CCCIV. THE RELATION OF MICRO-ORGANISMS TO CAROTENOIDS AND VITAMIN A.

II. THE PRODUCTION OF CAROTENOIDS BY *MYCOBACTERIUM PHLEI*.[1]

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Although great advances have been made in our knowledge of the chemistry of the carotenoid pigments little is known concerning the physiology of their production or their rôle in living cells. Ingraham and Baumann [1934] reported that many bacteria were capable of synthesising carotene when grown on simple media in the dark. Since that time a more detailed study has been made of the production of carotene and associated pigments by one of these organisms. *M. phlei* was selected because it grows well on a variety of synthetic media and because its chemical composition and physiological reactions have received more than ordinary attention.

Many factors were found to affect the pigment content of *M. phlei*. This made it advisable to define arbitrarily a set of standard conditions and then to compare the growth and pigmentation obtained in various experiments with those observed under these standard conditions. The medium used contained per litre of distilled water: asparagine 5·0 g., glucose 50·0 g., K_2HPO_4 1·0 g., $MgSO_4, 7H_2O$ 1·0 g., Na citrate 0·5 g., Fe citrate 0·05 g. It was bottled in 25 ml. amounts in 6 oz. signet bottles which were then capped and autoclaved. Cultures of 5-day cells grown under standard conditions were used as the inoculum. The temperature of incubation was 37°. Our first observations were made on the gross pigmentation produced when the carbon source, the nitrogen source, the mineral salts, the temperature of incubation and a number of other factors were modified. In later studies we also concerned ourselves with the nature of the different pigments produced.

The cells were collected on filter-paper, washed with distilled water and dried in thin layers at 37° for 48 hours. This procedure did not result in any appreciable loss of pigments. The weighed dried cells were treated with hot 95% alcohol and the extracted pigments were then estimated quantitatively with a Lovibond tintometer. The hydrogen ion concentration of the medium was determined colorimetrically. The Shaffer-Hartmann method was used in following the rate of utilisation of glucose. The nitrogen in the medium was determined by the Kjeldahl method. Lipoids were roughly estimated by weighing the ether-soluble portion of an absolute alcohol extract of dried cells.

Growth under standard conditions has been quantitatively reproducible over a period of two years. Typical curves for the cell weight and total pigment production are given in Fig. 1. The weight of cellular material increased rapidly to a maximum at 9 days and then autolytic processes set in. Pigmentation

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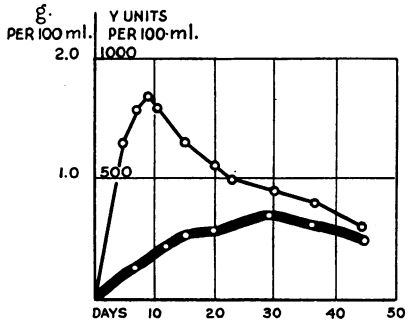
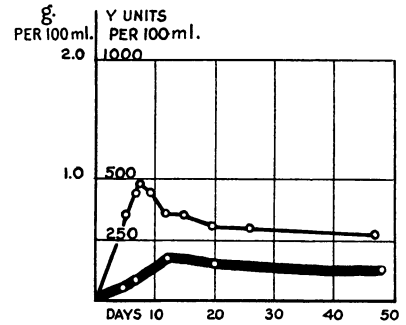
increased during the growth period and continued to increase long after autolytic processes had been initiated. Glucose was found to disappear from the medium at about the 9th day. At the end of 6 days 95% of the asparagine-N had been absorbed by the cells. It was therefore not surprising to find that the increase in cell weight from the 6th to the 9th day was largely due to the production of lipoids which were quickly destroyed when the sugar was exhausted.



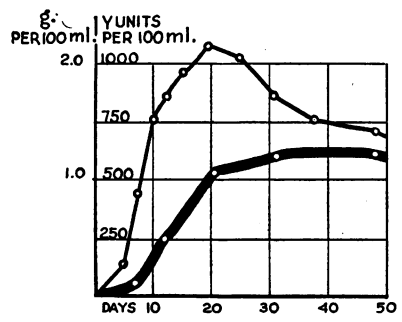
GROWTH AND PIGMENTATION ON STANDARD 5% GLUCOSE MEDIUM.

Fig. 1.



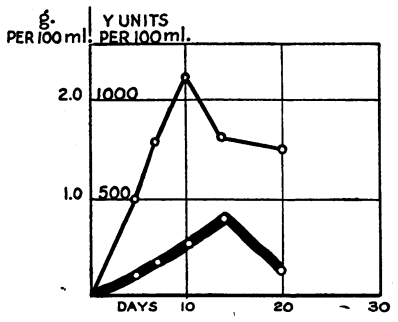
GROWTH AND PIGMENTATION ON 2% GLUCOSE

Fig. 2.



GROWTH AND PIGMENTATION ON 10% GLUCOSE

Fig. 3.



GROWTH AND PIGMENTATION ON 5% GLUCOSE WITH 1.0% ASPARAGINE

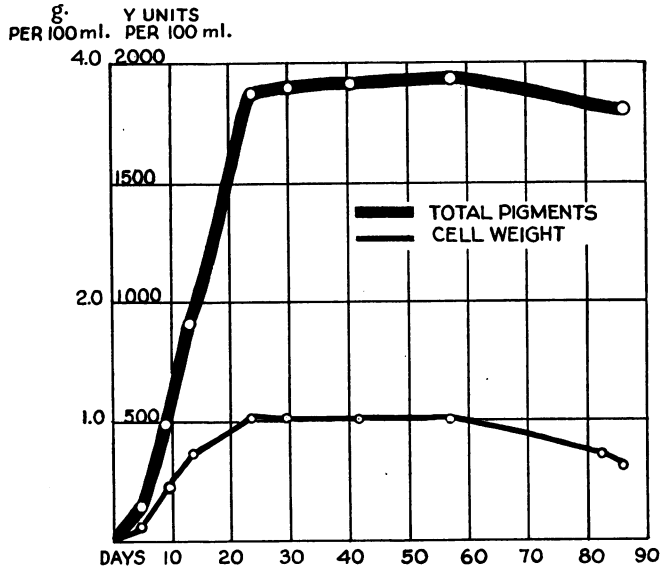
Fig. 4.

When yellow cells instead of white cells were used as the inoculum the growth was again only slightly pigmented until the second and third weeks, and the question therefore arose as to whether the progressive yellowing was an invariable consequence of age. This was found not to be the case, for when cultures which had been stunted by lowering the concentration of glucose to 2% were allowed to age, the cells remained white (Fig. 2). On the other hand when the glucose concentration was raised to 10% the old cells became very yellow (Fig. 3). In these experiments the pigment content of the cells varied directly with the amount of growth rather than with the age of the culture. However, when the amount of growth was varied independently of the concentration of sugar by changing the volume of medium used in each bottle, it was found that pigmentation was not affected. Therefore the thickness of the pellicle and the amount of growth per bottle were not determining factors in the pigmentation of the organism.

The possibilities existed that there was present in the fresh medium some substance inimical to pigment production which was removed as the cells grew,

or that some substance was produced by continued growth which stimulated pigment production. Evidence in favour of the former concept was obtained from an experiment in which cells were grown in a medium containing every constituent of the standard medium in double strength. With large inocula excellent growth was secured but the maximum pigmentation per g. of cells remained slightly lower than on the standard medium and very much lower than in a control series in which only the concentration of the glucose had been doubled. The specific factors involved will be discussed later.

The substitution of glycerol for glucose in the standard medium brought about striking changes in the growth and pigmentation of *M. phlei*. The rate of growth (Fig. 5) was considerably slower on glycerol than on glucose and the



GROWTH AND PIGMENTATION ON 5% GLYCEROL

Fig. 5.

maximum cell weight per 100 ml. of medium was very much less. However, the cells grown on glycerol became yellow early in the growth period and their pigment content during the later stages was many times that obtained on glucose. This is particularly interesting in view of the close chemical relationship between glycerol and glucose as contrasted with the carotenoid pigments. There is a possibility that some glycerol may be formed as an intermediate in the breakdown of glucose, but unfortunately little is known concerning the steps in the utilisation of either of these compounds by *M. phlei*. That glycerol might serve as a precursor of the carotenoid pigments in one way or another was further indicated by the fact that additions of small amounts of glycerol to standard medium greatly increased the pigment content of the cells.

The effects of a large number of carbon compounds were tested by adding them to standard glucose medium. Pigment production was not increased by the addition of 1 % of fructose, arabinose, xylose, sucrose, maltose, lactose, galactose, mannitol, arabitol, inositol, erythritol, quercitol, persietol or starch. The sodium and ammonium salts of formic, acetic, propionic, butyric, oxalic, lactic, citric, caproic, stearic and sebasic acids yielded negative results. A number of miscel-

laneous compounds such as acetone, methylglyoxal, ethyl propionate, acetaldehyde, aldol, allyl sulphate, β-ionone, phytol and a vitamin-A concentrate failed to stimulate pigmentation. On the other hand the alcohols, methyl, ethyl, *n*-propyl, *n*-butyl, *iso*butyl, *n*-amyl and particularly *iso*propyl favour pigment production. Ethyleneglycol, propyleneglycol, trimethyleneglycol, tetramethyleneglycol and *cyclo*hexane-1 : 3-diol when added to the medium in 1 % amounts produced very yellow cells (Table I).

Table I. *The effect of various carbon compounds on growth and pigmentation when added in 1 % amounts to standard medium.*

Carbon compound	Cell weight g./100 ml.	10 day cultures p_H	Pigmentation Y/g.
Glucose	1·8	7·0	100
Methyl alcohol	1·2	7·0	186
Ethyl alcohol	1·6	7·0	149
*iso*Propyl alcohol	1·6	7·0	508
n-Butyl alcohol	1·7	7·0	231
*iso*Butyl alcohol	1·3	7·0	158
Amyl alcohol	1·0	7·0	296
Ethyleneglycol	1·5	7·0	720
Trimethyleneglycol	1·2	7·0	340
Tetramethyleneglycol	1·2	7·0	820
*cyclo*Hexanediol	1·2	7·0	344
Ethyl propionate	1·6	7·0	134
Ammonium acetate	0·7	8·4	14
Ammonium lactate	0·9	8·6	44
Acetone	0·9	7·0	124
Sodium glycerophosphate	1·6	7·1	205
Glycerol *plus* Na_2HPO	1·8	7·2	362
Glycerol	1·7	7·0	550

The similarities and differences which exist between the behaviour of *M. phlei* towards acetic acid and towards ethyleneglycol were found to be particularly interesting. Neither of these 2-carbon atom compounds is capable of supporting growth when supplied as the sole source of carbon but both are utilised in the presence of glucose. Stephenson and Whetham [1922] demonstrated that when acetates were added to a glucose medium there was an increase in the percentage of lipoids in the cells. However, as evidenced by the data in Table I and by additional experiments in which the hydrogen ion concentration was maintained around p_H 7·0, no increase in pigments was observed when acetic acid was metabolised. In contrast to this is the marked increase in pigmentation observed in the presence of ethyleneglycol (Table II). The addition of this compound did not increase the yield of cells but as little as 0·2 % definitely stimulated pigment production.

Table II. *The effect of ethyleneglycol on growth and pigmentation.*

Glucose g./100 ml.	Glycol g./100 ml.	Maximum cell weight g./100 ml.	Maximum pigmentation Y/100 ml.
1·0	0·0	0·41	50
1·0	1·0	0·41	250
5·0	0·0	1·65	326
5·0	0·2	1·42	896
5·0	1·0	1·45	1920
5·0	2·0	1·32	3240
5·0	5·0	1·09	3280

It is of course impossible to change one constituent of a medium without tending to modify a number of its properties. Thus when the source of carbon was

changed it was frequently found that the medium became very alkaline with the growth of the organisms. The explanation was apparent when sodium salts of organic acids had been used. Alkalinity was also noted when the concentration of glucose or glycerol had been reduced, as well as during the early stages of growth on glycerol. In both of these cases the source of alkalinity was largely attributable to ammonia liberated from asparagine. These data lend a new interpretation to the results of such workers as Weinzirl and Knapton [1927], who attached great significance to the fact that certain mycobacteria turned a 1% glucose medium alkaline whereas a 5% glycerol medium remained acid. Their results undoubtedly depended upon concentrations used rather than upon differences in the end-products formed from the two compounds. The early rise in the hydrogen ion concentration on the glycerol medium indicated to us that the asparagine was attacked preferentially. Total nitrogen determinations showed that during this period considerable quantities of nitrogen were lost to the atmosphere and that this loss might account for the lower yields observed on this medium. By inoculating a glycerol medium very heavily it was possible to accelerate the rate of growth so that nitrogen was rapidly tied up in the cells. Under these conditions it was observed that the yield was fully as great on glycerol as on glucose.

Many other factors besides the source of available carbon were found to affect the production of pigments by *M. phlei*. The first to be considered was the source of nitrogen. When the concentration of asparagine in the standard medium was lowered from 0·5 to 0·1 or 0·2%, growth and pigmentation were checked as they had been when glucose was the limiting factor. Therefore the increased pigment production observed with an excess of glucose could not have been due to a changed C : N ratio. When the concentration of asparagine was increased, the young growth was similar to that obtained under standard conditions but the pigment content reached a maximum at an earlier period and was then rapidly lowered as autolysis proceeded (Fig. 4). A similar autolysis involving rapid loss of pigments was produced by adding asparagine to 15-day standard glucose cultures. Ammonium salts, urea, peptone, caseinogen and a number of other compounds were substituted for asparagine, but the type of compound which served as a source of nitrogen had little effect on pigmentation, provided that care was taken to maintain the hydrogen ion concentration around neutrality.

The concentration of potassium in the medium is of considerable importance. In the first subculture from standard medium to an ammonium phosphate-glucose medium without potassium or other salt additions, growth was very slight but the pigment content per g. of cells was remarkably high (Table III). The addition of magnesium sulphate increased growth without affecting the colour of the cells but when potassium was added as the phosphate, pigment production was greatly depressed. The absence of potassium when glycerol was used instead of glucose had no effect upon the pigment content of the cells which was already very high. These results suggested: first, that the constituent of the medium which had prevented the accumulation of pigments on standard glucose might have been potassium, and second, that possibly glycerol or a similar compound was produced as an intermediate in the utilisation of glucose when potassium was not present.

The fact that pigmentation on glucose media was increased by lowering the concentration of potassium was confirmed in an experiment reported in Table IV. The data also show that lowering the concentration of phosphate favours pigment production on glycerol as well as on glucose as is suggested by the last row of figures in Table I. It will be seen from Table IV that lowering the concentra-

Table III. *Growth and pigmentation on an ammonium phosphate medium with additions of potassium, magnesium sulphate and ferric citrate.*

Constituents of medium			g./100 ml.	Cell weight g./100 ml. 10 days	Pigmentation Y/g. 10 days
1.	$(NH_4)_2HPO_4$ + Glucose		0·2 5·0	0·05	980
2.	,,	,, $MgSO_4, 7H_2O$	0·1	0·09	1080
3.	,,	,, K_2HPO_4	0·1	0·24	119
4.	,,	,, $MgSO_4, 7H_2O$ K_2HPO_4	0·1 0·1	0·30	202
5.	,,	,, $MgSO_4, 7H_2O$ K_2HPO_4 Fe citrate	0·1 0·1 0·01	0·27	181
6.	,,	+ Glycerol	5·0	0·03	1780
7.	,,	,, $MgSO_4, 7H_2O$	0·1	0·05	1550
8.	,,	,, K_2HPO_4	0·1	0·10	1680
9.	,,	,, $MgSO_4, 7H_2O$ K_2HPO_4	0·1 0·1	0·09	1400
10.	,,	,, $MgSO_4, 7H_2O$ K_2HPO_4 Fe citrate	0·1 0·1 0·01	0·12	885

Table IV. *The effect of the concentration of potassium and of phosphate on growth and pigmentation.*

K and PO_4 salts (M)		Cell weight g./100 ml.	Pigmentation Y/g.	Reaction p_H	Glucose g./100 ml.
K_2SO_4	$(NH_4)_2HPO_4$				
0·001	0·01	0·93	266	6·2	0·7
0·01	0·01	0·84	156	6·1	—
0·1	0·01	1·01	114	6·4	—
K_2HPO_4	$(NH_4)_2SO_4$				
0·01	0·001	0·89	117	6·0	—
0·01	0·01	0·90	186	6·7	—
0·01	0·1	0·99	156	6·2	—
K_2HPO_4	$(NH_4)_2SO_4$				
0·001	0·01	0·41	274	8·4	2·3
0·01	0·01	0·97	222	6·0	—
0·1	0·01	1·52	100	6·8	0·3
K_2SO_4	$(NH_4)_2HPO_4$				
0·01	0·001	0·41	214	8·4	2·2
0·01	0·01	0·87	188	6·0	0·5
0·01	0·1	0·99	156	6·9	—

tion of phosphate, but not that of potassium, interfered with glucolysis, which in turn resulted in the liberation of ammonia from asparagine and the production of alkalinity. Determinations of the oxygen uptake on these media showed that lowering the concentration of phosphate limited the oxygen uptake but that within the range investigated the concentration of potassium had no effect.

The data in Table III indicate that increasing the concentration of iron decreased pigmentation. In Table V are given data which confirm this observation. Although ferric salts in moderate concentrations led to better growth, particularly on glycerol, concentrations of 0·1% ferric acetate definitely decreased the pigment content of the cells. Similar results were obtained with cupric salts.

Table V. *The effect of additions of ferric citrate on growth and pigmentation in a 5% glycerol medium inoculated heavily.*

Ferric citrate g./100 ml.	Cell weight, g./100 ml.			Pigmentation, Y/100 ml.		
	8 days	12 days	21 days	8 days	12 days	21 days
0·000	0·28	0·68	0·72	240	480	520
0·005	0·72	1·56	1·48	880	1600	1800
0·025	0·60	1·52	1·80	120	1400	2000
0·100	0·52	1·28	1·56	140	880	536

Table VI. *The oxidising action of cells grown in the presence of ferric citrate.*

Ferric citrate g./100 ml.	ml. of 0·005 *N* thiosulphate		
	4 days	8 days	12 days
0·000	0·00	0·00	0·00
0·005	0·42	0·00	0·00
0·025	3·00	2·59	0·00
0·100	11·02	11·08	0·00

The rôle of iron in the metabolism of *M. phlei* presents many problems. In Table VI are given data obtained by shaking washed cells in an acid solution of potassium iodide and titrating the liberated iodine with sodium thiosulphate. The oxidising agent, very possibly ferric ions, could be removed from the cell surface by washing with 95% alcohol. At the end of 4 days there was no trace of such an agent in any of the media. Those cultures which had contained the higher concentrations of ferric citrate were acid in reaction. It is not impossible that the lower pigment values observed in the presence of ferric and cupric salts, which were also adsorbed on the cells, were due to direct oxidation of the pigments.

A number of factors were studied which appeared to have no specific effect on pigmentation. These included ethylene gas[1] and salts of sodium, lithium, calcium, magnesium, selenium,[1] the oxidation-reduction potential, the light intensity and the temperature of incubation. The optimum reaction was found to be between p_H 6·0 and 7·0. When the p_H was higher than 8·6 pigmentation was very poor in any medium.

As yet little has been done by way of correlating pigment content with the presence of other substances in the cell, but a correlation has been noted between the pigment content of the cells and catalase. Our studies have not been sufficiently exhaustive to establish this as an absolute parallelism but it is quite conceivable that an increase in catalase content might help to protect pigments from oxidation.

So far we have no direct evidence as to whether the pigment content of the cells increases because the pigments are required in certain physiological processes, or because certain agents stimulate synthetic processes, or because processes of destruction are inhibited. Until facts of this character are known, it is difficult to draw conclusions as to the function of the carotenoids in bacterial metabolism.

Analysis of pigments.

The pigments of *M. phlei* have already been studied by Chargaff [1930; 1933] who used the chromatographic technique. In 1930 he resolved the mixed pigments with calcium carbonate into three bands, one of which he identified as β-carotene. In 1933 he separated the pigments into carotenes, xanthophylls and

[1] Agents which brings about a chlorotic condition in higher plants.

xanthophyll esters, and put each fraction through columns of aluminium oxide. Four bands were revealed when the hydrocarbons were examined, but Chargaff concluded from spectrophotometric data that three of them were due to β-carotene. Evidently he did not realise that spectrophotometric data do not differentiate between β-carotene and kryptoxanthin, both of which we found upon repeating his work. From the xanthophyll esters he obtained a pigment similar to lutein and two bands which were not identified.

In our experience the best method of revealing the pigments of *M. phlei* was to put a light petroleum solution of the mixed pigments through a magnesium oxide column, and then to wash the column first with 20% ether in light petroleum and then with chloroform. About a dozen bands could be revealed by this procedure. α-Carotene, β-carotene, kryptoxanthin and esters of lutein, zeaxanthin and azafrin have been isolated and identified spectrophotometrically. The identity of the remaining pigments is being studied.

It was possible to separate the carotenes, their monohydroxy-derivatives and the esters of lutein and zeaxanthin and azafrin from the other pigments. 20 ml. of the original 95% alcohol extract were pipetted into a small separating funnel to which 2·5 ml. of 5% KOH were added. The mixture was extracted with light petroleum and the light petroleum fraction estimated quantitatively with a Lovibond tintometer. Chromatographic examination of this fraction (known as the PE-1 fraction) showed that it contained the carotenes, kryptoxanthin, a pigment which appeared to be the monohydroxy-derivative of α-carotene and esters of lutein, zeaxanthin and azafrin. The other pigments remained in the alkaline alcohol. In Fig. 6 are given the quantitative data obtained for the PE-1

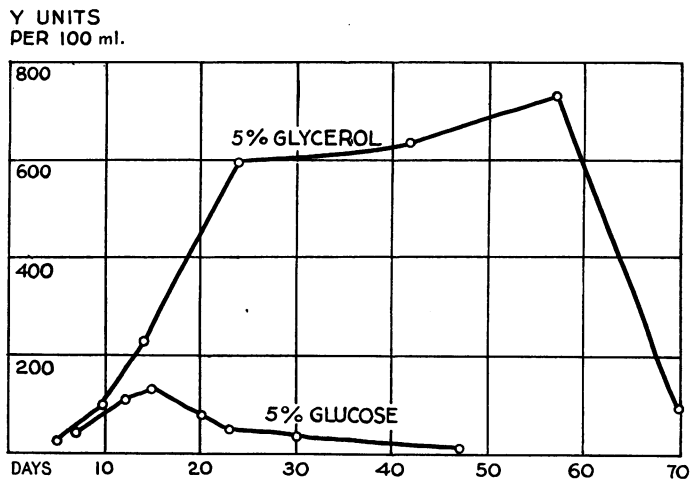


Fig. 6.

fractions from the cells used in Figs. 1 and 5. It will be seen that, although the values for the PE-1 fraction were high when total pigmentation was good, they were not high enough to account for more than half of the observed increase. Further, the PE-1 fraction reached a maximum at a much earlier point than did the remaining pigments. The percentage of pigments in the PE-1 fraction was estimated in all the experiments which have been reported in this paper, and in

general it was noted that whenever an agent such as glycol or potassium produced a change in total pigments, there was a change in the same direction in the carotenoid fraction. The highest percentages of pigments in the PE-1 fraction were obtained on glucose media where the absolute values were relatively low. It is difficult to interpret these results without a knowledge of the identity of the remaining pigments.

When glycerol or glycols were added to a glucose medium there was a tremendous increase in one pigment which has not yet been identified. In many respects the behaviour of this compound resembles that of phthiocol, the pigment isolated from *M. tuberculosis* by Anderson and Newman [1933]. It was yellow and ether-soluble in acid solution, but turned deep red and became water-soluble in alkaline solutions. In the chromatographic column it appeared above xanthophyll as a deep red line which turned purple when the column was washed with chloroform. In the presence of traces of water a blue colour was developed. When ferric salts were added to the medium the amount of this pigment was decreased. Quantitative estimates were made by measuring the red Lovibond units in the alkaline alcohol solution from which the PE-1 fraction had been extracted. It was found that the absolute amount of this pigment increased long after cell autolysis had been initiated, suggesting that it was an end-product in metabolism.

Summary.

The effects of a number of factors on the gross pigmentation of *M. phlei* have been studied. On a synthetic glucose-asparagine medium the pigment content of the cells was relatively low. As growth proceeded the cells became increasingly yellow and this was found not to be due to their age or to the influence of the heavy pellicle. A similar increase in pigmentation was induced by lowering the concentration of potassium or phosphate ions in the medium. Increasing the concentration of ferric salt tended to prevent pigment formation. When glycerol was substituted for glucose pigmentation was greatly increased. The concentration of potassium was without effect on pigmentation in the presence of glycerol, but phosphates and ferric or cupric salts decreased the colour of the cells. The addition of alcohols or glycols but not of sugars, sugar alcohols, acids or many other carbon compounds to the standard medium increased pigment production. An excess of asparagine in the medium led to rapid cell autolysis and destruction of the carotenoids. The substitution of ammonium salts, urea, peptones and other sources of nitrogen for asparagine had no effect upon pigmentation when the reaction of the medium was controlled. However, if the p_H was allowed to rise above 8·6, the cells were never highly coloured. Ethylene and the salts of sodium, lithium, calcium, magnesium and selenium, the oxidation-reduction potential, the light intensity and the temperature of incubation were without specific effect upon pigmentation.

In the absence of suitable sources of energy *M. phlei* attacked asparagine with the liberation of ammonia and the production of alkalinity. This was observed when the concentration of glucose or of phosphate limited glucolysis, or in the early stages of growth on glycerol. The absence of potassium checked growth but not the oxygen uptake of the cells or glucolysis. Cells grown in the presence of ferric or cupric salts were capable of oxidising potassium iodide to iodine.

At least a dozen different pigment bands were observed when the pigments were resolved on a magnesium oxide column. Of these α-carotene, β-carotene and kryptoxanthin were identified and the presence of esters of lutein, zeaxanthin and azafrin was established. A method was devised for separating this group of

known pigments and determining them quantitatively. They were found to vary directly with gross pigmentation but no close parallelism was noted and their increase in the presence of glycerol *etc.* was never enough to account for the total increase in pigments. A pigment somewhat resembling phthiocol was largely responsible for the increased pigmentation observed in the presence of glycerol and the glycols. This pigment, as contrasted with the carotenoids which generally reached a maximum before the cells began to autolyse, appeared to be an end-product since it increased in absolute amount until the cells were almost completely autolysed.

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