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The Role of Vitamin B_{12} in the Metabolism of *Euglena gracilis* var. *bacillaris*

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1. The concentrations of RNA, DNA and protein are decreased in cells of *Euglena gracilis* var. *bacillaris* grown on suboptimum concentrations of vitamin B_{12}. 2. The addition of vitamin B_{12} to deficient cells stimulates the incorporation of [^{14}C]formate into the above cell components as well as into thymine of DNA and serine and methionine of protein. 3. In a cell-free system from vitamin B_{12}-deficient cells, the incorporation of labelled formate into thymidylate is decreased to a greater extent with uridine than with deoxyuridine as the substrate. 4. The addition of unlabelled glutamate dilutes the radioactivity incorporated into thymine from labelled formate. 5. These results are interpreted to mean that, in DNA synthesis, vitamin B_{12} has a greater role in the reduction of ribotides to deoxyribotides than in the reduction of formate to thymine methyl and that the vitamin B_{12}-dependent conversion of glutamate into β-methylaspartate also contributes to thymine synthesis.

Vitamin B_{12} has been implicated in a wide variety of metabolic pathways in animals and in micro-organisms (Arnstein, 1955; Netrawali, Pradhan & Sreenivasan, 1961). It has been claimed by Dinning & Young (1959, 1960) that the vitamin takes part in the synthesis of the methyl group of thymine from C_1–tetrahydrofolate compounds at the formate level but not at the formaldehyde level. In support of this hypothesis, a lowering in the activity of methylenetetrahydrofolate dehydrogenase was observed in vitamin B_{12} deficiency (Henderson & Dinning, 1963). A major advance in the field has been the identification of the coenzyme form of the vitamin in *Clostridium tetanomorphum* by Barker, Weissbach & Smyth (1958) and in several other micro-organisms (Volcani, Toohey & Barker, 1961) as well as in animal tissues (Toohey & Barker, 1961). This has led to an extensive examination of the role of the coenzyme form of the vitamin. The coenzyme has been shown to participate in the glutamate-isomerase reaction (Smith & Monty, 1959), in the methylmalonyl-isomerase reaction (Gurnani, Mistry & Johnson, 1960; Stadtman, Overath, Eggerer & Lynen, 1960), in the conversion of ethylene glycol into acetaldehyde (Abeles & Lee, 1961) and in lysine degradation in anaerobic bacteria (Stadtman, 1964). Besides the above reactions, the vitamin coenzyme has also been shown to take part in the transfer of the methyl group from methyltetrahydrofolate to homocysteine in certain strains of *Escherichia coli* (Guest, Helleiner, Cross & Woods, 1960; Takeyama, Hatch & Buchanan, 1961; Larrabee, Rosenthal, Cathou & Buchanan, 1963). Recent evidence also suggests that the vitamin takes part in the reduction of ribotides to deoxyribotides (Downing & Schweigert, 1956; Wacker, Kirschfield & Trager, 1959; Manson, 1960; Blakley & Barker, 1964) in the biosynthesis of nucleic acids, although its role in protein biosynthesis as suggested by Wagle, Mehta & Johnson (1958) could not be confirmed by later workers (Arnstein & Simkin, 1959; Fraser & Holdsworth, 1959).

Although the vitamin B_{12}-requiring algal flagellate, *Euglena gracilis*, has been extensively used as an assay organism for the vitamin, studies of metabolic reactions known to require the vitamin have so far not been reported, unlike numerous observations on *Lactobacillus leichmannii*, *E. coli* and *Ochromonas malhamensis* (Johnson, Holdsworth, Porter & Kon, 1957; Dalal, Rege & Sreenivasan, 1961; Arnstein & White, 1961). It was therefore decided to study the possible functions of the vitamin in the metabolism of this organism.

EXPERIMENTAL

The strain *Euglena gracilis* var. *bacillaris* was used. The basal medium (double strength) used was as described by Hutner, Bach & Ross (1956) and contained (per l.): KH_2PO_4,

Table 1. *Effect of vitamin B_{12} on* [^{14}C]*formate uptake by* E. gracilis *cells*

Cells, grown on medium (100ml.) containing different concentrations of vitamin B_{12} at room temperature (25–27°) for 1 week, were harvested and suspended in 5ml. of fresh medium (10mg. dry wt./ml.) with and without added vitamin B_{12} (1mμg./ml.) and incubated with sodium [^{14}C]formate (0·187μc; specific activity 0·94mc/m-mole) for 30min. The cells, after separation from the medium, were washed repeatedly with non-radioactive 0·9% sodium formate and plated, and their radioactivity was determined. Results are averages of four independent experiments.

Vitamin B_{12} content of growth medium (μμg./ml.)	[^{14}C]Formate uptake (counts/min./mg. dry wt.)		Stimulation (%)
	Vitamin B_{12} absent	Vitamin B_{12} present	
5	200	370	85
10	244	358	46·7
20	280	346	23·6
40	342	344	—
50	349	351	—

1g.; L-glutamic acid, 10g.; DL-malic acid, 2g.; $MgSO_4,7H_2O$, 1g.; $ZnSO_4,7H_2O$, 88mg.; $CaCO_3$, 100mg.; $FeSO_4,7H_2O$, 30mg.; $MnSO_4,4H_2O$, 16·2mg.; $CuSO_4,5H_2O$, 3·92mg.; $CoSO_4,7H_2O$, 1·72mg.; $Na_2MoO_4,2H_2O$, 1·01mg.; H_3BO_3, 28·2mg.; thiamine hydrochloride, 2mg. The suboptimum concentration of vitamin B_{12} that yielded deficient cells was chosen based on percentage stimulation by the vitamin of [^{14}C]formate uptake by whole cells. For this, the organism was grown on basal medium (100ml.) containing concentrations ranging from 5 to 50μμg. of vitamin B_{12}/ml. in 250ml. conical flasks at 25–27° and under exposure to light from a fluorescent lamp source for 1 week. At the end of the period, the cells were harvested and suspended in 5ml. (10mg. dry wt./ml.) of fresh basal medium with and without exogenous vitamin B_{12} (1mμg./ml.). The suspensions were then incubated with sodium [^{14}C]formate (0·187μc; specific activity 0·94mc/m-mole; The Radiochemical Centre, Amersham, Bucks.) for 30min. The uptake of radioactivity by the whole cells was then determined by plating the washed cells and counting them in a Tracerlab SC–16 gas-flow counter (30% efficiency checked against standard $Ba^{14}CO_3$) in conjunction with an SC–51 autoscaler. The results presented in Table 1 give an indication of the 'vitamin B_{12} status' of the cells at the time of harvest. A stimulation of [^{14}C]formate incorporation to the extent of 85% was observed with cells grown on medium containing 5μμg. of vitamin B_{12}/ml. There is no stimulation of uptake of radioactivity at a concentration of 40μμg./ml. Cells harvested from medium containing the lower concentration of the vitamin therefore represent vitamin B_{12}-deficient cells. For further studies, cells were grown in larger volume (500ml.) of the medium under the above conditions.

Incorporation of [^{14}C]*formate into RNA, DNA and protein.* Suspensions of deficient cells (5ml.) were incubated with and without added vitamin B_{12} (1μg./ml.) with labelled formate (0·5μc; specific activity 0·94mc/m-mole) for 2hr. at 25°. At the end of the period the cells were separated from the medium by centrifugation and washed with cold non-radioactive 0·9% sodium formate solution. The cells were then fractionated into RNA, DNA and protein fractions by the method of Schmidt & Thannhauser (1945). Thymine was isolated from the DNA fraction after hydrolysis with 90% formic acid at 170° for 30min. in a sealed tube and by paper chromatography with propan-2-ol–conc. HCl (sp.gr. 1·19)–water (170:41:39, by vol.). The spot was identified on the dried paper by scanning under an ultraviolet lamp and with reference to a standard run. The spot corresponding to thymine was cut out and eluted with 0·1N-HCl.

The protein fraction was hydrolysed in 6N-HCl at 105° overnight, and serine and methionine were isolated from the protein hydrolysate by two-dimensional paper chromatography with butanol–acetic acid–water (4:1:5, by vol.) and phenol–water (1:1, v/v). The spots corresponding to serine and methionine were eluted from the paper with ethanol and estimated by the ninhydrin colour reaction (Yemm & Cocking, 1955).

RNA (Albaum & Umbreit, 1947), DNA (Seibert, 1940) and protein (Lowry, Rosebrough, Farr & Randall, 1951) were estimated by the methods cited, with yeast RNA, calf-thymus DNA and bovine serum albumin as the respective standards.

Incorporation of [^{14}C]*formate into thymidylic acid in cell-free extracts.* Normal and vitamin B_{12}-deficient cells were harvested from media and crushed by grinding with alumina in a chilled mortar with 0·1M-tris–HCl buffer, pH7·8. Unbroken cells were removed by low-speed centrifugation (200*g*) and the cell-free supernatant was dialysed against the same buffer for several hours in the cold. Dialysed extracts were incubated with either uridine or deoxyuridine (5mM), ATP (10mM) and tetrahydrofolic acid (5mM) in 0·1M-tris–HCl buffer, pH7·8, in an atmosphere of N_2 at 37° for 2hr. [^{14}C]Formate (0·5μc; specific activity 0·94mc/m-mole) was used as the labelled precursor. At the end of the incubation, the reaction was stopped by the addition of trichloroacetic acid (12%, w/v) and the supernatant fraction was subjected to paper chromatography. The spots corresponding to thymidine and thymidylate were isolated from the paper by the method described by McDougall & Blakley (1961).

Glutamate–β-methylaspartate conversion reaction. Cells grown for 1 week in media containing low and optimum concentrations of the vitamin were harvested and made into a suspension in water. Cells (50mg. of protein) were then incubated in a reaction mixture containing monosodium L-glutamate (10mM), tris–HCl buffer, pH8·2 (50mM), KCl (10mM), $MgCl_2$ (1mM) and αα′-bipyridyl (10mM) in a total volume of 5ml. at 37° for 1hr. Suitable blanks without the addition of the substrate were also used. At the end of the incubation, the reaction was stopped by the addition of 0·5N-H_2SO_4 in 95% (v/v) ethanol and the supernatant, after being passed through a 2cm. × 1cm. column of Dowex 50 (8% cross-linked; H^+ form; 200–400 mesh), was assayed for mesaconate by measurement of the extinction at 240mμ in a Beckman model DU spectrophotometer, as described by Barker *et al.* (1960).

Effect of exogenous glutamate on the incorporation of [^{14}C]*formate into DNA bases.* Cells were grown in medium containing peptone (Difco) (0·5%), yeast extract (0·2%) and sodium acetate (0·1%) under the conditions described

above. These cells were then harvested and suspended in fresh medium (10mg. dry wt./ml.). Cell suspensions (5ml.) were then incubated with 0·192μmole of [^{14}C]formate (specific activity 0·94mc/m-mole) at room temperature for 2hr. with and without monosodium L-glutamate (10mg.). At the end, the cells were separated and the DNA fraction was hydrolysed to bases by the method outlined above.

Suitable portions of the various samples were plated on glass planchets and counted at infinite thinness.

RESULTS AND DISCUSSION

RNA, DNA and protein contents of normal and vitamin B_{12}-deficient *E. gracilis* cells are shown in Table 2. The concentrations of these macromolecules are decreased in the vitamin deficiency. Rege & Sreenivasan (1954) had observed that *L. leichmannii* grown with optimum concentration of vitamin B_{12} are richer in DNA than when grown with suboptimum amounts. Decreases in RNA and DNA have also been reported in various animal tissues in deficiency of the vitamin (Rose & Schweigert, 1951; Schweigert, Scheid & Downing, 1954).

The incorporation of radioactivity from [^{14}C]-formate into RNA, DNA and protein fractions as well as into thymine of DNA and serine and methionine of protein is presented in Table 3. The addition of vitamin B_{12} to deficient cells stimulates the incorporation in all cases, although to different extents. The increase in specific activity of the protein fraction may be secondary to the effect on amino acid metabolism and particularly to the synthesis of methionine.

Although the stimulatory effect of vitamin B_{12} on RNA synthesis may not be due to its participation in purine biosynthesis, it may result from its effect on purine reserves of the cell. A rapid dissipation of guanine to adenine, which is excreted into the medium, has been reported to result from vitamin B_{12} deficiency in *L. leichmannii* (Floyd, Whitehead & Gould, 1962).

The role of the vitamin in DNA metabolism has been examined further. Analysis of the radioactivity incorporated into DNA thymine (Table 3) reveals a very significant stimulatory effect due to the vitamin. The incorporation of [^{14}C]formate into thymidylate in a cell-free system has been studied (Table 4). The incorporation of radioactivity is diminished to a greater extent in vitamin B_{12}-deficient cells when uridine serves as the C_1 acceptor than with deoxyuridine. These results

Table 2. *Effect of the vitamin B_{12} concentration of the growth medium on RNA, DNA and protein contents of* E. gracilis *cells*

Cells were grown on the basal medium (500ml.) of Hutner *et al.* (1956) with different concentrations of vitamin B_{12} at room temperature (25–27°) for 1 week. The cells were harvested by centrifugation and 100mg. dry wt. equivalents were extracted for determination of DNA, RNA and protein fractions according to the methods described in the text. Results are averages ± S.E.M. of four independent determinations.

Vitamin B_{12} content of growth medium ($\mu\mu$g./ml.)	Cell dry wt. from 100ml. of medium (mg.)	RNA (mg./100mg. dry wt.)	DNA (mg./100mg. dry wt.)	Protein (mg./100mg. dry wt.)
5	51·8	1·28±0·07	0·30±0·016	29·8±2·00
10	73·2	1·39±0·09	0·31±0·020	31·2±1·91
20	96·7	1·46±0·11	0·33±0·019	34·1±1·87
40	120·9	1·52±0·13	0·36±0·022	35·9±2·34
50	122·9	1·54±0·12	0·37±0·025	36·4±2·47

Table 3. *Effect of the addition of vitamin B_{12} to vitamin B_{12}-deficient cells of* E. gracilis *on* [^{14}C]*formate metabolism*

Cells, grown on a suboptimum concentration of vitamin B_{12} (5$\mu\mu$g./ml.) at 25° for 1 week, were harvested and resuspended in water (approx. 10mg. of protein/ml.). Cell suspensions (5ml.) were incubated with [^{14}C]formate (0·5μc; specific activity 0·94mc/m-mole) for 2hr. at room temperature with and without the addition of 1μg. of vitamin B_{12}. The cells were then centrifuged and fractionated into the various components as described in the text. Results are averages ± S.E.M. of four independent determinations.

Vitamin B_{12} (1μg./ml.)	$10^{-3}\times$ Sp. activity (counts/min./mg.)					
	RNA	DNA	Protein	Serine	Methionine	Thymine
–	27·2±1·9	32·5±2·1	8·1±0·9	14·2±1·1	10·2±0·8	14·6±1·5
+	38·1±2·5	51·8±4·3	11·8±0·8	19·4±1·6	18·7±1·3	26·3±1·9

Table 4. *Effect of vitamin B_{12} on thymidylic acid synthesis in* E. gracilis *cell-free system*

Dialysed cell-free extracts (10mg. of protein/ml.) of *E. gracilis*, grown on optimum and suboptimum concentrations of vitamin B_{12}, were incubated with either uridine or deoxyuridine (5mM) as indicated. Other additions include ATP (10mM) and tetrahydrofolic acid (5mM) in 0·1M-tris–HCl buffer, pH7·8. Incubations were carried out with [^{14}C]formate (0·5μC; specific activity 0·94mc/m-mole) at 37° for 2hr. in an atmosphere of N_2. The total volume of the reaction mixture was 1ml. Thymidine and thymidylic acid were isolated from the reaction products and counted by the methods detailed in the text. Results are averages ±S.E.M. of four independent experiments.

Vitamin B_{12} status of cells	Sp. activity of thymidylate (counts/min./mg. of protein) From uridine	From deoxyuridine
B_{12}-deficient	287±17·5	348±29·7
B_{12}-supplemented	465±22·0	398±37·5

indicate that the vitamin has a greater role in deoxyribotide synthesis than in the reduction of formate to thymine methyl, as reported with *L. leichmannii* and bone-marrow preparations (Dinning & Young, 1960). Wacker *et al.* (1959) have also observed increased conversion of [^{14}C]-guanosine into [^{14}C]deoxyguanosine in *L. leichmannii* in the presence of vitamin B_{12}. The possibility of a decreased apoenzyme concentration in the vitamin B_{12}-deficient cells may be ruled out from the results (Table 3) that show an increased incorporation of [^{14}C]formate into thymine of DNA by the deficient cells when exogenous vitamin B_{12} is added.

Another possible role of vitamin B_{12} in thymine biosynthesis may be through its intervention in the glutamate–β-methylaspartate conversion. A new pathway of thymine synthesis from β-methylaspartate has been found in certain micro-organisms (Seifter, Isenberg, Sawitsky & Berkman, 1960; Woolley & Koehelik, 1961). The existence of such a pathway has therefore been examined in *E. gracilis*. The effect of vitamin B_{12} deficiency on the conversion of glutamate into β-methylaspartate has been studied (Table 5): this conversion is affected in the vitamin B_{12}-deficient cells, indicating that glutamate may also contribute to the synthesis of thymine. This is further borne out from experiments where addition of a large amount of non-radioactive glutamate has shown an isotope-dilution effect on the incorporation of [^{14}C]formate into DNA thymine (Table 6). It seems possible that the further metabolism of β-methylaspartate to thymine causes the dilution of thymine formed from [^{14}C]formate and deoxyuridine. The results

Table 5. *Effect of vitamin B_{12} on glutamate–β-methylaspartate conversion in* E. gracilis

The reaction mixture contained *E. gracilis* cell suspension (10mg. of protein/ml.) grown on vitamin B_{12}-containing and -deficient media, monosodium L-glutamate (10mM), tris–HCl buffer, pH8·2 (50mM), KCl (10mM), $MgCl_2$ (1mM) and $\alpha\alpha'$-bipyridyl (10mM) in a total volume of 5ml. Incubation was carried out at 37° for 1hr. and the mesaconate formed in the reaction mixture was assayed in a Beckman model DU spectrophotometer at 240mμ against a similarly treated endogenous control as the blank. Results are averages ±S.E.M. of four independent observations.

Vitamin B_{12} status of cells	Mesaconate formed (mμmoles/hr./mg. of protein)
B_{12}-deficient	6·2±0·8
B_{12}-supplemented	10·8±1·0

Table 6. *Effect of exogenous glutamate on the incorporation of* [^{14}C]*formate into DNA bases*

Cells of *E. gracilis* were grown on a medium containing Bactopeptone (0·5%), yeast extract (0·2%) and sodium acetate (0·1%) for 1 week at room temperature. The cells were harvested and suspended in 5ml. of fresh medium (10mg. dry wt./ml.) and incubated with [^{14}C]formate (0·181μC; specific activity 0·94mc/m-mole) for 2hr. at room temperature with and without the addition of L-glutamate (10mg.). DNA isolated from the cells was fractionated into bases, which were eluted and counted. Results are averages ±S.E.M. of four sets of experiments.

Glutamate addition (10mg.)	Sp. activity (counts/min./μg.) Adenine	Guanine	Cytosine	Thymine
−	21·1 ±1·75	12·4 ±1·05	9·7 ±1·00	38·9 ±2·66
+	22·8 ±1·91	13·1 ±1·13	9·5 ±0·69	29·8 ±2·43

thus show that, in this organism, vitamin B_{12} has a role in the reduction of ribose to deoxyribose as well as in the synthesis of thymine by way of β-methylaspartate formation.

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