PHYSIOLOGICAL STUDIES ON SPORE GERMINATION, WITH SPECIAL REFERENCE TO CLOSTRIDIUM BOTULINUM

III. Carbon Dioxide and Germination, with a Note on Carbon Dioxide and Aerobic Spores[1]

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The requirement of CO_2 for vegetative cell development of bacteria is common knowledge and needs no review here, but scarcely anything is known of the relation of CO_2 to the process of bacterial spore germination as distinct from subsequent vegetative development. One might consider the latter in the sense of transition from heat-stable to heat-labile form (Wynne and Foster, 1948*a*). Relevant is the incidental observation that spores of one out of three strains of *Clostridium botulinum* failed to produce colonies in 72 hours when incubated in a vacuum (Morrison and Rettger, 1930).

It is rather common to discover that special efforts to eliminate CO_2 from the culture system, and to minimize the formation of CO_2 by the cells in the inoculum by supplying a low nutrition level medium, result in a retardation of growth that may extend indefinitely.

Our study of factors determinant in the germination process itself (as distinct from subsequent vegetative development) of *Clostridium botulinum*, begun in two previous papers (Wynne and Foster, 1948*a*, *b*), has included examination of the CO_2 effect. This stems from the finding that anaerobiosis secured by alkaline pyrogallol seems to delay germination of botulinum spores. Background information and general methodology are covered in the first of these papers and need not be reiterated here. To secure anaerobic conditions free of CO_2, vacuum desiccators containing the culture tubes were evacuated with a Cenco Hyvac pump for 30 to 60 minutes and refilled with natural (illuminating) gas (CH_4) cleansed of CO_2 by slow passage through a gas-washing train consisting of three bottles of NaOH and one of N/10 $Ba(OH)_2$. The latter was second last in the chain, functioning as a CO_2 indicator. As an added precaution normal NaOH was always placed in the bottom of the desiccator. Where a CO_2 atmosphere was required, it was added from a cylinder or generated in the desiccator by mixing excess acid with the calculated amount of solid $NaHCO_3$. Unless otherwise specified germination always took place in Difco brain-heart infusion broth with BBL thioglycolate supplement, and always the inoculum was about 500 spores per ml of medium. Table 1 compares the spore germination in atmospheres con-

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taining 0, 1, and 5 per cent CO_2. The CO_2 effect is striking. Germination is negligible in the absence of CO_2, whereas almost all the spores germinated in the presence of CO_2, the higher CO_2 tension being somewhat better. The difference between the two CO_2 treatments actually was greater than it appears; turbidity developed in 15 hours in the 5 per cent CO_2 desiccator and in 19 hours in 1 per cent CO_2. No turbidity appeared in the zero CO_2 control at 22 hours, the termination of the experiment.

However, a CO_2 effect could not be obtained for four other species of anaerobic sporeformers tested similarly: *Clostridium chauvei*, *Clostridium histolyticum*, *Clostridium perfringens*, and the well-known food spoilage organism designated as putrefactive no. 3679. This was true even at pH 6.0, which was chosen to reduce the solubility of CO_2 in the medium and which was the lowest pH supporting germination of these anaerobes. Thus under identical conditions germination of *C. botulinum* spores is inhibited by lack of CO_2, and germination of

TABLE 1

Effect of CO_2 concentration on germination

INCUBATION	CO_2 TENSION	RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
hr	%			%
0	—	560	—	—
22	0	520	40	7
22	1	90	470	84
22	5	18	540	97

the other four anaerobes is not. A possible interpretation of this is given in the discussion below.

The *C. botulinum* experiment described above (see also table 1) demonstrates only a rate effect under the conditions used, for whereas only 7 per cent germination occurred in the CO_2-free control at the 22-hour period, prolongation of incubation always resulted in high germination and pronounced turbidity. Failure to demonstrate an absolute CO_2 effect in brain-heart medium, even with several painstaking experiments involving modification of pH, exhaustive pumping, omission of the colloidal agar of the anaerobic supplement, continuous gassing with N_2, etc., was considered on two counts to be probably tied up with the complex nature of the brain-heart medium: (1) The CO_2 effect can be accentuated by eliminating complex media in favor of synthetic (Gladstone, Fildes, and Richardson, 1935) or by employing complex media at a minimal nutritional concentration, i.e., with respect to carbohydrate and protein content (Rockwell and Highberger, 1926, 1927). The first count may be considered to anticipate the issue of the second. (2) CO_2 should be dispensable so long as certain organic substances are present in whose synthesis CO_2 participates. The presence of such substances is likely in complex media of biological origin, and in such media, therefore, the need for CO_2 should be obviated. While our attack on these lines

was under way other reports appeared that confirm the logic of this approach (C_4 dicarboxylic acids, Ajl, White, and Werkman, 1947; aspartic acid, Lardy *et al.*, 1947; Lardy, 1947).

A synthetic medium similar to that devised by Roessler[2] (1946) for the growth of *C. botulinum* was used as a starting point, but with only one-tenth the regular concentration of amino acids. (Results were similar, however, with the full medium, which contained 1 per cent amino acids.) This medium supported abundant vegetative development of our strain of *C. botulinum*, and spore germination was much slower than in complex media, seemingly opening an approach to factors essential for germination, including those involving CO_2 and those not. For good anaerobic growth it was expeditious to add 0.2 per cent glucose to the synthetic media, as germination is negligible in its absence.

With an inoculum of around 500 spores per ml, clear-cut turbidity developed in the presence of CO_2 at about 72 hours, but counts at 87 hours showed that

TABLE 2

Quantitative indefinite inhibition of germination of C. botulinum spores due to the absence of CO_2

CO_2 IN ATMOSPHERE	INCUBATION	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
%	*days*			%
—	1	560	—	—
0	17	500	60	11
1	5	220	340	61

* Counts corrected for volume loss of 9 per cent during prolonged incubation over NaOH. No turbidity developed in any of the tubes in this series.

only about 15 per cent of the spores had germinated. In subsequent work a 5-day incubation period was employed for positive CO_2 controls, for in this time germination counts were well over 50 per cent. In such a medium it is possible to approach an absolute CO_2 requirement for germination. Thus in an experiment in which the positive (1 per cent) CO_2 control showed 61 per cent germination in 5 days, the CO_2-free treatment showed only 11 per cent germination and no turbidity even after 17 days (table 2). Indeed, the figure of 11 per cent may not be significant at all owing to the fact that the spore-counting method has an over-all accuracy of ± 9 per cent and occasionally has spread wider than this (Wynne and Foster, 1948*a*). Under these conditions clear-cut turbidity always

[2] This medium had the following composition: *dl*-leucine, 0.0083 M; *dl*-phenylalanine, 0.0132 M; *l*-arginine, 0.0065 M; *dl*-valine, 0.0083 M; *dl*-isoleucine, 0.004 M; *l*-tryptophane, 0.11 M; *l*-tyrosine, 0.0003 M; *dl*-methionine, 0.002 M; *dl*-threonine, 0.0067 M; *dl*-serine, 0.01 M; *l*-histidine, 0.0013 M; biotin, 5 mμg per ml; PABA, 0.02 μg per ml; nicotinamide, 1 μg per ml; thiamine, 0.2 μg per ml; yeast nucleic acid, 0.01 per cent; Na-thioglycolate, 0.05 per cent; $MgSO_4$, 0.0002 M; $MnSO_4$, 0.0001 M; $CaCl_2$, 0.0001 M; $FeSO_4$, 0.00005 M; K_2HPO_4, 0.015 M; KH_2PO_4, 0.015 M.

follows germination within a few hours. Thus probably no germination at all occurred in the CO_2-free tube, the 11 per cent value doubtless being an experimental counting error.

It seems safe, therefore, to conclude that CO_2 is absolutely essential for spore germination of *C. botulinum* in a medium otherwise adequate for that process. This apparently is the first demonstration of CO_2 requirement specifically for the germination process, and apart from subsequent vegetative development.

TABLE 3

Effect of oxalacetate on germination in CO_2-free gas phase

Experiment A

INCUBATION	CO_2 IN ATMOSPHERE	OAA, 10^{-3} M	EXHAUSTION PERIOD	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
hr	%		*minutes*			%
0	—	−		535	—	—
20	0	−		460	75	14
20	1	−		340	195	36
20	0	+		74	460	86
			Experiment B			
0	—	−	—	520	—	—
23	0	0	30	470	50	10
23	0	+	30	35	485	93
23	0	+	270	65	455	88
23	1	0	30	21	500	96

BY-PASSING CARBON DIOXIDE

Oxalacetic acid. Along the lines discussed under count (2) above, the C_4 dicarboxylic acids were tested for their ability to permit germination in the absence of CO_2, as the universality of the Wood-Werkman reaction via pyruvate fixation of CO_2 indicates the likelihood of their being involved here. The primary fixation product, oxalacetic acid (OAA), is generally in biological equilibrium with malic, fumaric, and succinic acids, all vital catalysts or intermediates in cells. OAA in brain-heart media definitely promotes the germination rate of spores in the absence of gaseous CO_2 (table 3) and apparently by-passes CO_2. Experiment A in table 3 shows that the OAA induced spore germination at a rate appreciably faster than a 1 per cent CO_2 gas tension, and in experiment B it was equal to the CO_2 in promoting germination. The chances are that OAA would have induced faster germination in experiment B also had the counts been made at a shorter incubation period.

The OAA effect might, to a certain extent, be ascribed to CO_2 resulting from the spontaneous decomposition of OAA to CO_2 and pyruvic acid (Krampitz and Werkman, 1941; Krebs, 1942). OAA in solution at 37 C has a very short half-life and its decomposition is catalyzed by amino groups and by traces of cationic

metals. However, since OAA gives a germination rate exceeding that of CO_2, the effect seemingly is due to the OAA per se, though CO_2 may contribute to the rate partially. Germination by OAA was not retarded when the medium was continuously exhausted with a Hyvac pump for 4.5 hours after OAA addition, the idea being to remove quickly any CO_2 generated from OAA (exp. B., table 3). As no lessening of the OAA effect by this continuous CO_2 removal was observed, the probability of a direct OAA participation seems good.

Maybe a brief contact with CO_2, such as would occur in the pumping experiment mentioned above, would suffice for germination, but other experiments showed that contact with a 1 per cent CO_2 atmosphere for the initial 4 hours, followed by removal ("hyvac") and replacement with CO_2-free gas had an insignificant effect on germination.

Stable C_4 dicarboxylic acids. A mixture of *l*-malic, fumaric, and succinic acids (Na salts), each at a concentration of 3.3×10^{-4} M, was shown repeatedly to have a definite acceleration on germination rate in the absence of CO_2. These acids were not as effective as CO_2 (or OAA) in promoting germination. The efficacy of these acids in promoting spore germination was roughly about one-third that of a 1 per cent CO_2 gas phase. The inability of the acids to substitute fully for OAA has been encountered previously (Shive and Rogers, 1947, and others) and probably relates to membrane penetration at pH values in physiological range, in which these acids are almost 100 per cent dissociated. It will be recalled that OAA itself does not penetrate unaltered cells of *Micrococcus lysodeikticus* (Krampitz and Werkman, 1941), and several other examples could be given. If these acids diffuse in the molecular (undissociated) form as do the free acids, it would be expected that diffusion would be greatest at pH 3 to 4, as the acids are almost entirely in molecular form in this range as contrasted to a negligible percentage at pH 6 or above. It was not possible to test this with *C. botulinum*, as germination is inhibited at pH values below 6. It will be recalled that Ajl, White, and Werkman (1947) found that the C_4 dicarboxylic acids or their respiratory precursors by-passed the CO_2 requirements for coliform bacteria.

The specificity of the effect for the C_4 dicarboxylic acids on botulinum germination is exemplified by the fact that no demonstrable action was given by α-ketoglutarate, glutarate, valerate, butyrate, propionate, lactate, or pyruvate. On the other hand, a striking stimulation in vegetative development was induced by all these acids (except pyruvic) at 10^{-3} M. So marked was this that cultures with well-advanced turbidities showed surprisingly small germination percentages. This is a fine example of the fallacy of judging germination rates by the intensity of vegetative turbidity.

Aspartic acid. As OAA is converted to aspartic acid by transamination, one might expect that this amino acid also would by-pass the CO_2 requirement, the latter participating in the synthesis of aspartate. This has indeed been demonstrated for *Lactobacillus arabinosis* (Lardy *et al.*, 1947; Lardy, 1947), in which case aspartate is apparently the only constituent of cell material in the synthesis of which CO_2 participates, excepting perhaps for relatively insignificant amounts

of other components. This was proved by isotopic CO_2, substantially the entire content of the labeled C in the cells being in the carboxyl groups of the cellular aspartate. It is likely that the other C_4 dicarboxylic acids are converted to aspartate via OAA.

The germination tests were conducted in Roessler's synthetic medium (1/10 strength amino acids) which, as a basal medium, lacked $NaHCO_3$, biotin, and aspartic acid. Preliminary experiments indicated that neither biotin + aspartate nor sodium oleate + aspartate could by-pass CO_2. (Oleate was tested because of its known biotin-sparing action.) Mixtures of biotin (5 mμg per ml), aspartate (10^{-3} M or 10^{-4} M), and oleate (1, 10, or 100 mμg per ml) were also tested, but germination was insignificant in the absence of CO_2 after 14 days' incuba-

TABLE 4

Effect of complex supplements on germination

INCUBATION	1% CO_2	SUPPLEMENT ADDED	AVG COUNT RESIDUAL SPORES	GERMINATED SPORES	GERMINATION
days					%
0			450		
1	+	1% yeast extract	15	435	97
1	+	1% liver extract	230	220	49
1	+	1% brain heart	35	415	92
2	−	1% yeast extract	45	405	90
2	−	0.1% yeast extract	120	330	73
2	−	1% liver extract*	385	65	14
2	−	1% brain heart*	50	400	89
5	+	None	160	290	64
15	−	None	425	25	5
15	−	1% liver extract†	475	0	0
15	−	0.1% liver extract	475	0	0
15	−	1% brain heart†	430	20	4
15	−	0.1% brain heart	410	40	9

* One out of three tubes, of which † represents remaining two.

tion. The control medium in the presence of CO_2 showed 50 per cent germination at 7 days.

Finally, the following known or suspected by-passing substances and available participants in the tricarboxylic acid respiratory system were tested in combination, all at 10^{-3} M in basal synthetic medium, in the presence and in the absence of CO_2: aspartate, malate, fumarate, succinate, α-ketoglutarate, glutamic acid, glutarate, and *cis*-aconitate. These were entirely unsuccessful in by-passing CO_2. When CO_2 was present, the germination rate was unaffected in this medium, a fact indicating no toxicity caused by the supplements.

Complex supplements. Also, the following complex organic supplements were tested in triplicate tubes at 0.1 and 1.0 per cent levels in the basal synthetic medium, again in the absence and in the presence of CO_2: brain-heart infusion, liver extract,[3] and yeast extract, all Difco. The CO_2-free yeast and liver treat-

[3] Extract of 0.1 and 1.0 per cent dried liver.

ments were incubated in one desiccator, the CO_2-free brain-heart in another, and the CO_2-free synthetic medium in another. Within 40 hours in the absence of CO_2 all the yeast tubes, a single 1 per cent liver tube, and a single 1 per cent brain-heart tube developed marked turbidity. To avoid contaminating the other tubes with fermentation CO_2, these turbid tubes were removed, pasteurized, and held for spore counts. All the tubes of synthetic medium in CO_2 showed turbidity at 3 to 4 days and were removed for counting on the fifth day. The CO_2-free synthetic medium showed no turbidity even after 15 days, the termination of the experiment, and the remaining liver and brain-heart tubes in CO_2-free atmosphere behaved similarly. Residual spore counts for this experiment are in table 4.

It is clear that yeast contains CO_2 by-passing factor (s) that are not identical with the supplements added to the basal medium, because CO_2 was necessary for germination in the latter treatment but not in the yeast. Yeast apparently is richest in the unknown by-passing factor(s), as the liver and the brain-heart were greatly inferior in this respect. The rapid growth in the yeast tubes in the same desiccator as the negative liver tubes shows that the effect resides specifically in their contents of CO_2 by-passing substances and not in a CO_2 leak or other artifact leading to the unintentional presence of CO_2, for the smallest amounts of CO_2 induce rapid germination in the liver (and brain-heart medium). One will note that even the amounts of CO_2 generated by the turbid yeast tubes were insufficient to induce significant germination in brain-heart medium.

AEROBIC SPOREFORMERS

Some testing of a survey nature was done with four species of aerobic sporeformers: *Bacillus brevis*, *Bacillus megatherium*, *Bacillus mesentericus*, and *Bacillus subtilis*. Germination occurred in Difco nutrient broth in shallow layers in 50-ml Erlenmeyer flasks at room temperature. CO_2-free treatments were conducted in desiccators with air as the gas phase. In no case was it possible to retard germination of these organisms in a CO_2-free atmosphere. Evidently this is due to the presence in the nutrient broth of organic substances by-passing the CO_2, although no attempt was made to confirm this with synthetic media. Interestingly enough, though CO_2 did not enhance the germination of any of these four aerobes, in one, *B. mesentericus*, the stable C_4 dicarboxylic acids mixture (3.3×10^{-4} M each) distinctly accelerated the germination. Thus in a typical experiment with an inoculum of 3,040 spores per ml, 29 per cent germination was obtained in the CO_2-free treatment after 25 hours and 74 per cent in the C_4 treatment. This organism presumably was inefficient in the conversion of CO_2 to C_4 dicarboxylic acids.

The behavior of each of these aerobes in respect to vegetative development in relation to CO_2 is in decided contrast to that of the spores, for with each aerobe CO_2 induced a marked acceleration. This again emphasizes the distinction between the germination process and the subsequent vegetative activity of sporeforming bacteria.

DISCUSSION AND SUMMARY

The germination process and vegetative cells are not affected alike by CO_2 and the C_4 dicarboxylic acids. The fact that germination in four out of five anaerobes tested failed to respond to CO_2, whereas in *Clostridium botulinum* it did, indicates species or strain differences. This applied also to the differences described for the four aerobic sporeformers.

A clue to the nature of these effects comes from the fact that in complex media (i.e., brain-heart infusion) CO_2 deprivation only slowed down the rate of germination but did not stop it, whereas in a synthetic medium germination could be entirely suppressed indefinitely without CO_2. Judging from the evidence available, this could mean that present in complex media are substances as yet unknown that can by-pass CO_2. Some such substances are known (see above, C_4 dicarboxylic acids and aspartic acid), but these could not substitute for CO_2 in the germination of *C. botulinum* spores in a synthetic medium that is otherwise adequate for germination and growth. Does this mean, then, that present in complex natural materials are additional new substances capable of by-passing the CO_2 requirement, in whose synthesis CO_2 participates when they are not supplied artificially to the medium? Seemingly the positive germination results obtained in a CO_2-free system upon addition of small amounts of yeast extract to the basal synthetic medium speak in this behalf. After this work was completed, the recent report of Lwoff and Monod (1947) was received. These authors, working with *Escherichia coli*, found C_4 and C_5 dicarboxylic acids and their amino derivatives to be effective CO_2 by-passing agents, but that they alone did not suffice; and they came to exactly the same conclusions as those given above: namely, other essential CO_2 by-passing agents are present in complex natural materials.

One may suspect that CO_2 is involved in the synthesis of biological substances other than C_4 and C_5 acids and the derived aspartic and glutamic acids, and, indeed, at least one other system is already known, viz., carboxylation of α-ketoglutaric acid to oxalsuccinic acid (Ochoa, 1945). Others are under suspicion, and new ones not only are a distinct possibility but on the basis of the foregoing evidence must exist.

Variations in response to C_4 dicarboxylic acids mean that these are required in different degrees by different organisms. Thus, the response by *C. botulinum*, in the complex medium, to added C_4 acids indicates that these were limiting or near limiting in germination. Complete lack of response to CO_2 by the other clostridia indicates that whatever by-passing agents (presumably including the C_4 acids) were present they were sufficient to by-pass CO_2 entirely. Similar differences showed up in the aerobes: though removal of CO_2 did not retard germination in any of the four species, C_4 acids significantly stimulated germination in *Bacillus mesentericus* and were therefore limiting even in the presence of CO_2. It is possible that the C_4 acids may fully by-pass CO_2 in this organism. If C_4 acids play a role in the by-passing of CO_2 in the other three aerobes, the concentration present in nutrient broth must be adequate, though other substances may be involved.

The main conclusion obtainable from all these observations is that organisms differ widely in the extent to which medium components enable them to by-pass their CO_2 requirements and that some hitherto-unrecognized CO_2 by-passing substances exist. A corollary is that a complete diet of organic compounds renders CO_2 dispensable for germination and initiation of growth.

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