aureus 709	0·9	0·9	—	0·9
Aureus 719	0·9	0·9	—	0·9

Medium unless otherwise stated is nutrient agar.

Effect of pH.

To test the possibility that CO_2 exerted its effect by an alteration of pH, nutrient agar plates with a pH range from 5·8 to 8·4 were poured. Duplicate plates were seeded with the variant; one incubated aerobically and the other in the presence of CO_2. Table II shows that over the whole pH range tested, CO_2 caused an increase in the colony size of the variant. It is, therefore, improbable that stimulation of the variant's growth by CO_2 is the result of pH changes.

TABLE II.—*The Effect of pH on the Growth of the Variant.*

Nutrient agar pH.	Mean diam. of colonies (mm.). Incubation in air.	Mean diam. of colonies (mm.). Incubation in 10% CO_2.	Hours' incubation.
5·8	0·02	0·25	17
6·0	0·03	0·3	17
6·4	0·03	0·3	17
6·8	1·0	3·0	48
7·4	0·3	1·2	17
7·6	0·3	1·2	17
8·0	1·1	3·2	48
8·4	1·4	3·5	48

Attempts at replacement of the CO_2 requirement by other metabolites.

The use of enriched media, serum or yeast extract agar did not improve the growth of the variant. Pappenheimer and Hottle (1940) demonstrated an interesting relationship between adenylic acid and CO_2 tension. They found that a Lancefield Group A streptococcus required adenine or adenylic acid for growth, but that an absence of these factors could be offset by an increase in the CO_2 tension. In view of this finding, adenylic acid was added to media used for the growth of the variant, but it was without effect.

METABOLIC EXPERIMENTS.

Table III shows that with the substrates tested, the oxygen uptake of the variant is lower than that of the "wild type" when both have been grown aerobically. Growth in the presence of CO_2 has little effect on the oxygen uptake of the "wild type," whereas in the case of the variant, oxygen uptakes are restored to values that approach those exhibited by the "wild type." Thus it would appear that the variant strain, when grown in CO_2, elaborates enzyme systems identical with those of the "wild type," although subsequent culture in the absence of CO_2 results in a loss of one or more enzyme systems so that this abnormal small colony type of growth is resumed.

Krebs and Johnson (1937) have shown that fumarate stimulates the respiration of certain tissues through the agency of a tri-carboxylic acid cycle. When pyruvate is used as the substrate, fumarate stimulates the oxygen uptake both of the "wild type" and the variant grown in CO_2, but is without effect in the case of the variant grown in air.

TABLE III.—*Oxygen Uptake of the Variant and "Wild Type."*

	QO_2 of suspensions.			
	Variant grown in		"Wild type" grown in	
Substrate.	10% CO_2.	Air.	10% CO_2.	Air.
Glucose 0·025 M	−157·5	−57·9	−178·8	−144·6
Peptone 0·1%	−206	−117·2	−220·4	−197·2
Pyruvate 0·043 M	−82·8	−33	−116	−111·7
Fumarate 0·043 M	−40·1	−11·7	−41·6	−39·9
Pyruvate 0·043 M + Fumarate 0·043 M	−136·3	−35·5	−152·9	−147·5

Dismutation of pyruvic acid.

Krebs (1937) demonstrated in staphylococci a dismutation of pyruvate to lactate and acetate with the liberation of CO_2. Table IV demonstrates the relative capacity of the variant when grown in air to carry out this reaction. After growth in CO_2 this capacity was restored. Some increased activity was also observed with the "wild type" grown in CO_2. Lipmann (1937) and Hills (1938) studied this reaction in *Lactobacillus delbrückii* and staphylococci and found that co-carboxylase (a derivative of Vitamin B_1), adenine dinucleotide, magnesium or manganese ions and inorganic phosphate were required as co-factors. The addition of vitamin B_1 to suspensions of the variant did not stimulate pyruvic acid dismutation, therefore there was probably no deficiency of co-carboxylase Yeast extract added to the reaction mixtures increased the velocity of the dismutation, both with the variant and the "wild type." Although the velocity increased in both cases, the difference between the variant grown in air and in the presence of CO_2 remained obvious. In preparation of cell suspensions, coenzymes and accessory factors are probably lost during the washing, so that their replacement by the addition of yeast extract would account for the general stimulation. The loss of activity shown by cells stored in the refrigerator is also restored by the addition of yeast extract. Thus it appears that the variant grown in air is deficient in the actual apoenzyme and not in coenzymes or accessory factors.

TABLE IV.—*Anaerobic Dismutation of Pyruvic Acid and Decarboxylation of Oxaloacetic Acid by the Variant and "Wild Type" Staphylococcus.*

	QCO_2 of suspensions.			
	Variant grown in		"Wild type" grown in	
Substrate.	10% CO_2.	Air.	10% CO_2.	Air.
Pyruvate 0·172 M	+28	+15·7	+40	+31
Oxaloacetic 0·172 M	+14·7	+4	+10·6	+9·6

Decarboxylation of oxaloacetate and α-ketoglutarate.

Krampitz and Werkman (1941) isolated an enzyme from *Micrococcus lysodeikticus* that decarboxylated oxaloacetate to pyruvate. This enzyme, working in the reverse direction, was considered by many workers to be responsible for

CO_2 fixation in organisms (Wood and Werkman, 1935, 1936, 1940*a*, *b* ; Werkman and Wood, 1942 ; Wood *et al.*, 1941). Both the variant and the " wild type " decarboxylated oxaloacetate, but, as Table IV shows, the same differences are seen as when pyruvic acid is decarboxylated. The decarboxylation of α-ketoglutarate is one of the reactions in the tricarboxylic acid cycle postulated by Krebs and Johnson (1937), but both the variant and " wild type " failed to decarboxylate this compound.

Inadequate utilization of pyruvate by variant strain.

The anaerobic glycolysis of the variant, whether grown in air or in the presence of CO_2, was found to be the same as that of the " wild type." Pyruvic acid production should be the same when the suspensions of the variant, grown either in air or in 10 per cent CO_2, are incubated with glucose. As the subsequent dismutation of the pyruvic acid would be slower with cells grown in air, it should be possible to demonstrate a greater accumulation of pyruvic acid than with cells grown in CO_2. In order to test this hypothesis the variant staphylococcus was seeded on blood-agar plates, some of which were incubated in air and others in CO_2. After harvesting in the usual way, 0·5 ml. of each suspension was pipetted into flasks containing 9·5 ml. of 0·25 per cent glucose in isotonic phosphate buffer pH 7·2, which were incubated for 1 hour at 37°. At the end of this period 0·5 and 1·0 ml. samples were removed from each flask, the cells removed by centrifuging and the keto acids present in the samples estimated by the technique described by LePage (1950). In the mixture containing the variant grown aerobically, 3 μg. pyruvate per mg. of dried cells was detected. But when CO_2 was used, no trace of pyruvate was found. In neither instance was oxaloacetate or α-ketoglutarate detected in the samples.

DISCUSSION.

The small colony variant described was isolated in pure culture from a typical staphylococcal lesion and was also carried in the patient's nose. It is therefore probable that conditions in the body gave the variant a survival value over the parent strain. The case reported is probably not an isolated example, and the use of antibiotics may well provide selective conditions for a wide range of small variants. The growth of such variants, however, is so scanty that they could easily be missed in routine cultures from lesions where they were the sole invading organisms, unless the possibility of their existence were borne in mind.

The experiments reported above show that the small variant has a diminished capacity to bring about the dismutation of pyruvic acid and decarboxylation of oxaloacetate. When the variant is grown in the presence of CO_2 these capacities are restored to normal.

Smyth (1940), working in Krebs' laboratory, confirmed the work of Hills (1938) that vitamin B_1—deficient staphylococci exhibited a diminished capacity for pyruvic dismutation. He further demonstrated that this reaction could not only be restored by the addition of vitamin B_1, but also by oxaloacetate or fumarate. The function of the oxalocetate or fumarate was, he thought, to act as a carrier:

$$\text{Pyruvate} + \text{Oxaloacetate} \longrightarrow \text{Malate} + \text{Acetate}$$
$$\text{Pyruvate} + \text{Malate} \longrightarrow \text{Lactate} + \text{Oxaloacetate},$$

the function of the vitamin B_1 being to catalyse the reaction,

Pyruvate + Carbon Dioxide —→ Oxaloacetate,

and thus supply the carrier.

Addition of oxaloacetate to the pyruvate failed to restore the ability of the variant grown in air to bring about the dismutation of the pyruvate. The failure to restore activity by use of these compounds or the addition of known coenzymes is very suggestive that the deficiency is one of the apoenzymes.

Smyth (1940) was of the opinion that the tricarboxylic acid cycle did not function in the staphylococcus, but that there was a modified Szent Gyorgyi cycle in operation. The results presented confirm this view since no decarboxylation of α-ketoglutarate could be demonstrated. Smyth apparently believes that the same enzyme functions both in the dismutation of the pyruvate and in the production of the oxaloacetate. On the other hand Krampitz and Werkman (1941) suggest that the enzyme bringing about the dismutation of pyruvate is quite distinct from the enzyme they obtained, which decarboxylated oxaloacetate. This enzyme, working in reverse, is, they state, responsible for carbon dioxide fixation and oxaloacetate production.

It is possible to advance a tentative hypothesis that will account for the finding that, after growth in the presence of carbon dioxide the variant cells have the same enzymic content as the " wild type." The increase in carbon dioxide tension will probably facilitate the reaction,

Pyruvate + CO_2 —→ Oxaloacetate,

by swinging it in favour of oxaloacetate production. In so doing the enzyme concerned may be adaptively increased and, accepting the view of Smyth that this enzyme is also concerned in the dismutation of pyruvate, then this (metabolic mechanism) will also be restored. As the capacity to produce this enzyme is dependent on an increased carbon dioxide tension, the stimulus to production of the enzyme will cease to operate under aerobic conditions, so that the cells will again become deficient.

The normal staphylococcus grown in air must be able to synthesize adequate amounts of the enzyme or enzymes postulated. In the case of the variant, the genetic control of the enzymes may be altered in such a fashion that enzyme synthesis is impaired, but still capable of adaptive increase under the stimulus of carbon dioxide. If the view of Krampitz and Werkman that there are two different enzymes involved, one decarboxylating oxaloacetate and the other causing pyruvate dismutation, is correct, then the above reasoning explains only the restoration of oxaloacetate decarboxylation. Since this work was completed, however, Watt and Werkman (1950) have shown that the reaction,

Pyruvate —→ Acetate + Lactate + CO_2,

is reversible and is a method of CO_2 fixation in the staphylococci. The hypothesis postulated can equally well be applied to this reaction.

SUMMARY.

1. A small colony variant of staphylococcus isolated in pure culture from an abscess was found to give the normal large colony type of growth in the presence of 10 per cent CO_2.

2. This variant was found to bring about dismutation of pyruvate much more slowly than did normal staphylococci, and addition of coenzymes and accessory factors did not restore this ability.

3. After growth in carbon dioxide pyruvate dismutation proceeded normally, and therefore the cells must build up a full enzymic content in the presence of this gas, but fail to do so in its absence.

4. The dismutation of pyruvate is also a reaction by which CO_2 is fixed, and it is tentatively suggested that increased CO_2 tension favours this reverse reaction and in so doing results in an adaptive increase of the enzyme concerned.

5. The significance and the implications of the isolation from a patient of a small colony variant in pure culture are discussed.

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