STUDIES ON THE CARBON DIOXIDE REQUIREMENT OF NEISSERIA MENINGITIDIS[1]

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Received for publication January 11, 1952

An excellent review of the earlier literature was presented by Valley (1928) by which time it gradually had been proved and accepted that all microorganisms require CO_2 for growth and reproduction. Walker (1932), Winslow *et al.* (1932), and Gladstone *et al.* (1935) related the length of the lag phase to the CO_2 content of the medium or of the cells themselves. Wood and Werkman (1935) opened an entirely new approach to the study of CO_2 utilization when they advanced the concept of heterotrophic assimilation. Since 1935 many papers have appeared dealing with various aspects of the Wood-Werkman and related reactions [reviewed most recently by Utter and Wood (1951)].

Pappenheimer and Hottle (1940) and Lyman *et al.* (1946) considered the role of CO_2 in metabolism in relation to other known growth factors, namely, purines and amino acids and pyridoxine. Lwoff and Monod (1947) found that glutamic acid did not change the growth rate of *Escherichia coli* under optimal CO_2 pressure. If the CO_2 tension was lowered, the growth rate decreased; glutamic acid and other C_4 and C_5 dicarboxylic acids compensated for this effect partially, but at very low CO_2 tensions these compounds were without action. They suggested that other "heterocarboxylic essential metabolites" must be essential for growth. Ajl and Werkman (1948, 1949) reported that glutamic acid, as well as other C_4 and C_5 dicarboxylic acids, would substitute for CO_2 and permit growth of *E. coli* and *Aerobacter aerogenes* in its absence. Gerhardt and Wilson (1950) found that none of the compounds reported as substitutes for CO_2 by Ajl and Werkman (1948) replaced the effect of an atmosphere containing 10 per cent CO_2 for the strains of *Brucella abortus* tested.

It has long been recognized that most strains of *Neisseria meningitidis* require, or benefit by, a concentration of CO_2 greater than atmospheric (Gates, 1919). This organism, therefore, is especially suitable for the study of the CO_2 effect. The present paper concerns the fulfillment of the need of the meningococcus for supplemental CO_2 by various combinations of nutrients and their probable mode of action.

[1] Most of the data presented in this paper were taken from a thesis submitted in June, 1947, by Dorothy M. Tuttle to the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy. The study was supported in part by a contract (N6-ori-126, Task III) between the University of Rochester and the Office of Naval Research. This contract stipulates that "reproduction in whole or in part is permitted for any purpose of the United States Government". Part of the material was presented before the 50th General Meeting of the Society of American Bacteriologists in Baltimore, Maryland, in May, 1950 (Tuttle and Scherp, 1950).

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MATERIALS AND METHODS

Medium. The basic medium used throughout was a slight modification of the one developed by Frantz (1942) and had the following composition: L-glutamic acid hydrochloride, 1.3 g; L-cystine, 0.012 g; $NaH_2PO_4 \cdot 2H_2O$, 2.5 g; KCl, 0.09 g; NaCl, 6.0 g; NH_4Cl, 1.25 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; glucose, 5.0 g; and distilled water to make 1,000 ml. A stock solution of all ingredients except cystine and $MgSO_4 \cdot 7H_2O$ was prepared in one-fifth the final volume. A 0.12 per cent stock solution of cystine was made in 0.1 N HCl. These two stock solutions were combined in the proper proportions, distilled water was added (making allowance for the amount of 1 N NaOH needed to adjust the pH), the pH was adjusted to 7.3, and the volume was adjusted to the correct amount. The medium was sterilized either by autoclaving at 10 pounds for 10 minutes or by filtration through a sterile Selas 02 candle. The $MgSO_4 \cdot 7H_2O$ was made up separately as a 6 per cent solution, sterilized by autoclaving, and added 1 per cent by volume to the rest of the sterilized medium.

All the inorganic salts and the glucose used were Baker's "CP Analyzed" grade. Glutamic acid (Eastman) was purified by converting it to the hydrochloride and recrystallizing it three times from 6 N HCl. Cystine (Pfanstiehl) was dissolved in the minimal quantity of 3 N HCl and recrystallized by pouring the solution into an excess of distilled water. This process was repeated three times. All other amino acids used were from Merck. The casein hydrolyzate used was a "vitamin-free" Smaco product. The following vitamins of the B complex were from Merck: thiamin hydrochloride, riboflavin, pyridoxine hydrochloride, pantothenic acid, and nicotinic acid. A solution of crystalline biotin was generously supplied by Dr. R. R. Sealock. Since pure folic acid was not available at the time of the present experiments, a folic acid concentrate obtained from Dr. R. J. Williams was used. The adenine sulfate and guanine hydrochloride were Eastman products; the guanine was purified by recrystallization. The uracil, thymine, and cytosine were synthesized according to conventional methods. The yeast extract was a Difco product, bacto-yeast extract, which is the water soluble portion of autolyzed yeast.

Cultures. A total of 10 different strains of meningococci were tested in a preliminary survey of the nutritional requirements of this organism. (The customary criteria were used to identify these organisms as strains of *N. meningitidis:* staining as gram negative diplococci, a positive oxidase reaction, the fermentation of maltose and glucose but not of sucrose in phenol red agar (Difco) and serological tests.) A tabulation of these strains, their types, and the least number of organisms necessary for growth in air and in air plus 3 per cent CO_2 appears in table 1. The 520M96 and 69 strains were old laboratory strains, the latter having changed gradually into the avirulent "stock" form (Rake, 1933). All other strains were considered to be freshly isolated since they had been lyophilized after only a few transfers from the primary culture.

As can be seen from table 1, these strains varied widely in the number of organisms necessary for growth in the Frantz medium incubated in air and in the effect of additional CO_2 in reducing this number. Although these data in each case are from a single comparative experiment and it is, therefore, not

implied that the absolute values were precisely reproducible, the over-all pattern was consistent, e.g., in experiments done at different times large inocula were always necessary for the growth of the Campbell, Herrington, and Paolino strains, and the number was not reduced by supplemental CO_2. Strain no. 520M96 was selected as the test organism for the present study because of the consistently great difference between the number of organisms needed to initiate growth in the Frantz medium in air and in 3 per cent CO_2. This allowed a wide range in which to test the substitution of various growth factors for the supplemental CO_2 requirement.

Cultures were preserved by desiccation from the frozen state (lyophile), as reported by Flosdorf and Mudd (1935), to maintain the organism in its original condition. Routinely a new lyophile tube was opened for each experiment. The dried material was taken up in a small amount of sterile distilled water and streaked on a blood agar plate which was incubated in 3 per cent CO_2 at 37.5 C for 24 hours. A "starter culture" was made then by subculturing from a confluent

TABLE 1

A summary of the least number of organisms of strains of Neisseria meningitidis initiating growth in 5 ml of Frantz medium in air and air + 3 per cent CO_2

STRAIN NO.	TYPE	AIR	AIR + 3% CO_2
520M96	I	1.4×10^7	1.4×10
69	I ("stock")	1.8×10^6	1.8×10^2
Card	I	7.8×10^4	7.8×10^3
Freeman	I	4.8×10^5	4.8×10^4
Robarge	I	3.8×10^6	3.8×10^5
43377	II α	3.8×10^7	3.8×10^6
Campbell	I	1.3×10^7	1.3×10^7
Herrington 54	II	1.6×10^6	1.6×10^6
Paolino	I	1.5×10^6	1.5×10^6
Chilson	I	5.3×10^3	5.3×10^3

portion of the growth into Frantz medium which was also incubated under the same conditions. An aliquot of this culture was used to make a faintly turbid suspension of organisms in Frantz medium. Serial dilutions of such a suspension were used in all experiments.

The lyophile cultures were put up in batches of 24 tubes. When one batch was almost depleted, a new one was prepared by passing the organisms from a blood agar plate subculture through a chick embryo. It was thought that this procedure would maintain the uniformity of the cultures, for it had been shown by Buddingh and Polk (1939) that no change in the type specificity, fermentation reactions, or the original virulence for the chick embryo of a strain of *N. meningitidis* took place during 100 daily passages from membrane to membrane. The chick embryo usually was dead in 24 hours at which time the chorioallantoic fluid was removed, checked for contamination by gram stain, and then lyophilized. This menstruum was very satisfactory for lyophile cultures.

Experimental procedures. Two methods were used for testing the effect of

added growth factors on the CO_2 requirement of the 520M96 strain of *N. meningitidis*. In the majority of experiments the serial dilution method used by Gillespie (1913) and Smith (1926) was followed. Parallel series of the various media being tested were run. That is (see table 2), in each experiment two control series, the Frantz medium alone and the Frantz medium plus 3 per cent CO_2, were set up as well as the series of Frantz media to which had been added other growth factors such as yeast extract, the purines and pyrimidines, etc. The effectiveness of a substance in replacing the need for supplemental CO_2 (i.e., the addition, at the start, of a concentration of CO_2 greater than the 0.03 per cent normally present in air) was determined by the degree of reduction in the number of organisms necessary for growth to occur in air. A completely effective growth factor in this respect, then, would be one that allowed growth in air from as few organisms as would grow in 3 per cent CO_2.

After the serial dilutions were made and before incubation of the cultures, 0.1 ml portions of both the 10^5 and 10^6 dilutions were plated on blood agar. These plates along with the CO_2 series of tubes were incubated at 37.5 C for 24 hours in closed jars in an atmosphere of 3 per cent CO_2 added from a commercial tank. This procedure gave an estimate of the number of viable organisms present and also served as a check on the purity of the starter culture. In the earlier experiments, eight serial tenfold dilutions of a faintly turbid suspension of the organisms in Frantz medium were made in each of the media being tested. In all of the later experiments, the appropriate dilutions of organisms were added to the different series from a master set of dilutions made in the Frantz medium. The number of organisms present in the 10^5 and 10^6 dilutions in series prepared by each method checked closely as shown by plate counts.

All series of tubes were incubated in the same large incubator room at 37.5 C in order to ensure uniformity of the atmosphere. Growth was judged by the presence or absence of turbidity at the end of 24 and 48 hours. The end point of each series was defined as the least number of organisms able to initiate growth in 48 hours.

The final volume of medium in each tube was 5 ml. The tubes were 6 by $\frac{3}{4}$ inches in size and cotton stoppered. It was thought important that the same size cotton stoppered test tubes and the same final volume of medium per tube be used routinely because the growth of *N. meningitidis* is influenced by changes in the ratio of volume of medium to surface exposed as well as by the type of stopper used. Since this method uses a comparison of the least number of organisms necessary to initiate growth as a means of evaluating the ability of other growth substances to replace the supplemental CO_2 requirement, any mechanical factors which influence the number of organisms needed for growth to occur must be carefully controlled.

The second method used to test the effect of added growth factors was to measure the survival time of *N. meningitidis* when it was deprived of metabolic CO_2 by aeration with air or air freed of CO_2. In all experiments the air was first passed through a sterile, cotton filled tube. Air freed of CO_2 was obtained by passage through a column packed with small sticks of NaOH, and complete

absorption was indicated by the lack of precipitation as the CO_2-free air was bubbled through a solution of $Ba(OH)_2$. For these experiments, 7 by $\frac{7}{8}$ inch test tubes were used. These were fitted with two-hole rubber stoppers carrying cotton stoppered inlet and outlet tubes. The inlet tube reached almost to the bottom of the test tube to allow for maximum aeration of the entire culture. Five ml of the respective media containing a 10^2 dilution of a faintly turbid suspension of organisms in Frantz medium were used in each tube. This dilution was chosen because previous experiments had shown that growth invariably resulted from such a large inoculum in nonaerated tubes without supplemental CO_2. Aeration was carried on for varying lengths of time and was adjusted to the fastest rate of flow which would still form a steady stream of discrete bubbles. At the end of each period of bubbling, the respective tubes were removed from the 37.5 C water bath and solid rubber stoppers were inserted in place of the stoppers used during aeration. Growth as indicated by turbidity was determined after an additional 24 and 48 hours' incubation at 37.5 C.

Throughout these aeration experiments an electric coil rather than a Bunsen burner was used for sterilizing the mouths of tubes and flasks in order to avoid introducing any additional CO_2 into the tubes of media being tested.

RESULTS

Replacement of supplemental CO_2 by yeast extract and various chemically defined compounds. a. *Serial dilution experiments.* A large number of preliminary experiments were carried out to test the ability of yeast extract and of various growth factors which might be present in yeast extract to substitute for the supplemental CO_2 requirement of strain no. 520M96 of *N. meningitidis*. Several critical experiments were done then in each of which various combinations of these growth factors were tested in concentrations that had been found to be optimal in the preliminary experiments. The results of a typical critical experiment are shown in table 2. From this table it can be seen that the least number of bacteria able to initiate growth in the basic Frantz medium was 2.0×10^6. The number of organisms necessary for growth was reduced by a factor of 10^6 in a similar series grown in 3 per cent CO_2. Yeast extract in a final concentration of 0.01 per cent proved to be a very effective substitute since it permitted growth in air from as few organisms as did the addition of 3 per cent CO_2. In other tests this was found to be the minimal fully effective concentration of yeast extract, although as little as 0.001 per cent was partially effective. In these experiments the comparative differences in the number of organisms needed to initiate growth under the conditions described above were relatively constant from experiment to experiment, seldom varying by more than one dilution.

It was found that a purine-pyrimidine mixture containing guanine, uracil, and cytosine (adenine and thymine were ineffective), casein hydrolyzate, and a combination of B vitamins (concentrations as shown in table 2) varied from one experiment to another in their ability to replace the supplemental CO_2 requirement. In any one experiment, however, a combination of two of these groups of substances resulted in growth from fewer organisms than with either group

alone. For example, in the experiment shown in table 2, the guanine, uracil, and cytosine mixture and casein hydrolyzate each reduced the number of organisms needed to initiate growth in air by a factor of 10^3. When the purine-pyrimidine mixture and casein hydrolyzate were combined, the number of organisms necessary for growth in air was reduced by a factor of 10^5. However, only when all substances were added was the replacement value as high as that of the yeast extract. A combination of all factors proved to be a completely effective substitute for supplemental CO_2 in all experiments. The same results were obtained when the casein hydrolyzate was replaced by an equivalent mixture of pure amino acids in the concentrations given by Schmidt (1938).

TABLE 2

Substitutes for supplemental CO_2 in the growth of a strain of Neisseria meningitidis in the chemically defined medium of Frantz

SUPPLEMENT	NUMBER OF ORGANISMS INOCULATED PER 5 ML (× 2.0)							
	10^7	10^6	10^5	10^4	10^3	10^2	10^1	10^0
None	+	+	−	−	−	−	−	−
3% CO_2	+	+	+	+	+	+	+	+
0.01% yeast extract	+	+	+	+	+	+	+	+
0.03% GUC (total)†	+	+	+	+	*	−	−	−
0.5% casein hydrolyzate	+	+	+	+	*	−	−	−
B vitamins‡	+	+	−	−	−	−	−	−
0.03% GUC + 0.5% casein hydrolyzate	+	+	+	+	*	*	*	−
0.03% GUC + B vitamins	+	+	+	+	*	*	−	−
0.5% casein hydrolyzate + B vitamins	+	+	+	+	+	+	*	−
0.03% GUC + 0.5% casein hydrolyzate + B vitamins	+	+	+	+	+	+	+	+

+ = growth in 24 hours.
− = no growth in 48 hours.
* = growth in 48 hours but not in 24 hours.
† GUC = 0.004% guanine, 0.013% uracil, and 0.013% cytosine.
‡ B vitamins = thiamin, pantothenic acid, pyridoxine, and nicotinic acid, 1 μg/ml; riboflavin, 0.5 μg/ml; biotin, 0.001 μg/ml, and folic acid concentrate, 0.01 μg/ml.

The variation in replacement value of the growth factors when tested singly was studied further with the guanine, uracil, and cytosine mixture. In a series of experiments with single colony isolates it was found that this variation was a function of the number of organisms in the original inoculum which possessed the ability to utilize these factors in place of supplemental CO_2. These experiments, representative examples of which are presented in table 3, were carried out in two ways. In experiments 1 and 5, ten starter cultures inoculated from different single colonies, isolated on a blood agar plate directly from a lyophile tube, were tested. In experiments 2, 3, and 4, starter cultures were first made from the confluent portion of growth obtained on a blood agar plate from a lyophile tube. These were tested and also streaked on blood agar plates, from which 10, 7, and 10 single colonies, respectively, were subcultured to Frantz

medium and then tested. The spectrum of variation obtained in these 5 experiments is summarized in table 3 in terms of the reduction by the guanine-uracil-cytosine mixture of the number of organisms necessary for growth without supplemental CO_2 (i.e., in air). It can be seen that the proportion of colonies yielding cultures in which the reduction was at least 10^2 varied from 70 per cent in experiment 1 to zero in experiment 3. This difference in the proportion of organisms able to utilize the purine-pyrimidine mixture in place of supplemental CO_2 was consistent with the results obtained with the starter cultures from confluent growth, the reduction being 10^3, 10^0, and 10^2 in experiments 2, 3, and 4, respectively. It seems, therefore, that the smaller the proportion of these organisms, the sooner they are diluted out and consequently the smaller the

TABLE 3

Reduction by the guanine-uracil-cytosine mixture of the number of organisms from single colony isolates necessary for growth without supplemental carbon dioxide

STARTER FROM COLONY	REDUCTION IN NUMBER OF ORGANISMS NECESSARY FOR GROWTH				
	Experiment				
	1	2	3	4	5
1	10^1	10^3	10^0	10^2	10^2
2	10^3	10^1	10^0	10^1	10^0
3	10^2	10^3	10^0	10^0	10^1
4	10^1	10^4	10^0	10^0	10^0
5	10^1	10^0	10^0	10^0	10^0
6	10^5	10^0	10^0	10^1	10^1
7	10^6	10^1	10^0	10^1	10^1
8	10^5	10^0	—	10^4	10^4
9	10^2	10^1	—	10^1	10^0
10	10^2	10^1	—	10^1	10^2
Percentage of starters with a reduction of 10^2 or greater	70	30	0	20	30
Starter culture from confluent growth	—	10^3	10^0	10^2	—

reduction in the number of organisms needed to initiate growth in air. When all growth factors were present fluctuations from culture to culture in the number of organisms able to utilize any one growth factor or group of similar growth factors appeared to be of no importance, and the high replacement value of the combination of all growth factors remained constant. Attempts to stabilize the ability to utilize the guanine, uracil, and cytosine mixture in place of supplemental CO_2 by continual selection of colonies with which high replacement values were obtained were unsuccessful.

Since the B vitamins, in the experiment in table 2, had no effect by themselves, but enhanced the replacement value of the purine-pyrimidine mixture and the casein hydrolyzate singly and in combination, it would appear that they aid in the utilization of these substances.

None of the following compounds associated with the Krebs cycle showed

any replacement value for the supplemental CO_2 requirement of this strain of *N. meningitidis:* acetate (10 and 1 mg per ml); citrate (10 and 1 mg per ml); *alpha*-ketoglutarate (10 and 1 mg per ml); pyruvate (10 and 1 mg per ml); succinate (10, 1, and 0.1 mg per ml). Choline chloride (0.1 and 0.01 mg per ml) and *p*-aminobenzoic acid (0.001 M and 0.0001 M) also were ineffective.

b. *Aeration experiments.* Aeration experiments with air and CO_2-free air were carried out in parallel with most of the critical experiments described above using aliquots of the same media and starter cultures. It was thought that not only might the effect of the various growth factors be compared by another method, but also that the mechanism by which these factors are utilized in place of additional CO_2 might be elucidated. Aeration of cultures resulted in the death of the organisms after varying lengths of time as shown by their failure to grow when subcultured. Yeast extract and the combination of all growth factors proved to be the most effective in prolonging the survival time of the organisms. Cultures in the basic medium alone did not survive 1 hour of aeration, whereas those in which yeast extract or the combination of all growth factors was present survived 3 but not 6 hours of aeration. Similar relative results were obtained when CO_2-free air was used although the survival times in all instances were markedly reduced. The cultures in the basic medium alone did not survive 15 minutes of aeration with CO_2-free air, whereas those in which yeast extract or the combination of all growth factors was present survived 15 but not 30 minutes.

Replacement of supplemental CO_2 by an unknown growth factor (or factors) in yeast extract. Since the yeast extract was more effective on an equivalent weight basis than any combination of chemically defined growth factors, it seemed possible that a substance (or substances) other than those tested could be responsible for its replacement value. Accordingly, an attempt was made to define the active principle in yeast extract by chemical fractionation. This principle can be extracted from aqueous solution with alcohol or acetone, is not destroyed by autoclaving at pH 1.8 or pH 11.7 for 1 hour at 121 C, and is inactivated by ultraviolet irradiation. It has not been isolated yet or identified. Despite its similarity in solubility and in resistance to acid and alkali, it does not appear to be the pyruvate oxidation factor (POF) (O'Kane and Gunsalus, 1948). Two preparations of pyruvate oxidation factor purified 357- and 943-fold, respectively, were generously supplied by Dr. I. C. Gunsalus. They were tested by the serial dilution method in amounts equivalent to 0.01 and 0.1 per cent yeast extract, both with and without supplementation by casein hydrolyzate in concentrations of 0.01 and 0.1 per cent. In no case did growth occur without added CO_2. A sample of the same yeast extract (Fleischman no. 3) used as the source of the pyruvate oxidation factor was fully effective in a concentration of 0.01 per cent.

Other yeast extracts (Baltimore Biological Laboratories, Difco Supplement B and a freshly prepared autolysate of National Grain Yeast) were of comparable potency on a dry weight basis. "V" factor, prepared according to the directions of Lwoff and Lwoff (1937), was essentially ineffective. Beef extract (Difco) and liver extract (Lilly) were only partial replacements. Malt sprout

extracts were inactive. The allantoic fluid from 12 day old chick embryos, after removal of carbonate, served as a complete replacement for CO_2 when added to the Frantz medium in a minimal proportion of 10 per cent by volume. Similar fluids from embryos infected with mumps virus, and from which the virus had been removed by precipitation with methanol at a low temperature, were generously supplied by Dr. Herald R. Cox as a possible source of the factor, but they were inactive.

DISCUSSION

It has been shown that yeast extract or a combination of chemically defined growth factors can replace the supplemental CO_2 requirement of a strain of *N. meningitidis*. Before considering how these growth substances function as supplemental CO_2 substitutes, the relationship to growth of CO_2 itself will be discussed.

Gladstone *et al.* (1935) concluded that all organisms need a certain, but not necessarily a similar, level of CO_2 for growth and reproduction. The period during which this level is being increased corresponds to the lag phase. The organism is unable to divide until the critical concentration of CO_2 is reached, and death occurs if this necessary level of CO_2 is not obtained. The production of this level of CO_2 is determined by the rate of respiration of the organism balanced by the rate of removal of CO_2 (by diffusion and chemical combination). Any condition which will affect either of these rates consequently will affect growth.

Following this line of reasoning in the serial dilution experiments, the number of organisms generally present in the first 2 tubes in the basic Frantz medium series in air was usually sufficient to produce the critical level of CO_2. When the concentration of organisms in this series was smaller, however, the numbers present were not able to build up adequate amounts of CO_2 for growth and reproduction. The lack of sufficient CO_2 was bactericidal rather than bacteriostatic as shown by the failure of the organisms to grow when subcultured to a blood agar plate and incubated in an atmosphere of 3 per cent CO_2. If an identical series was incubated in 3 per cent CO_2 at the start, enough CO_2 was immediately present for growth and reproduction. In a number of experiments not reported in this paper, similar results were obtained if such series were incubated in closed jars without added CO_2. The organisms in the higher concentrations presumably produced a high enough level of CO_2 in the jar by their metabolism to initiate growth in the tubes with the smaller inocula since the removal of the respired CO_2 by diffusion was prevented. The CO_2 concentration in the closed jar after 48 hours' incubation of a series of eight tubes was found to be about 0.4 per cent.

It can be seen, therefore, that if the lag period is unduly prolonged by the inability of small inocula to reach the critical level of CO_2, the organisms die. The addition of various growth factors to the basic Frantz medium allows growth with as few organisms as with the addition of 3 per cent CO_2. The growth factors, then, in some way influence the CO_2 metabolism of these organisms. According to Gladstone any condition which will affect either the rate of pro-

duction of CO_2 by the organism or the rate of its removal by diffusion and chemical combination consequently will affect growth. It seems more likely that the function of the growth factors would be a stimulation of the rate of production rather than a decrease in the rate of removal of CO_2.

That the growth factors tested cannot completely replace the need for CO_2 was shown by the inability of the organism to survive and grow when a 0.03 per cent concentration of CO_2 was maintained in the culture medium by aeration with air for longer than 3 hours. Similarly, the organisms died when the CO_2 was swept out with CO_2-free air for more than 15 minutes. The fact that the growth factors increased the length of survival time further substantiates an explanation of their action as being a stimulation of the rate of CO_2 production. Since aeration with CO_2-free air shortened the survival time in comparison with aeration with air, it appears that the faster the CO_2 concentration in the medium is reduced, the shorter the survival time will be, and *vice versa*. In other words, Gladstone's hypothesis on the relationship of CO_2 to the lag period can be applied here to the survival time. It was concluded, therefore, that the effect of the growth factors was to influence the rate of production of CO_2.

The inability of any of the growth factors tested to replace completely the need for CO_2 of strain no. 520M96 of *N. meningitidis* is in agreement with the results obtained by Gerhardt and Wilson (1950) and Lwoff and Monod (1947) using other microorganisms. Lwoff and Monod state that CO_2 is essential for the growth of microorganisms because the synthesis of certain essential metabolites is carried out only by carboxylation and that the C_4 and C_5 dicarboxylic acids are among the essential heterocarboxylic metabolites. These authors feel that without a doubt there are other essential heterocarboxylic compounds and that certain of these at least appear to be present in yeast extract, casein hydrolyzate, and peptone. Ajl and Werkman (1948), on the other hand, report that a number of compounds, all of which are constituents of the Krebs cycle, or their metabolic precursors substitute for CO_2 in the case of *E. coli*. Lwoff and Monod (1949) have criticized these results on the basis of the aeration methods used, which they feel were not adequate to remove CO_2 completely from the culture medium.

The heat stable substance (or substances) in yeast extract, which is much more active on a weight basis than a combination of chemically defined growth factors as a substitute for supplemental CO_2, may be similar to the B.R. factor described by Kline and Barker (1950). Its relationship to this factor as well as to vitamin B_{12} (Novak and Hauge, 1948), protogen (Stokstad *et al.*, 1949) and the acetate replacing factor (Guirard, Snell, and Williams, 1946) must still be considered. It does not appear to be identical with the pyruvate oxidation factor (O'Kane and Gunsalus, 1948; Lytle *et al.*, 1951[3]), for when this factor

[3] In view of the results of the aeration experiments described herein, the statement in the latter paper that "Tuttle and Scherp (1950) have reported that the requirement of *Neisseria meningitidis* for CO_2 for growth may be replaced by a substance in yeast extract having properties similar to those of POF" should have read "for supplemental CO_2" instead of "for CO_2".

was tested in equivalent amounts it did not replace the need for supplemental CO_2. Snell and Broquist (1949) suggest that the pyruvate oxidation factor, protogen and the acetate replacing factor are identical. Because of the influence of this heat stable factor on CO_2 metabolism, it might be postulated to be one of the unknown essential heterocarboxylic compounds thought to be present in yeast extract by Lwoff and Monod (1947).

SUMMARY

The supplemental CO_2 required for the growth of a strain of *Neisseria meningitidis* in a chemically defined medium could be replaced completely by adding 0.01 per cent yeast extract or a combination of chemically defined growth factors.

As shown by the failure of the organism to survive aeration, none of the growth factors tested could substitute completely for CO_2. The results indicate that these substances act by stimulating the rate of production of CO_2 by the organism rather than by replacing the need for additional CO_2.

The active substance (or substances) in yeast extract is extractable from aqueous solution with alcohol or acetone, resists autoclaving at 121 C for one hour at pH 1.8 or 11.7, and is inactivated by ultraviolet light.

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