REFERENCES

Block, R. J. & Mitchell, H. H. (1946–7). *Nutr. Abstr. Rev.* **16**, 249.
de Loureiro, A. (1931). *Arch. Pat., Lisboa*, **3**, 72.
Folley, S. J., Ikin, E. W., Kon, S. K. & Watson, H. M. S. (1938). *Biochem. J.* **32**, 1988.
Forbes, R. M. & Yohe, M. (1955). *J. Nutr.* **55**, 499.
Frost, D. V. (1950). In *Protein and Amino Acid Requirements of Mammals*, p. 33. [A. A. Albanese, editor.] New York: Academic Press Inc.
Hartsook, E. W. & Mitchell, H. H. (1956). *J. Nutr.* **60**, 173.
Henry, K. M. & Kon, S. K. (1952). *J. Dairy Res.* **19**, 216.
Henry, K. M. & Kon, S. K. (1956). *Brit. J. Nutr.* **10**, 39.
Henry, K. M., Kon, S. K., Lea, C. H. & White, J. C. D. (1947–8). *J. Dairy Res.* **15**, 292.
Henry, K. M., Kon, S. K. & Watson, M. B. (1937). *Milk and Nutrition*, Part I, p. 37. Reading: National Institute for Research in Dairying.
Kon, S. K. & Henry, K. M. (1951). *J. Dairy Res.* **18**, 317.
Kon, S. K. & Henry, K. M. (1954). *J. Dairy Res.* **21**, 245.
Mitchell, H. H. (1923–4*a*). *J. biol. Chem.* **58**, 873.
Mitchell, H. H. (1923–4*b*). *J. biol. Chem.* **58**, 905.
Mitchell, H. H. (1947). *Arch. Biochem.* **12**, 293.
Mitchell, H. H. (1950). In *Protein and Amino Acid Requirements of Mammals*, p. 1. [A. A. Albanese, editor.] New York: Academic Press Inc.
Mitchell, H. H. & Beadles, J. R. (1950). *J. Nutr.* **40**, 25.
Mitchell, H. H. & Beadles, J. R. (1952). *J. Nutr.* **47**, 133.
Mitchell, H. H. & Carman, G. G. (1926). *J. biol. Chem.* **68**, 183.
Nasset, E. S. & Ely, M. T. (1952). *J. Nutr.* **48**, 391.
Neuberger, A. & Webster, T. A. (1945). *Biochem. J.* **39**, 200.
Osborne, T. B., Mendel, L. B. & Ferry, E. L. (1919). *J. biol. Chem.* **37**, 223.
Womack, M., Harlin, H. A. & Lin, P.-H. (1953). *J. Nutr.* **49**, 513.

Vitamin B_{12} and methyl-group synthesis

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Work with vitamin B_{12}-deficient and normal baby pigs has shown that vitamin B_{12} takes no part in transmethylation from choline or betaine to homocysteine or homocystine to form methionine (Mistry, Firth & Johnson, 1955; Firth, Mistry, James & Johnson, 1954) or in the conversion of glycine to serine (Chang & Johnson, 1955), but that vitamin B_{12} is required for methyl synthesis from glycine and from serine (Johnson, Firth & Mistry, 1955; Chang & Johnson, 1955; Johnson & Mistry, 1955).

Thus it was shown that the place of vitamin B_{12} in methionine formation is similar in the pig to that established in the rat (Arnstein & Neuberger, 1953; Stekol, Weiss, Smith & Weiss, 1953; Henry & Kon, 1956) for methyl synthesis, although it differs

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from that reported for vitamin B_{12} in transmethylation in the rat (Williams, Monson, Harper & Elvehjem, 1953).

In the hope that the protozoan *Ochromonas malhamensis* which has an absolute requirement for vitamin B_{12} (Hutner, Provasoli & Filfus, 1953; Ford, 1953) might serve as a guide to further investigations into the function of vitamin B_{12} in higher animals and man, the problem of methyl-group synthesis and the role in it of vitamin B_{12} has been investigated with this organism. A preliminary report of our findings has been published (Johnson, Holdsworth, Ford, Porter & Kon, 1955).

EXPERIMENTAL

Growth experiments

All growth experiments were replicated at least twice, 5 ml. medium (1 ml. basal medium + 4 ml. water containing test materials) being used in test tubes. The tubes were sterilized by autoclaving at 10 lb. for 10 min and when cool were inoculated with one drop from a 3- to 7-day culture of *Ochromonas malhamensis* which was maintained in a casein-hydrolysate medium containing 0·4 μg vitamin B_{12}/l. (Ford, 1953). The inoculated tubes were kept in the dark for the stated period in a shaking machine in an incubator at 28°, and were then steamed. When cool, the culture was diluted by addition of 5 ml. distilled water and the optical density measured in a Lumetron colorimeter.

Table 1. *Composition of basal medium for* Ochromonas malhamensis

(Amount/200 ml. medium five times strength)

NH_4Cl (g)	3	Glucose (g)	10
Arginine (g)	0·4	Tween 80 (ml.)	1
Methionine* (g)	0·6	Trace-mineral solution† (ml.)	10
$CaCl_2$ (g)	0·15	Thiamine (mg)	2
$MgSO_4.7H_2O$ (g)	0·2	Biotin (μg)	10
Ammonium molybdate (g)	0·05	NaCN (mg)	1
KH_2PO_4 (g)	0·3		
	Adjust pH to 5·0–5·5		

* Methionine, though necessary for an optimum medium, was omitted in all experiments on methionine and its precursors.

† Consisting of (g/1000 ml.):

Ethylenediamine tetra-acetic acid	5	$CoSO_4.7H_2O$	0·3
$MnSO_4.H_2O$	6·15	$CuSO_4.5H_2O$	0·04
$ZnSO_4.7H_2O$	11	H_3BO_3	0·06
$FeSO_4.7H_2O$	1	KI	0·001

Medium. The defined medium is given in Table 1 and was a modification of that of Hutner *et al.* (1953). This medium formed the basis for an investigation of a comparison of various nitrogen sources and the effect of various compounds on the growth of *Ochromonas*. Since methionine can stimulate the growth of this organism in simple media (Hutner *et al.* 1953) the effect of replacing the methionine with some possible precursors was studied.

Antimetabolite experiments

The specific antimetabolite of methionine metabolism, ethionine, inhibited growth of *Ochromonas* at 0·2 and 0·6 g/l. medium. Methionine and certain of its precursors

or methyl-group precursors were added to the medium containing ethionine to measure their ability to reverse the growth inhibition. These materials were tested in the absence and in the presence of vitamin B_{12}, and growth was measured at 3 and 5 days.

Respiration studies

The oxygen uptake of *Ochromonas* cells was studied in the conventional Warburg apparatus. Three types of cell were used: (*a*) cells grown on the basal medium without vitamin B_{12} but containing 0·6 g/l. methionine; (*b*) those grown with a suboptimal amount of vitamin B_{12} (0·1 μg/l.); and (*c*) those grown with excess of the vitamin (10 μg/l.). The cells were centrifuged off and washed with sterile basal medium containing no vitamin B_{12} and no nitrogen source, i.e. neither ammonium chloride nor arginine. For the study of the effect of the vitamin on respiration, glucose was the substrate in basal medium (without nitrogen source). With cells grown with excess vitamin, the oxygen uptake of *Ochromonas* without substrate, or with glycine (10 g/l.), was compared with that of glucose, and the effect of leaving Tween 80 (polyoxyethylenesorbitan mono-oleate, Atlas Powder Co., Wilmington, Delaware) out of the substrate medium was examined. The oxygen uptake per unit of optical density was obtained by taking 1 ml. of the suspension from the Warburg flask, diluting to 10 ml. and measuring the optical density in the colorimeter.

Chromatography and autoradiography

No. 7 Whatman paper was used in a descending system with sec.-butanol/ammonia (three parts sec.-butanol, one part of 3% (v/v) conc. ammonia (sp.gr. 0·88) in water) or with water-saturated phenol as solvents. For isolation of methionine, the preparation was streaked across a sheet of Whatman 3MM paper and, after development and detection of the methionine zone, this portion was cut out and eluted with water.

Autoradiographs were prepared with Ilfex (Ilford Ltd, London) No-screen X-ray film, exposed for from 3 to 14 days.

Isolation of methionine sulphoxide

To the radioactive eluate from the chromatogram (approx. 10 ml.) were added 2 mg inactive DL-methionine and 1 ml. hydrogen peroxide (100 vol.). The mixture was concentrated under reduced pressure to 2 ml. and crystallized from aqueous ethanol. The sulphoxide was repeatedly recrystallized and the specific activity of each batch of crystals determined.

Incorporation of ^{14}C-glycine into growing Ochromonas

To a total volume of 25 ml. of the basal medium containing 10 μg vitamin B_{12}/l. were added 26 mg α-^{14}C-glycine (25 mg unlabelled and 1 mg labelled = 10 μc) and 15 mg homocysteine; a similar experiment was conducted with only 1 mg (= 10 μc) ^{14}C-glycine. The cells were grown on these media for 5 days and then harvested. Three fractions were obtained. The cells were centrifuged (1500 ***g***) leaving the clear supernatant medium (fraction 1); the cells were then ruptured by repeated freezing and thawing with vibration in a mechanical shaker (Towers, Widnes) with glass beads

The ruptured cells were centrifuged at high speed (10,000 ***g***) to give a clear cell extract (cell contents, fraction 2) and cell debris. The cell debris was hydrolysed with 5N-HCl in a sealed tube to liberate the amino-acids from the cell proteins (fraction 3). These fractions were examined for methionine, by chromatography followed by autoradiography.

One hundred ml. medium containing no vitamin B_{12} but with the same concentrations of labelled glycine and homocysteine gave too few cells for an adequate control experiment. Because of this, more cells deficient in vitamin B_{12} were obtained by growing cells in a total of 600 ml. of medium containing 0·04 μg vitamin B_{12}/l. After 5 days the cells were harvested by centrifuging aseptically and suspended in 100 ml. vitamin B_{12}-free basal medium containing added homocysteine (60 mg) and ^{14}C-glycine (60 mg containing 25 μC α-^{14}C-glycine). Portions of this culture were taken after 2 days', and the rest after 5 days', incubation for examination by chromatography.

Methionine synthesis in cell-free preparations

Three types of cell were used: (*a*) for cells grown with vitamin B_{12} a 3-day culture from 300 ml. medium containing 10 μg vitamin B_{12}/l.; (*b*) for cells deficient in the vitamin 400 ml. of medium containing 0·04 μg vitamin B_{12}/l.; and (*c*) cells grown with excess vitamin B_{12} but deficient in thiamine were obtained from 500 ml. medium containing 10 μg vitamin B_{12}/l. and 2 μg thiamine/l., this last medium being inoculated from a thiamine-low culture.

The cells were centrifuged and the supernatant liquid was rejected; small glass beads were added and the cells ruptured by repeated freezing, thawing and shaking. The cell debris was removed by centrifuging, and to the cell-free extract were added 1 mg homocysteine and 1 mg α-^{14}C-glycine (10 μC, volume 1 ml.). Samples (10 μl.) were taken at zero time, at hourly intervals for 5 h, and finally after 18 h incubation at 28°. The remainder of the preparation was steamed and further samples were taken for chromatography. The chromatograms were examined by autoradiography. For isolation of the radioactive methionine, the enzyme preparation from cells grown with excess vitamin B_{12} was applied to a sheet of Whatman 3MM paper (see p. 315).

RESULTS

In the development of a simple growth medium for *Ochromonas*, a number of nitrogen sources were examined, and the results are shown in Table 2. In addition to the general basic nitrogen sources, several other compounds were tried as supplements to ammonium chloride; the additions that had the greatest effect besides methionine were arginine, lysine and malate. Though these were definitely stimulatory at 3 days' incubation, they had less effect at 5 days' incubation. The methionine stimulation was clearly shown on all methionine-free media but appeared more striking on the simple medium used. Thus, for most of the work, the basal medium containing ammonium chloride and arginine but not lysine or malate (i.e. the medium given in Table 1) with the methionine omitted was used.

The growth-stimulating effect of methionine for *Ochromonas* was studied with

several different basal media and at various levels of vitamin B_{12} supplementation. The results are summarized in Table 3. From them it is evident that methionine at the proper levels gives an added growth response over that obtained with vitamin B_{12} and

Table 2. *Growth of* Ochromonas malhamensis *in various simple media*

Source of nitrogen		Optical density (× 100)* after incubation in presence of vitamin B_{12} at 10 μg/l. final medium for	
Description	Amount (g/l. final medium)	3 days	5 days
Ammonium citrate	3 (to 5)†	30	46
Ammonium chloride	3	30	68
Asparagine	3	15	38
Glutamic acid	3	—	18
Glycine	3	5	22
Hydrolysed casein	3	57	73

* i.e. Lumetron readings of diluted (1:1) cultures × 10.

† Increases over 3 g/l. did not increase growth (3 g ammonium citrate supplies approx. the same amount of nitrogen as 5 g casein hydrolystate).

Table 3. *Effect of vitamin B_{12} and methionine on the growth of* Ochromonas malhamensis

Total methionine in final medium (g/l.)	Optical density (× 100)* of growth medium when the vitamin B_{12} concentration in the final medium was					
	Nil	0·1 g/l.	0·2 g/l.	0·4 g/l.	0·8 g/l.	1·0 g/l.
Basal medium with asparagine; 5 days' incubation period						
0	10	26	34	43	60	—
0·1	9	28	37	43	62	—
0·2	10	30	40	55	66	—
0·4	17	45	49	64	66	—
0·8	30	45	45	45	45	—
Basal medium with ammonium citrate; 5 days' incubation period						
0	1·0	3	—	—	—	31
0·1	3·5	7·5	—	—	—	49
0·2	7	13·5	—	—	—	63
0·4	14	30	—	—	—	71
0·6	19	36	—	—	—	75
0·8	17	32	—	—	—	50
1·0	15	31	—	—	—	50
2·0	10	14	—	—	—	13
Basal medium with ammonium chloride; 4 days' incubation period						
0	2·5	—	—	10	29	—
0·2	2·6	—	—	14	44	—
0·7	7	—	—	18	48	—
1·4	5·5	—	—	19	43	—
Basal medium with hydrolysed casein (3 g/l.); 4 days' incubation period						
0·1	6	—	—	11	53	—
0·2	6	—	—	19	56	—
0·7	9·5	—	—	22	63	—
1·4	9	—	—	27	57	—

at all levels of vitamin B_{12} used. It also appears that high levels of methionine are definitely inhibitory.

The growth stimulation by methionine (Hutner *et al.* 1953) being thus confirmed, in both the presence and absence of vitamin B_{12}, the possibility of replacing methionine by a metabolic precursor in the presence of vitamin B_{12} was next investigated. Because of the animal work on glycine already cited, glycine plus homocysteine was tried first in 3-day growth experiments, but without effect. However, with a longer (5-day) incubation period it was found that glycine plus homocysteine would replace methionine for the growth stimulation of the organism, in the presence of vitamin B_{12} but not in its absence. The results of these experiments for 3- and 5-day incubation periods are given in Table 4. Since glycine in the presence of vitamin B_{12}, although not in its absence, would replace methionine in the growth stimulation of *Ochromonas* after a 5-day incubation period, a number of possible methionine precursors were tried in this same assay system. The results are summarized in Table 5, from which it is clear that glycine, serine, threonine and homoserine will, to varying degrees, serve as precursors of methyl groups for methionine synthesis for the growth of *Ochromonas* and that it is not necessary to supply the homocysteine part of the molecule either as homocysteine or as homocystine. The values shown in Tables 4 and 5 are complicated by the inhibitory effect sometimes shown when glycine was included in the medium, particularly in the absence of vitamin B_{12}, but the marked stimulation when glycine and the vitamin were both present is always evident.

Table 4. *Effect of period of incubation on growth response of* Ochromonas malhamensis *to methionine and its precursors*

(Triplicate experiments, three tubes at each level in each experiment)

Supplement to basal medium (without methionine)		Relative turbidity*			
		3-day incubation period		5-day incubation period	
Description	Amount (g/l. final medium)	Without vitamin B_{12}	With vitamin B_{12}	Without vitamin B_{12}	With vitamin B_{12}
None	—	100 (0·25)	100 (2·0)	100 (0·6)	100 (6·5)
Methionine	0·6	152	175	183	150
Homocysteine	0·6	100	100	83	104
Homocysteine + glycine	0·6 + 1·0	83	91	50	155

* Figures in parentheses are Lumetron scale readings.

In order to demonstrate further that this growth stimulation in the absence of methionine, but in the presence of vitamin B_{12}, was indeed due to methionine synthesis from the precursors involved, experiments were carried out with the specific methionine antimetabolite ethionine. From Table 6 it is evident that the inhibition caused by ethionine at 0·6 g/l. can be reversed by 0·6 g/l. of methionine. However, at this level of antimetabolite, glycine with or without vitamin B_{12} was unable to cause significant reversal of inhibition. At the ethionine level of 0·2 g/l. glycine in the presence of vitamin B_{12} partially reversed the inhibition and the effect approached that brought about by 0·6 g/l. of methionine. In a system such as this the mechanism of reversal of inhibition must be complicated, therefore only marked reversal of the inhibition

Table 5. *Ability of possible precursors of the methyl group of methionine to replace methionine for the growth stimulation of* Ochromonas *in the presence and absence of vitamin B_{12}*

Supplement to basal medium (without methionine)		Relative turbidity*			
		3-day incubation		5-day incubation	
Description	Amount (g/l. final medium)	Without vitamin B_{12}	With vitamin B_{12}	Without vitamin B_{12}	With vitamin B_{12}
None	—	100 (0·4)	100 (3·0)	100 (0·7)	100 (6·6)
DL-Methionine	0·6	150	164	150	147
Glycine	1·0	90	120	100	150
Homocysteine: alone	0·6	100	100	80	104
+glycine	0·6+1·0	100	100	60	150
+DL-serine	0·6+1·0	100	83	60	120
+formate	0·6+1·0†	80	12	20	7
+choline	0·6+1·0	80	120	80	104
Homocystine: alone	0·6	100	145	100	122
+glycine	0·6+1·0	90	146	100	150
DL-Serine	1·0	100	100	100	128
L-Serine‡	1·0	100	120	100	130
D-Serine‡	1·0	100	80	100	116
DL-Histidine	1·0	90	90	100	108
DL-Threonine	1·0	90	80	100	119
DL-Homoserine	1·0	100	120	100	120
DL-Methionine+glycine	0·6+1·0	150	175	100	154

* Figures in parentheses are Lumetron scale readings.
† Formate was toxic at levels from 0·2 to 1·0 g/l. and ineffective below 0·2 g/l.
‡ Kindly supplied by Dr H. R. V. Arnstein, National Institute for Medical Research.

Table 6. *Effect of possible precursors of the methyl group of methionine on reversing the toxicity of ethionine for* Ochromonas

Supplement to basal medium (without methionine)		Optical density (×100)*			
		3-day incubation		5-day incubation	
Description	Amount (g/l. final medium)	Without vitamin B_{12}	With vitamin B_{12}	Without vitamin B_{12}	With vitamin B_{12}
None	—	1·6	12·0	7·2	75
Ethionine: alone	0·6	0·2	1·0	1·2	6·3
+methionine	0·6+0·6	0·5	11·0	5·3	74
+glycine	0·6+1·0	0·0	1·5	2·7	13
None	—	—	—	6·5	83
Ethionine: alone	0·2	—	—	6·7	11
+glycine	0·2+1·0	—	—	6·4	66
+DL-serine	0·2+1·0	—	—	6·3	63
+L-serine	0·2+1·0	—	—	6·4	79
+D-serine	0·2+1·0	—	—	6·4	42
+DL-threonine	0·2+1·0	—	—	6·0	28
+DL-homocystine	0·2+0·6	—	—	8·0	18
+DL-histidine	0·2+1·0	—	—	6·2	13
+DL-homoserine	0·2+1·0	—	—	6·0	47
+glycollic acid	0·2+1·0	—	—	4·0	9
+DL-methionine	0·2+0·6	—	—	7·4	83
+sodium glyoxylate	0·2+0·5	—	—	6·5	10
+sodium formate	0·2+0·1	—	—	4·5	8

* i.e. Lumetron readings of diluted (1:1) cultures × 10.

has been taken as evidence of the synthesis of methionine. Thus the effect of serine, particularly the L form, and homoserine seem unequivocal, whereas the effect of the other methyl-group precursors is doubtful.

To study directly the synthesis of the methyl groups of methionine from glycine, *Ochromonas* cells were grown in the presence of α-^{14}C-glycine and homocysteine with adequate or limiting amounts of vitamin B_{12}. Typical autoradiographs are shown in Pl. 1. No methionine was found in the cell contents of *Ochromonas* whether grown with or without vitamin B_{12} (Pl. 1, 1,2), but in the hydrolysed cells grown with vitamin B_{12} and ^{14}C-labelled glycine a substance appeared with the same R_F as methionine. This metabolite was proved to be methionine by repeated carrier crystallization with an authentic specimen of inactive methionine sulphoxide. Cell-free preparations, obtained by freezing and thawing to disrupt the cells and centrifugation, gave similar autoradiographs. Preparations from *Ochromonas* grown with limiting vitamin B_{12} were less able to metabolize glycine (Pl. 1, 3) and produced no detectable amount of ^{14}C-methionine. Since deficient cells may be less active metabolically and the effect of vitamin B_{12} deficiency on glycine may be due to some non-specific phenomena, cell-free preparations were studied from *Ochromonas* grown with excess vitamin B_{12}, but with limiting amounts of thiamine (also an absolute requirement for growth). These preparations were able to synthesize methionine from the α-^{14}C of glycine, which indicates that methyl-group formation is a specific function of vitamin B_{12}.

Since the sec. butanol/ammonium solvent did not separate glycine from serine, glycine bands were cut from the chromatograms used for the isolation of methionine. This material was then eluted and rechromatographed with water-saturated phenol. No ^{14}C-labelled serine could be detected by autoradiography.

Table 7. *Oxygen uptake of* Ochromonas *cells grown under different conditions of vitamin B_{12} nutrition*

Supplement added to medium used for growth of cells	Oxygen uptake (mm^3/h/unit optical density)*
Methionine (0·6 g/l.) (no vitamin B_{12})	88
Vitamin B_{12} at 0·1 μg/l.	89
Vitamin B_{12} at 10 μg/l.	92

* One unit optical density = Lumetron reading of a ten times diluted suspension from Warburg flask.

Although lacking the ability to synthesize methionine, vitamin B_{12}-deficient cells did not differ significantly, in their oxygen uptake, from cells grown with excess vitamin. These results are shown in Table 7. It can be seen from Table 8 that *Ochromonas* cells had a very high endogenous respiration, and it was found that this rate of respiration was constant for over 18 h, indicating the great amount of energy stored in the cell. Omission of Tween 80 from the medium for growing *Ochromonas* gave cells with approximately half the rate of respiration given in Table 8. These experiments also show that glycine is inefficient as a carbon (energy) source.

Table 8. *Oxygen uptake of* Ochromonas *cells grown on a complete medium (containing 10 μg/l. vitamin B_{12}) when deriving carbon from different substrates*

Substrate	Oxygen uptake (mm^3/h/unit optical density)*
None (endogenous)	81
Glycine (10 g/l.)	93
Glucose (10 g/l.)	135

* One unit optical density = Lumetron reading of a ten times diluted suspension from Warburg flask.

DISCUSSION

It has been stated that methionine stimulates the growth of *Ochromonas malhamensis* (Hutner *et al.* 1953) and our experiments both in the presence and absence of vitamin B_{12} confirm this finding. This effect was obtained on media with inorganic nitrogen, or with a complete amino-acid mixture. The methionine could be replaced by homocysteine (or homocystine) and glycine, but only in the presence of vitamin B_{12}. Similarly, homocysteine and serine, or to a lesser extent certain other methyl-group precursors, would give a stimulation similar to that of methionine when the vitamin was present. It is interesting that transmethylation appears to be ruled out, since no effect was obtained with choline. The cells appeared to contain sufficient methyl-group acceptor (without added homocysteine) since glycine or serine was effective alone. The response to homoserine and the ability of homoserine to reverse ethionine inhibition may be related to its metabolism to glycine (Woods, 1955) or its ability to accept sulphur and methyl groups for methionine synthesis (Wokes & Picard, 1955). The reversal of ethionine inhibition was perhaps the most striking proof of the conversion of glycine and serine to methionine and demonstrated the greater activity of L-serine.

Both growing cells and cell-free preparations from *Ochromonas* grown with adequate vitamin B_{12} used the ^{14}C from the α group of glycine for incorporation into methionine. Cells grown with limiting vitamin B_{12} did not have this ability. Deficient cells did not differ from normal cells in their rate of oxygen uptake with either glucose or glycine as substrate. Therefore the deficient cells were not moribund. Also, cells grown with adequate vitamin B_{12}, but limiting thiamine, could use glycine as the source of the methyl of methionine, which suggests that vitamin B_{12} acts specifically in methyl-group synthesis.

At least three possible pathways appear to exist for the synthesis of the methyl group of methionine from glycine. They involve: (1) the formation of serine hydroxymethyl (Arnstein, 1954, 1955) from the α-carbon of glycine and its transfer from serine to homocysteine followed by reduction to methyl to yield methionine; (2) the oxidative deamination of glycine to glyoxylic acid which is then decarboxylated to yield CO_2 and an 'active formaldehyde' which will directly give methionine via hydroxymethyl homocysteine (Nakada, Friedmann & Weinhouse, 1955); (3) the formation of δ-amino-laevulic acid and its conversion into α-keto-glutaraldehyde which then gives succinate and 'active formaldehyde' (Shemin, 1955). The fact that no serine was found

during metabolism of glycine in either the presence or absence of vitamin B_{12} would indicate that glycine provides a methyl group without serine serving as an intermediate. However, the serine may have been metabolized more rapidly than it was formed and thus escaped detection. Glyoxylic and glycollic acids did not reverse the growth inhibition produced by ethionine and would thus appear to be ruled out as the one-carbon precursors. However, glyoxylic acid is unstable and there may have been little of the compound present in the medium during the later stages of growth. Preliminary experiments with growing cells with δ-amino-laevulic acid showed that it was not capable of stimulating growth like methionine. Thus the steps in the conversion of the α-C of glycine into methionine need further investigation.

SUMMARY

1. On a simplified medium the protozoan *Ochromonas malhamensis* showed a growth response to methionine, both in the presence and in the absence of added vitamin B_{12}.

2. In the presence of vitamin B_{12}, but not in its absence, the methyl-group precursors, glycine and serine, gave a growth response similar to that of methionine.

3. Inhibition of growth of the organism by ethionine was completely reversed by methionine. Glycine, serine, and to a less extent certain other methyl precursors, reversed ethionine inhibition only if vitamin B_{12} was also present in the medium and a 5-day incubation period used.

4. Cell-free extracts prepared from cells grown with an adequate amount of vitamin B_{12} formed ^{14}C-methionine from α-^{14}C-glycine. Vitamin B_{12}-deficient cells did not have this property.

5. No difference in oxygen uptake was found between cell growth without, or with limiting, or with adequate vitamin B_{12}.

EXPLANATION OF PLATE

Autoradiographs of paper-strip chromatograms.

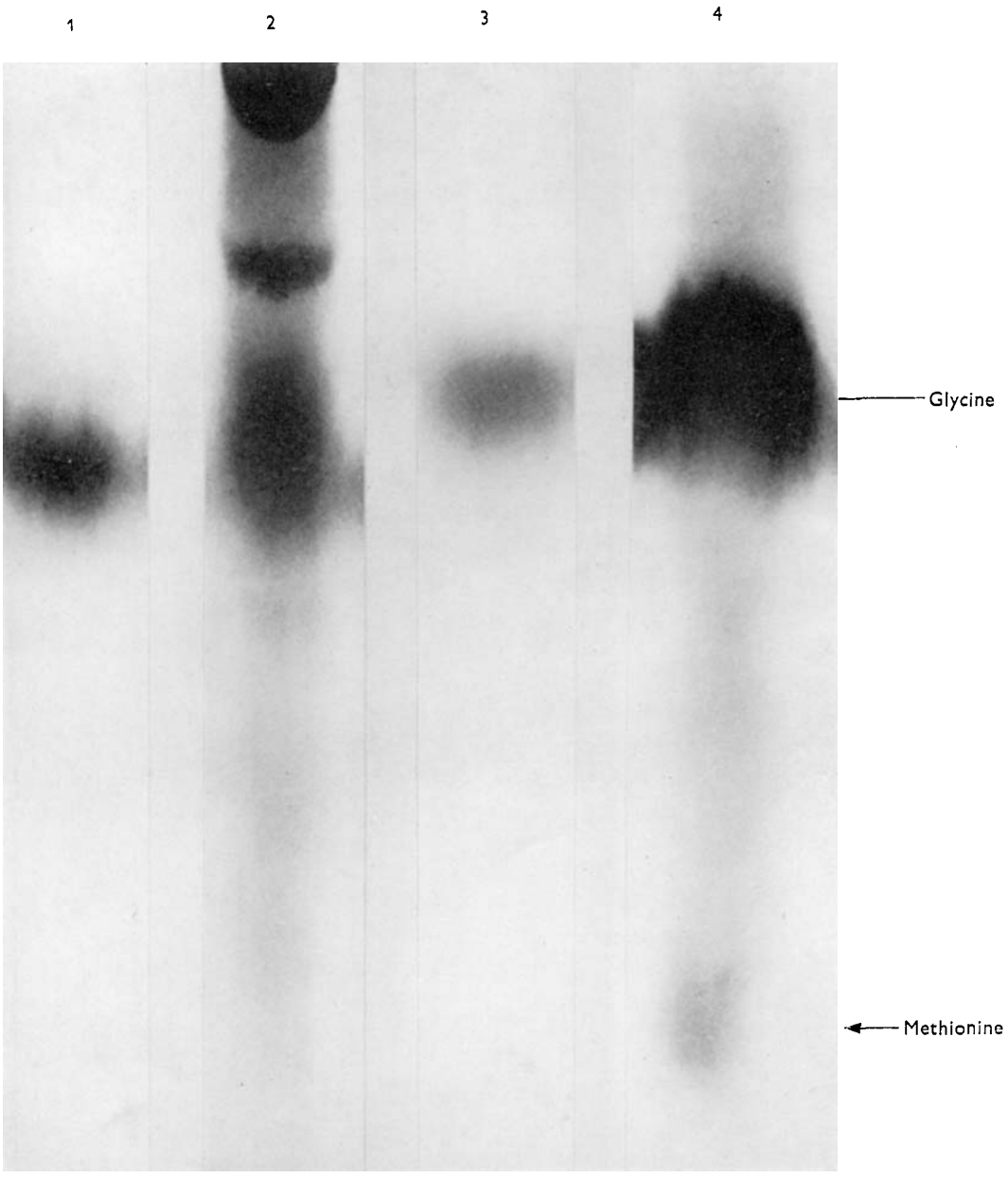
1. Cell extract (cell contents) of cells grown without vitamin B_{12} on a medium containing α-^{14}C-glycine.
2. Cell extract (cell contents) of cells grown with vitamin B_{12} and α-^{14}C-glycine.
3. Acid hydrolysate of cell debris from 1.
4. Acid hydrolysate of cell debris from 2.

Chromatographed on Whatman no. 7 filter-paper sheets in *sec.*-butanol (2 parts)–ammonia (1 part of 3% (v/v) conc. NH_4OH in water) solvent solution.

Significant amounts of methionine were found only in the debris of cells grown with vitamin B_{12}.

REFERENCES

Arnstein, H. R. V. (1954). *Advanc. Protein Chem.* **9**, 2.
Arnstein, H. R. V. (1955). *Biochem. J.* **60**, vii.
Arnstein, H. R. V. & Neuberger, A. (1953). *Biochem. J.* **55**, 259.
Chang, I. & Johnson, B. C. (1955). *Arch. Biochem. Biophys.* **55**, 151.
Firth, J., Mistry, S. P., James, M. F. & Johnson, B. C. (1954). *Proc. Soc. exp. Biol., N.Y.*, **85**, 307.
Ford, J. E. (1953). *Brit. J. Nutr.* **7**, 299.
Henry, S. K. & Kon, S. K. (1956). *Brit. J. Nutr.* **10**, 39.
Hutner, S. H., Provasoli, H. L. & Filfus, J. (1953). *Ann. N.Y. Acad. Sci.* **55**, 852.
Johnson, B. C., Firth, J. & Mistry, S. P. (1955). *Arch. Biochem. Biophys.* **54**, 467.



(*Facing p.* 322)

Johnson, B. C., Holdsworth, E. S., Ford, J. E., Porter, J. W. G. & Kon, S. K. (1955). *Biochem. J.* **60**, xxxix.
Johnson, B. C. & Mistry, S. P. (1955). *Congr. int. Biochim.* III. *Brussels. Résumé des Communications*, p. 116.
Mistry, S. P., Firth, J. & Johnson, B. C. (1955). *Fed. Proc.* **14**, 445.
Nakada, H. I., Friedmann, B. & Weinhouse, S. (1955). *J. biol. Chem.* **216**, 583.
Shemin, D. (1955). In *A Symposium on Amino Acid Metabolism*, p. 727. [W. D. McElroy and H. B. Glass, editors.] Baltimore: Johns Hopkins Press.
Stekol, J. A., Weiss, S., Smith, P. & Weiss, K. (1953). *J. biol. Chem.* **201**, 299.
Williams, J. N. Jr., Monson, W. J., Harper, A. E. & Elvehjem, C. A. (1953). *J. biol. Chem.* **202**, 607.
Wokes, F. & Picard, C. W. (1955). *Amer. J. clin. Nutr.* **3**, 383.
Woods, D. D. (1955). Personal communication.

Methionine sulphoximine and the growth of the wheat embryo

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Besides having a neurotoxic action methionine sulphoximine, the toxic principle produced by the action of nitrogen trichloride (agene) on the protein of wheat (Bentley, McDermott, Moran, Pace & Whitehead, 1950), has been reported to depress or inhibit the germination of seeds, the larval development of insects, the growth of certain bacteria (Pace & McDermott, 1952), the synthesis of protein by cells in culture (Rabinovitz, Olson & Greenberg, 1956), the growth in culture of bone rudiments and the development of chick embryo (Mellanby, 1956). It has also been shown that the depressing effect on the growth of bacteria and the synthesis of protein by cells in culture can be prevented by the provision of glutamine or methionine to the growing cells (Heathcote, 1949; Heathcote & Pace, 1950; Rabinovitz *et al.* 1956). The experiments described in this paper show that methionine sulphoximine is also markedly toxic to the wheat embryo and that this toxicity can be nullified by the presence of glutamine.

EXPERIMENTAL

The effects were demonstrated by growing the embryos on sterile media containing methionine sulphoximine. Grain, variety Squarehead's Master, of good germinating vigour was sterilized for 1 sec in 70% alcohol, allowed to dry and then placed in a moist chamber for 2 or 3 days until the embryos, inclusive of scutellum, were just moist enough to be removed cleanly without damage. They were allowed to dry and immediately before being placed on the medium were sterilized in 70% alcohol, the excess being removed on sterile filter-paper. Two types of medium were used, one a simple synthetic similar to that described by Purvis (1944), containing dextrose 2%,