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The Influence of Vitamin B₁₂ on Growth Rate and Cell Composition of the Flagellate *Ochromonas malhamensis*

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SUMMARY: In cultures of the flagellate *Ochromonas malhamensis* the rate of growth is determined by the concentration of cyanocobalamin up to a limit at which a maximum growth rate is achieved. In organisms grown exponentially with different rate-limiting concentrations of cyanocobalamin, the amounts of protein and nucleic acids were approximately proportional to the rates at which the organisms had grown. With increasing growth rate the concentrations of riboflavin, nicotinic acid, pantothenic acid and chlorophylls increased. Carbohydrate showed little change, whereas fat and 'free' amino-nitrogen diminished in concentration as the growth rate increased. In cultures of *O. malhamensis* grown with limiting or near-limiting concentrations of cyanocobalamin and with increasing concentrations of chloramphenicol, the rates of division declined asymptotically to a low value corresponding with 500 µg. chloramphenicol/ml. culture medium. Inhibition of growth caused by the antibiotic was annulled by increasing the concentration of cyanocobalamin in the culture medium.

A variety of cultural and nutritional factors can influence the balance of composition of living cells. Vitamin deficiencies, for example, may distort the cell metabolism and bring about characteristic 'biochemical lesions'. Vitamin B₁₂ plays presumably a part in diverse metabolic processes, but in some of these its action is probably indirect. No single fundamental role can as yet be assigned to this vitamin, although there are indications, mainly from work with animals and animal-tissue preparations, that its essential function is concerned with protein biosynthesis (see review by Smith, 1958). If indeed the vitamin acts in protein or nucleic acid synthesis, this action would give rise to a variety of secondary effects and explain an apparent multiplicity of functions. For example, it would explain the low concentrations of certain enzymes found in the tissues of deficient animals (Mistry *et al.* 1955; Wong & Schweigert, 1957; Murthy, Desikachar & Swaminathan, 1956); the sharp decrease in ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) in vitamin B₁₂-deficient *Euglena gracilis* (Soldo, 1955) and of DNA in *Lactobacillus leichmannii* (Wacker, Pfahl & Schröder, 1957); and the effect of vitamin B₁₂ in reversing the actions of aminopterin and aureomycin on the synthesis of DNA in *L. leichmannii* (Rege & Sreenivasan, 1954*a, b*).

In several species of bacteria the nucleic acid content of the organisms is related to the rate at which they have grown (Malmgren & Hedén, 1947). In *Bacterium lactis aerogenes* the growth rate under a variety of cultural conditions is indeed proportional to the cell content of RNA (Caldwell, Mackor & Hinshelwood, 1950). In cultures of the flagellate *Ochromonas malhamensis* the rate of growth is determined by the concentration of vitamin B₁₂ up to a

limit at which a maximum growth rate is achieved. This relationship is described by an equation of the form of an adsorption isotherm (Ford, 1958). The present paper is concerned with the concentrations of proteins, nucleic acids, fat, carbohydrate and of vitamins representative of major enzyme systems, in *O. malhamensis* grown with different limiting concentrations of vitamin B₁₂ and harvested within the period of exponential growth. An effect of vitamin B₁₂ in reversing inhibition of growth caused by chloramphenicol is also reported.

METHODS

Growth of organisms and preparation for analysis

The composition of the culture media, and the general methods used for maintaining the organism and for preparing inocula, were as described previously (Ford, 1953, 1958). Cultures used to provide the organisms for analysis were incubated in darkness at 30° in vitamin B₁₂-free basal medium supplemented with 0.05, 0.10, 0.20 or 4.0 μ g. cyanocobalamin/ml. At each of these concentrations ten replicate cultures were grown, in 100 ml. portions of medium contained in 10 oz. 'medicine flats'. The bottles of medium were plugged with cotton wool and autoclaved for 10 min. at 115°. After cooling to room temperature each was then inoculated with 1 ml. of a 4-day culture (cf. Ford, 1958), placed in a shaking machine in an air incubator operating at 29°, and incubated for 46 hr. After this relatively short period of incubation the yield of organisms was only about one-third of the maximum obtainable, but growth was strictly exponential only during these early hours of incubation, and after 48 hr. the rate of division began to decline markedly in the cultures grown with the higher concentrations of vitamin B₁₂. On being removed from the incubator the replicate cultures were combined and their optical densities at 580 μ determined in a Lumetron model 400 A photometer. At this stage there were four bulked cultures, each of them comprised of ten replicate 100 ml. portions. These bulked cultures were now centrifuged separately for 20 min. at 1500 g. The supernatant fluid was drained as completely as possible from the deposited organisms, which were resuspended in 2-3 ml. of water, frozen quickly and freeze-dried. The dried organisms were ground finely and stored in a desiccator over P₂O₅.

Ochromonas malhamensis is facultatively photosynthetic, and for one experiment the organism was grown in light, at different concentrations of cyanocobalamin in a culture medium containing NH₄Cl (0.3 g./100 ml.) as sole nitrogen source. Cultures were grown in 400 ml. portions of medium in 'penicillin bottles' at 27° and without shaking under uniform illumination at 180 foot-candles. Under these conditions growth was slower and a longer incubation period (9 days) was used. The organisms were otherwise prepared for analysis as described above.

Infra-red absorption spectra. The infra-red absorption spectrum of each of the dried cell preparations was examined by the method described by Goulden & Sharpe (1958).

Determination of cell constituents

Nitrogen. Total cell nitrogen was determined by micro-Kjeldahl analysis. 'Free' amino-nitrogen was extracted from the cells with cold 10 % (v/v) aqueous acetic acid (cf. Mattick *et al.* 1956) and estimated with ninhydrin reagent by the method of Yemm & Cocking (1955).

Nucleic acids. Total nucleic acid was measured by ultraviolet absorption at 268 m μ , and deoxyribonucleic acid by reaction with indole, with a purified preparation of calf thymus DNA as reference standard; ribonucleic acid was calculated by difference. The procedures followed were those of Ceriotti (1955) as modified by Paul (1958).

Carbohydrates. Total carbohydrate was determined by the anthrone method, as described by Trevelyan & Harrison (1952).

Fat. Fat was extracted from the dried organisms with diethyl ether, and was estimated by difference.

Vitamins of the B-complex. Pantothenic acid, nicotinic acid, riboflavin and the vitamin B₆ group were determined by microbiological assay. The methods used were as described by Gregory, Ford & Kon (1958).

Chlorophylls. Chlorophylls were determined spectrophotometrically by the method described by Richards (1952).

RESULTS

The values obtained for nitrogen, nucleic acids, carbohydrate and fat are set out in Table 1. With increasing concentration of cyanocobalamin the rate of growth of the organism increased, as did the content of nitrogen, RNA and DNA. Total carbohydrate showed little or no change; fat and 'free' amino-nitrogen diminished.

The relation between growth rate and cyanocobalamin concentrations in the culture medium is described by the equation, $k/k_{\max} = C/(C_1 + C)$, in which k is the relative growth rate and $k_{\max}$ the rate when $C = \infty$; C is the

Table 1. *Growth rate and composition of Ochromonas malhamensis grown at different concentrations of cyanocobalamin*

Conditions of cultivation	Cyanocobalamin in culture medium (m μ g./ml.)	Growth rate (k)*	Percentage organism dry wt.					
			Total nitrogen	'Free' amino-nitrogen	RNA	DNA	Carbohydrate	Fat
Grown in darkness in complete medium	0.05	2.55	2.83	0.30	1.61	0.95	52.0	7.0
	0.10	2.85	3.40	0.26	1.92	1.00	47.5	3.6
	0.20	3.02	3.90	0.23	2.13	1.02	52.0	1.3
	4.0	3.20	4.06	0.24	2.26	1.21	52.0	0
Grown in light in minimal medium	0.06	—	4.96	0.50	3.20	0.82	42.0	20.0
	0.60	—	5.60	0.35	3.72	0.82	40.5	15.6

* No. divisions/24 hr.

concentration of cyanocobalamin, and C_1 is the value of C at which $k = \frac{1}{2}k_{\max}$ (Ford, 1958). Some further possible relationships between growth rate in the 'complete' medium and cell composition are indicated in Table 2. These results indicate a close relation between the rate at which the organisms had grown and their content of nucleic acids and total nitrogen. A similar close proportionality was evident in the cell concentrations of nitrogen and nucleic acid.

Table 2. *Some correlations between growth rate (k), nitrogen and nucleic acids in *Ochromonas malhamensis* grown with different concentrations of cyanocobalamin*

Cyanocobalamin in culture medium ($\mu\text{g./ml.}$)	Nucleic acids	Nitrogen	Nitrogen
	k	k	Nucleic acids
0.05	1.00	1.10	1.10
0.10	1.02	1.19	1.16
0.20	1.04	1.29	1.24
4.0	1.08	1.27	1.17

It had been considered inadvisable to wash the organisms before drying and the deposited organisms obtained by centrifuging the cultures were simply drained of supernatant fluid as completely as possible. The residual entrained culture fluid might have introduced a systematic error into the figures representing 'free' amino-nitrogen in the organisms grown in complete medium. However, the values for 'free' amino-nitrogen in the organisms grown in light in minimal medium (NH_4Cl as nitrogen source) showed the same reciprocal relationship to growth rate and are not suspect for this reason.

There were clear differences in composition between organisms grown in darkness in complete medium and those grown in light in minimal medium. These latter were much higher in fat; the concentrations of nitrogen, RNA and B_2 -complex vitamins (see p. 272), were also comparatively high, whereas DNA and carbohydrate were lower. No increase in DNA concentration with increase in cyanocobalamin was observed, but in other respects the influence of the vitamin on cell composition was the same under both sets of cultural conditions. No estimates of growth rate were made because growth of the organism in minimal medium was relatively slow, and was probably not strictly exponential throughout the incubation period of 211 hr. used.

Tween 80 (polyoxyethylene sorbitol mono-oleate) stimulates the growth of *Ochromonas malhamensis* and was included in both minimal and complete culture media. It was observed that in the presence of this compound the requirement for biotin reported by Hutner, Provasoli & Filfus (1953) could not be demonstrated. The accumulation of fat in the vitamin B_{12} -deficient cultures was not related to the presence of Tween 80. There was apparently a reciprocal relation between growth rate and fat content, and, in fact, the omission of Tween 80 from the culture media caused a significant increase in the concentration of cell fat, together with a slight retardation of growth.

Infra-red absorption spectra. The relation between vitamin B₁₂ content and the fat and protein content of *Ochromonas malhamensis* is illustrated in a comparison of the infra-red absorption spectra of organisms grown at different concentrations of vitamin. Figure 1 shows the absorption, in the 2000 to 830 cm.⁻¹ region, of organisms grown in minimal medium in light (curves A and B), and of organisms grown in complete medium in darkness (curves C and D), with limiting amounts or excess of cyanocobalamin. The vitamin B₁₂-deficient organisms (curves B and D) showed higher absorption at 1730 cm.⁻¹

Fig. 1. Infra-red absorption spectra of dried *Ochromonas malhamensis*. Curves A, B: organisms grown in minimal medium in light, with limiting (B) or with excess (A) cyanocobalamin. Curves C, D: organisms grown in complete medium in darkness, with limiting (D) or with excess (C) cyanocobalamin.

and lower absorption in the 1650 and 1540 cm.⁻¹ bands. The absorption at 1730 cm.⁻¹ disappeared on extraction of the organisms with diethyl ether, and the spectrum of the extracted material suggested that it consisted largely of saturated triglyceride fats. The absorption at 1650 and 1540 cm.⁻¹ can be attributed in the main to the cell proteins (Rideal & Adams, 1957), and was of lower intensity in the organisms grown with limiting amounts of cyanocobalamin. The strong absorption bands in the 1050 cm.⁻¹ region were due mainly to polysaccharides (Rideal & Adams, 1957) and were of equal intensity in the organisms grown with limiting amounts or with excess of cyanocobalamin.

Vitamin content of organisms. The preparations of organisms grown in 'complete' medium (see Table 1) were analysed for riboflavin, nicotinic acid, pantothenic acid and the vitamin B₆ group. The values obtained are given in Table 3; for comparison, values are also given for organisms grown in light in minimal medium. The acceleration of growth brought about by cyanocobalamin

was matched by increased cell concentrations of nicotinic acid, riboflavin and pantothenic acid. 'Vitamin B₆', on the other hand, was lowest in organisms grown with cyanocobalamin in excess.

Table 3. *Vitamin content of Ochromonas malhamensis grown at different concentrations of cyanocobalamin*

Conditions of cultivation	Vitamin concentration ($\mu\text{g./g.}$ dried cells)			
	Nicotinic acid	Riboflavin	Pantothenic acid	'Vitamin B ₆ '
Complete medium* + cyanocobalamin ($\text{m}\mu\text{g./ml.}$)				
0.05	49	12	3.0	4.6
0.10	53	14	4.3	5.0
0.20	54	16	5.5	4.6
4.0	62	18	6.0	2.9
Minimal medium + cyanocobalamin ($\text{m}\mu\text{g./ml.}$)				
0.025	59	20	5.0	5.8
0.20	64	28	6.2	2.8

* Same organisms as described in Table 1.

Distribution of vitamins between organisms and culture fluid. Nicotinic acid, pantothenic acid and the vitamin B₆ group were measured in the organisms and culture fluids of a culture grown in complete medium. These three vitamins were initially absent from the medium, and the sum of the vitamin content of the organisms and that of the culture fluid in which they had grown represents net synthesis. The results showed that even during the period of exponential growth, loss of these vitamins from the organisms to the surrounding medium constituted the main burden on synthesis. The ratio, amounts of these vitamins in the organisms/ml. culture:amounts/ml. culture fluid, was about 1:6 for pantothenic acid, about 1:3 for nicotinic acid and about 1:15 for the vitamin B₆ group. These figures refer to a culture grown for 42 hr. with limiting amount of cyanocobalamin (0.2 $\text{m}\mu\text{g./ml.}$).

Chlorophylls. When grown in light in minimal medium, the concentration of chlorophylls in cyanocobalamin-deficient organisms was lower than in controls grown with the vitamin in excess of the requirement for maximum growth rate. The chlorophyll contents of the organisms grown in light with 0.06 and 0.60 $\text{m}\mu\text{g.}$ cyanocobalamin/ml. were 1.80 and 2.85 $\mu\text{g./mg.}$ dried organism, respectively.

Glutathione. Attempts to measure glutathione by reaction with nitroprusside (Grunert & Phillips, 1951) were unsuccessful. This method of assay was not sufficiently sensitive to reveal the presence of glutathione in the cell preparations, either before or after electrolytic reduction. It seems safe to presume that the dried organisms contained less than 0.2 $\mu\text{g.}$ glutathione/mg. dry wt.

Tests with chloramphenicol. Gale & Folkes (1953) showed that with *Staphylococcus aureus* chloramphenicol at suitable concentration acted selectively in

preventing protein synthesis; other cell processes, including nucleic acid synthesis and the accumulation of free amino acids, were not inhibited except at very high concentrations of the antibiotic. In cultures of *Ochromonas malhamensis* grown with limiting amounts of cyanocobalamin and with increasing concentrations of chloramphenicol, the rates of division declined asymptotically to a low value at 500 $\mu\text{g.}$ chloramphenicol/ml. culture medium. It seemed that the relation between growth rate and chloramphenicol concentration might be the inverse of that linking growth rate with cyanocobalamin concentration, but this possibility was not investigated in detail.

Fig. 2. Influence of chloramphenicol on the growth of *Ochromonas malhamensis* in media containing cyanocobalamin at: (1) 0.03 mμg./ml. (Δ—Δ); (2) 0.1 mμg./ml. (○—○); (3) 0.3 mμg./ml. (●—●).

Evidence that vitamin B₁₂ and chloramphenicol may be concerned in the same metabolic processes can be adduced from the finding (Fig. 2) that inhibition of growth caused by chloramphenicol was annulled by increasing the concentration of cyanocobalamin. This effect might, of course, be indirect. Miyamura (1958) showed with *Escherichia coli* an annulment of a growth-inhibitory effect of chloramphenicol by certain amino acids and peptides.

Effects of chloramphenicol on the composition of organisms. Analyses were made of dried *Ochromonas malhamensis* grown in complete medium with excess cyanocobalamin, with and without chloramphenicol (50 μg./ml.). Growth in the presence of chloramphenicol was slow and the organisms were relatively rich in fat (as judged by the absorption at 1730 cm.⁻¹), and in protein, RNA and DNA, all of which were about 12 % higher than in the control organisms. The carbohydrate content was markedly lower, being 44 % of the dry weight of organisms as compared with 58 % in the control organisms. 'Free' amino-nitrogen showed an increase of c. 5 %. Thus chloramphenicol and vitamin B₁₂-depletion had clearly different effects on the protein and nucleic acid composition of the organisms.

DISCUSSION

Vitamin B₁₂ depletion in *Ochromonas malhamensis* brings about changes in composition that have been recognized in various algal species as being highly characteristic of nitrogen deficiency (Collyer & Fogg, 1955; Spoehr & Milner, 1949; for a review see Fogg, 1959). This finding suggests that in *O. malhamensis* the vitamin is concerned with the elaboration of nitrogen compounds; the vitamin B₁₂-deficient organisms may be in a sense deficient in nitrogen. Wagle, Mehta & Johnson (1958) believe that vitamin B₁₂ functions essentially in the incorporation of amino acids into proteins; they reported a striking effect on this incorporation in preparations of tissues from vitamin B₁₂-deficient rats. Other workers (Fraser & Holdsworth, 1959; Arnstein & Simkin, 1959) were unable to confirm these findings, and suggested that the decreased protein synthesis in vitamin B₁₂ deficiency might be secondary to some other function of the vitamin. Apart from these equivocal findings of Wagle *et al.* (1958), most of the evidence which links vitamin B₁₂ with protein biosynthesis is circumstantial. However, the syndrome of vitamin B₁₂ deficiency in *O. malhamensis* seems altogether compatible with an over-riding function of the vitamin in protein biosynthesis, and the relationships between cyanocobalamin concentration, growth rate and cell composition suggest that a re-orientation of thinking around this problem may be helpful. To seek to demonstrate a dramatic potentiation of enzyme function upon the addition of cyanocobalamin to deficient systems might be to seek the proverbial mare's nest. Reference to Table 1 shows that in *O. malhamensis* the effect of severe deprivation of vitamin B₁₂ was to decrease the rate of cell division by only 20 %. The rate of protein biosynthesis, and so presumably of amino acid incorporation, was decreased by a slightly greater amount. If we consider two populations of *O. malhamensis* growing exponentially, a difference of 20 % in their relative growth rates would quickly lead to a wide divergence between their relative population densities. If this small difference in rates of amino acid incorporation were the essential difference between the normal and the vitamin B₁₂-deficient organism, the effect on growth would probably be a summation of small effects on many enzymes, and might not be demonstrable as an all-or-nothing effect on any one system.

Ochromonas malhamensis grown at low concentrations of cyanocobalamin was relatively low in protein and nucleic acids, whereas organisms whose growth had been inhibited by chloramphenicol were over-full of both these constituents. This observation argues against the view that chloramphenicol and vitamin B₁₂ exert their opposite effects on growth at the same site in protein synthesis. It seems to rule out the possibility that chloramphenicol blocks the action of the vitamin by saturating the vitamin B₁₂-binding mechanism (Ford, 1958).

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