ON THE USE OF CALCIUM CARBONATE IN NITROGEN FIXATION EXPERIMENTS[1]

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In reviewing the literature on nitrogen fixation by soil bacteria one is impressed with the great variety of media that have been employed by different investigators. Many of these media, while generally satisfactory, have not proved entirely so when employed by other investigators with slightly different environmental conditions.

It is not the purpose of this paper to enter into any discussion of the relative merits of different media, but rather to call attention to a frequent fundamental difference and its possible bearing upon the success attending their use. This difference is the presence or absence of calcium carbonate.

Winogradsky (*9*)[2] in his original experiments on nitrogen fixation by anaerobic bacteria used a dilute solution of the various salts necessary to furnish the elements essential to growth. To this was added a simple sugar as a source of energy and an excess of calcium carbonate to neutralize the acids formed from the sugar. Winogradsky's medium has been almost universally adopted for anaerobic nitrogen-fixing experiments.

Beijerinck (*2*), studying the aerobic Azotobacter group of nitrogen-fixing bacteria, found that a 0.02 per cent solution of K_2HPO_4 in "Leitungswasser," to which was added a source of energy, furnished the necessary conditions for good growth of these organisms. Either the water or the inoculum must have furnished the other essential elements in sufficient quantity. The reaction of this medium was unaltered, the statement being made that—

> Die Nährlösung reagiert durch das K_2HPO_4 schwach alkalisch

and that—

> Die Alkalisch Reacktion ist für den versuch günstig.

Beijerinck preferred mannite or a salt of propionic acid as a source of energy because—

> Mannit kann nur schwierig und langsam, Propionate durchaus nicht der Buttersäuregärung anheimfallen.

Beijerinck further states that—

> Die Produkte die Oxydation sind Kohlensäure und Wasser.

However, he realized that in impure cultures from soil, organic acids might be formed. Beijerinck failed to secure appreciable fixation of nitrogen by pure cultures.

Lipman (*3*) began a study of the Azotobacter group of nitrogen-fixing organisms shortly after Beijerinck. He demonstrated that Beijerinck's failure to secure fixation in pure cultures was due to the unfavorable re-

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[2] Reference is made by number (italic) to "Literature cited," pp. 189–190.

action of his media. The media adopted by Lipman was composed of tap water 1,000 cc., mannite 15 gm., K_2HPO_4 0.5 gm., $Mg.SO_4$ 0.2 gm., a drop of 10 per cent solution of ferric chlorid, and enough sodium hydroxid to make the solution slightly alkaline to phenolphthalein. Lipman showed that within certain limits the quantity of nitrogen fixed was proportional to the quantity of sodium hydroxid added. He further demonstrated that the addition of $CaCO_3$, even to the medium made alkaline to phenolphthalein with sodium hydroxid, rendered it more favorable for nitrogen fixation. With regard to the influence of calcium carbonate Lipman (*4*) says:

> It is clear therefore, that the presence of calcium carbonate stimulated growth either directly by furnishing calcium, or indirectly by making available more phosphorus, sulphur, and magnesium.

However, Lipman apparently lost sight of the value of calcium carbonate, for he did not recommend its use in his laboratory guide (*5*).

Ashby (*1*), apparently following the lead of Lipman, proposed that the acidity arising from the phosphate be neutralized with sodium hydroxid and in addition an excess of calcium carbonate be added. The medium proposed by Ashby has been more widely used than any other. It has the following composition: Distilled water 1,000 cc., mannite 12 or 20 gm., $Mg.SO_4$ 0.2 gm., KH_2PO_4 0.2 gm., NaCl 0.2 gm., $CaSO_4$ 0.1 gm., and 0.5 gm. of $CaCO_3$ to each culture of 75 or 100 c. c. The phosphate is dissolved separately in a little water and made neutral to phenolphthalein with sodium hydroxid. Ashby found that the presence of calcium carbonate favored nitrogen fixation and that Azotobacter would sometimes develop in the presence of calcium carbonate but would not form a film if the carbonate were left out. Ashby also found that magnesium carbonate was even more efficacious than calcium carbonate, thus showing that calcium was not the essential constituent. Other investigators have since shown that other basic compounds can be substituted for calcium carbonate.

In addition to the three types of media just mentioned Löhnis and his students have made rather extensive use of soil extract to which was added K_2HPO_4 and mannite or some other simple organic source of energy. In comparing the fixation of nitrogen in a medium of this type with and without the addition of calcium carbonate Löhnis and Pillai (*7*) found, as a rule, slightly greater fixation where the carbonate was added. However, Löhnis failed to adopt the use of calcium carbonate generally in his work, or to recommend its use in his laboratory guide (*6*).

It remained for Stoklasa (*8*) to produce the necessary evidence for a correct understanding of the function of calcium carbonate in nitrogen-fixation experiments by demonstrating quantitatively the formation of organic acids in cultures of Azotobacter. A survey of the accumulated literature on the subject will show, however, that many investigators failed to realize the significance of Stoklasa's results.

Practically all investigators agree that a neutral or alkaline reaction is desirable, if not essential, for the best development of nitrogen-fixing organisms. In most work some effort is made to adjust the medium to an alkaline reaction before inoculating, but in many cases no effort is made to maintain such a reaction. Even the influence of the inoculum upon the initial reaction has usually not been taken into consideration.

So far as the writer is aware no one has ever reported a detrimental effect upon nitrogen fixation from the presence of calcium carbonate in

the medium, even when present in large excess. This is an important consideration, however, since if it has no toxic effect upon the organisms it may be added in excess of initial requirements and thereby tend to maintain a favorable reaction throughout the experiment.

There are a number of isolated experiments such as those cited above showing the effect of calcium carbonate upon the growth of nitrogen-fixing organisms. In the course of some experiments, conducted by the writer, in which the relative growth of Azotobacter from a large number of different soils was compared there was an opportunity to observe the effects of $CaCO_3$ on nitrogen fixation.

METHODS

The medium employed had the following composition: Mannite 20 gm., K_2HPO_4 0.2 gm., $MgSO_4$ 0.2 gm., NaCl 0.5 gm., $FeCl_3$ trace, and water 1,000 cc. Two-hundredths gm. of $CaCl_2$ was sometimes added, although in most cases because of the high calcium content of local soils the $CaCl_2$ is not essential and was without effect. In those tests to which no $CaCO_3$ was added the medium was always rendered slightly alkaline to phenolphthalein with sodium hydroxid. When $CaCO_3$ was to be added the medium was sometimes first rendered slightly alkaline to phenolphthalein and at other times the reaction was unaltered prior to the addition of the $CaCO_3$. Fifty cc. of the medium were placed in 300-cc. Erlenmeyer flasks, and the $CaCO_3$ was added in the form of sterile powder just prior to inoculation. No superiority is claimed for this medium over a score of others that might have been used. Obviously a medium with as variable composition as that containing tap water or soil extract would be unsuited for comparative work that must extend over a long period of time.

Samples were always set up in duplicate and total nitrogen determinations made on the whole sample. Total nitrogen determinations were also made in duplicate upon the inoculum. The inoculum consisted of 10 cc. of the supernatant suspension prepared by shaking one part of soil (50 to 100 gm.) with two parts of water and allowing to settle long enough for the larger particles to sink to the bottom. It is believed that such an inoculum is more representative of a mass of soil than 5 gm. of soil, and at the same time the quantity of solid material added is not sufficient to interfere in the least with total nitrogen determinations. Incubation was at room temperature for three weeks. In estimating the quantity of nitrogen fixed that present in the inoculum was deducted. Only the average of check determinations were recorded.

Frequent examinations of the cultures were made, both macroscopically and microscopically to ascertain whether Azotobacter were present. If Azotobacter make an appreciable growth it can usually be recognized by the appearance of the film. A microscopic examination of an unstained mount from such a film will reveal an unmistakable picture. A film is sometimes encountered which at certain stages in its development resembles quite closely an Azotobacter film, which, under the mircoscope, is found to be composed almost entirely of filimentous fungi, no organisms typical of Azotobacter being observed. In other instances nontypical films examined under the microscope would be found to be composed largely of fruiting fungi, the spores of which often closely resembled individual cells of Azotobacter.

If these examinations failed to reveal organisms morphologically similar to Azotobacter they were regarded as absent. Owing to the above-mentioned complex conditions it is quite possible that Azotobacter were sometines reported present when in reality they were absent and vice versa. The end to be gained did not seem to justify the large amount of time that would be necessary to isolate and identify Azotobacter from the various soils. It is believed that if Azotobacter are not present in a soil in sufficient numbers and vigor to develop unmistakable evidence of their presence by the methods just described, for practical purposes they may as well be absent.

RESULTS

Several hundred samples of soil from Kansas and other States have been examined by the methods described above. The following is a comparison of the average quantity of nitrogen fixed by 200 soils.

All samples	5.87 mgm.
Presence of $CaCO_3$	7.10 mgm.
Absence of $CaCO_3$	4.60 mgm.
Azotobacter film formed	7.70 mgm.
No Azotobacter film formed	4.10 mgm.

There were only two samples that failed to show some nitrogen fixation, and both of these were in media containing no $CaCO_3$.

When calcium carbonate was added to the medium an Azotobacter film was formed from 117 samples, or 58 per cent of the soils. The average quantity of nitrogen fixed in these was 8.1 mgm. The average quantity of nitrogen fixed in the 83 samples having no Azotobacter film was 5.7 mgm.

When no calcium carbonate was added to the medium an Azotobacter film was formed from 75 samples, or 38 per cent. These had fixed on the average 7.1 mgm. of nitrogen. One hundred and twenty-four samples, or 62 per cent, produced no Azotobacter film, and the average nitrogen fixed for these was 3.1 mgm.

Twenty-seven samples, or 14 per cent of all soils examined, fixed more nitrogen in the samples to which no $CaCO_3$ was added, while 173 samples, or 86 per cent, fixed larger quantities of nitrogen in those samples receiving an addition of $CaCO_3$. The microscope revealed Azotobacter in cultures from 130 samples, or 65 per cent of all. No Azotobacter were observed in cultures from 70 samples, or 35 per cent of all. Some nitrogen fixation took place in practically all samples inoculated regardless of the source of the soil.

There were 12 samples containing Azotobacter or organisms resembling Azotobacter that failed to form an Azotobacter film. The average nitrogen fixed by these 12 soils where $CaCO_3$ was added was 6.2 mgm. The average in the absence of $CaCO_3$ was 3.1 mgm. This is 0.5 mgm. higher than the average fixed by those giving no film when $CaCO_3$ was added and exactly the same as those giving no film in the absence of $CaCO_3$. It is highly probable, therefore, that some soils contain Azotobacter but are incapable of initiating the growth of an Azotabacter film in a mannite culture solution.

Practically all soils that failed to produce Azotobacter films formed more or less heavy films of fungi in the medium containing $CaCO_3$. As a rule, no such films were formed in the medium containing no $CaCO_3$. Whether or not these fungi are associated with the increased

nitrogen fixation under these conditions is not known. It is possible that the films of aerobic fungi were a factor in maintaining anaerobic conditions and thereby stimulated nitrogen fixation by anaerobic organisms. The fungi were usually slow to develop, indicating that their development depended upon some subsequent change, possibly the accumulation of nitrogen or of calcium salts of some organic acids. The number of samples that failed to develop fungi films were hardly sufficient to give a comparison of the quantity of nitrogen fixed in the presence and in the absence of a film. It is perhaps significant, however, that the average quantity of nitrogen fixed by the 7 samples which failed to grow films of fungi in the presence of $CaCO_3$ was only 2.6 mgm., compared with 6.0 mgm. for the 76 samples producing a film. This would indicate that the fungus growth is in some way associated with the fixation of nitrogen either as a factor or as a result.

It is evident from the preceding data that practically all soils will bring about the fixation of appreciable quantities of nitrogen under the conditions of these experiments. A large percentage of the soils examined however, failed to initiate the growth of Azotobacter. There are, therefore, other organisms which are capable of fixing appreciable quantities of nitrogen. Such organisms seem to be quite widely distributed in nature.

CONCLUSIONS

(1) The quantity of nitrogen fixed in the presence of Azotobacter is greater than when it fails to develop.

(2) The number of soils capable of initiating the growth of Azotobacter under the experimental conditions here described is greater by 20 per cent if $CaCO_3$ is added to the medium than if it is omitted.

(3) The quantity of nitrogen fixed in a medium containing $CaCO_3$ is, for practical purposes, always equal to and in most cases greater than when $CaCO_3$ is not present in the medium.

(4) The presence of $CaCO_3$ exerts a greater beneficial effect upon those organisms, other than Azotobacter, that bring about the fixation of nitrogen than upon Azotobacter itself.

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