MUTANTS OF THE FORMYLTETRAHYDROFOLATE INTERCONVERSION PATHWAY OF *SACCHAROMYCES CEREVISIAE*

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Manuscript received October 19, 1976
Revised copy received January 13, 1977

ABSTRACT

Thirteen mutants of *Saccharomyces cerevisiae* that lack one or more of the three enzyme activities of the pathway for interconversion of tetrahydrofolate coenzymes at the formate level of oxidation have been isolated. They do not require adenine. All fail to complement mutations in the *ade3* locus. Mutations that greatly reduce activity for one enzyme also reduce activity for the other two interconversion enzymes. The three enzyme activities cochromatograph on TEAE-cellulose columns. A mutation that eliminates synthetase activity also alters the chromatographic behavior of the remaining cyclohydrolase and dehydrogenase activities. It is suggested that the three activities reside in an enzyme complex encoded by the *ade3* locus.

POINT mutations at the *ade3* locus of *Saccharomyces cerevisiae* may result in a requirement for adenine and histidine (ROMAN 1956), for adenine only (LOMAX, GROSS and WOODS 1971; LAM and JONES 1973; JONES 1977) or for adenine or histidine (LOMAX, GROSS and WOODS 1971). At least 70% of the mutations of the *ade3* locus isolated as white or pink derivatives of red *ade2*-bearing strains are nonsense mutations (JONES 1972b).

The *ade3* mutants have been shown to be deficient for formyltetrahydrofolate synthetase (EC6.3.4.3), and methenyltetrahydrofolate cyclohydrolase (EC3.5.4.9), and to lack one of two isoenzymes of methylenetetrahydrofolate dehydrogenase (EC1.5.1.5) (JONES and MAGASANIK 1967a; LAZOWSKA and LUZZATTI 1970a,b; LOMAX, GROSS and WOODS 1971; LAM and JONES 1973; JONES 1977). LOMAX, GROSS and WOODS (1971) found some heterogeneity in the levels of formyltetrahydrofolate synthetase among the *ade3* mutants, depending on the particular nutritional phenotype of the mutant strain.

The pathway for the interconversion of single carbon derivatives of tetrahydrofolate (THFA) can be entered from two main directions (Figure 1). THFA may be directly charged with formate to give 10-formylTHFA or the β-carbon of serine may be donated to give 5,10-methyleneTHFA. Mutations in the *ade3* locus that reduce the activities for all three interconversion enzymes will result in a dearth of 10-formylTHFA and 5,10-methenylTHFA. This reduction in level

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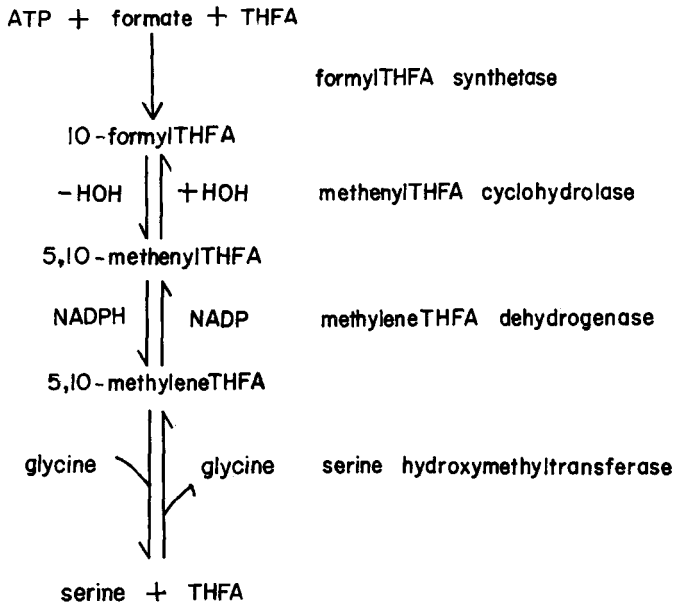

FIGURE 1.—Pathway and enzymes involved in interconversion of selected coenzymes of tetrahydrofolic acid.

of these two co-factors leads to the inability of the cells to carry out the reactions which convert 5′-phosphoribosylglycinamide to 5′-phosphoribosyl-N-formylglycinamide and 5′-phosphoribosyl-5-amino-4-imidazolecarboxamide to 5′-phosphoribosyl-5-formamido-4-imidazolecarboxamide (JONES and MAGASANIK 1967b) and provides an explanation for the adenine requirement. A mutation which causes a deficiency for only one of the enzymatic activities would not be expected to cause a decreased level of charged THFA coenzymes and should not, by itself, cause a nutritional requirement.

In order to isolate mutants lacking activity for only one enzyme in the interconversion pathway given in Figure 1, it is necessary to establish conditions such that integrity of the pathway is essential for growth. The nutritional requirement of serine auxotrophs can be met by serine or by high levels (100 μg/ml) of glycine. If the concentration of glycine is reduced to 20 μg/ml, serine auxotrophs do not grow. Apparently this low level of glycine is insufficient to supply the glycine, serine and single carbons needed for growth. The addition of formate to the medium containing low levels of glycine restores growth of serine auxotrophs. Under this growth condition (glycine plus formate) the integrity of the interconversion pathway has become essential for growth. Among mutants isolated for the inability to satisfy their serine requirement by glycine plus formate should be strains which have reduced levels of one or more of the three interconversion enzymes.

This paper reports results of genetic, physiological and enzymatic studies of 13 mutants which lack one or more of the interconversion enzymes. Fine structure mapping of these mutations within the *ade3* locus will be reported sub-

sequently (JONES and MCKENZIE, in preparation). A preliminary report of this work was presented (MCKENZIE and JONES 1973).

MATERIALS AND METHODS

Yeast strains: Mutations affecting interconversion enzymes were isolated in M16–14C of genotype **a** *leu1 ser1*. The *ser1* mutation causes a deficiency for phosphoserine aminotransferase (EC2.6.1.52) (ULANE and OGUR 1972). New mutant strains were outcrossed to M16–14D of genotype α *ser1 ura1*. *ade3–41*, *ade3–118* and *ade3–130* have been previously described (JONES 1972a,b; LAM and JONES 1973; JONES 1977). They have been crossed to *ser1*-bearing strains to generate the appropriate **a** *leu1 ade3-x ser1* and α *ade3-x ser1 ura1* strains.

Media: YEPD and sporulation media have been previously described (JONES 1972a). Minimal medium (MV) was according to the recipe for yeast nitrogen base without amino acids as described in the DIFCO manual (1953) with 20 g/l glucose.

Synthetic complete: MV with following nutrients (in mg/l): adenine (20), histidine (20), leucine (60), uracil (10), serine (20) and glycine (100).

Omission medium: Synthetic complete medium from which one or more nutrients have been omitted.

Supplemented MV: MV to which one or more nutrients have been added. When added the concentrations (in mg/1) are leucine (60), serine (50), uracil (10), adenine (20), histidine (20), methionine (40), glycine (20), formate (500 for solid media, 1000 for liquid media).

Low glycine-formate: MV with 20 mg/l glycine, 500 mg/l Na formate (solid medium) or 1000 mg/l (liquid medium). Media were solidified when necessary by addition of 15 g/l DIFCO Bacto-agar.

Genetic methods: Diploid strains were isolated by prototrophic selection (POMPER and BURKHOLDER 1949). Ascus dissection was according to JOHNSTON and MORTIMER (1959). Cells from the ascosporal clones were streaked on YEPD. After two days, the clones were replica plated (LEDERBERG and LEDERBERG 1952) to appropriate media to determine their phenotypes.

For complementation tests, haploid strains were mated and the diploids were isolated by prototrophic selection. The diploid strains were then tested by replica plating to low glycine-formate medium. If the diploid grows on this medium within 48 hours the two mutations complement; if no growth occurs the two mutations do not complement.

M16–14C (**a** *ser1 leu1*) was mutagenized by a 60-minute exposure to ethyl methanesulfonate (EMS), using the procedure of LINDEGREN *et al.* (1965). Following termination of the exposure, appropriate dilutions were plated on YEPD. After 48 hours' incubation at 30°, these plates were replica plated to MV + leu + ser and to MV + leu, low gly, formate. These plates were read at 24 and 48 hours. Colonies which failed to grow on the latter plate but grew on the former plate were isolated for further testing.

Growth experiments: Cultures were grown at 30° in 300 ml nephelometric flasks in a shaking water bath. Inocula for growth experiments were grown in MV + serine (50 mg/l) to a cell density of 4–8 $\times$ 10^6 cells/ml (50–90 Klett units). The cells were concentrated and resuspended in a lesser volume of MV. 0.5 ml of this cell suspension was inoculated into 10 ml of one of a variety of media. Growth was monitored nephelometrically in a Klett-Summerson photoelectric colorimeter equipped with a 520 nm filter.

Preparation of cell free extracts: Cell were grown and harvested and extracts prepared as described in LAM and JONES (1973). Protein concentration was estimated according to LOWRY *et al.* (1951).

Preparation of permeabilized cells: Cells which had been grown in YEPD were harvested and washed once with distilled water. The cells were resuspended in 2 ml of 0.01 M phosphate buffer, pH 7.5. After addition of two drops of toluene (about 0.06 ml) the cell suspension was gently rotated for 20 minutes at room temperature. The suspensions were then centrifuged, the pellet was rinsed with cold buffer, was resuspended in cold buffer, and then placed on ice.

Enzyme assays: Crude extracts were assayed for formylTHFA synthetase, methenylTHFA cyclohydrolase and methyleneTHFA dehydrogenase by the methods given in LAM and JONES

(1973) except that the synthetase reaction and dehydrogenase reaction were performed in tubes from which 0.1 ml aliquots were removed into 0.9 ml of 7% perchloric acid (PCA) at specified time intervals.

Screening assays for the presence of formylTHFA synthetase and methyleneTHFA dehydrogenase in the formate nonutilizer mutants was according to the descriptions given above except that permeabilized cells replaced the crude extract. MethenylTHFA cyclohydrolase assays could not be performed using permeabilized cells because of the high absorbance of the cells.

Enzyme assays for column fractions: FormylTHFA synthetase: To a tube on ice the following were added: 50 μmoles Tris buffer pH 7.5, 4 μmoles $MgCl_2$, 150 μmoles sodium formate, 50 μmoles 2-mercaptoethanol, 145 μmoles NH_4Cl, 5 μmoles potassium 3-phosphoglycerate, 1.25 μmoles adenosine triphosphate (ATP), 0.36 μmoles d,l-L-THFA in a volume of 0.07 ml. After addition of 0.04 ml of column fraction the tubes were placed at 37°. After 10 minutes' incubation the reaction was terminated by addition of 0.9 ml of 7% PCA. After standing at least 10 minutes on ice the tubes were centrifuged to precipitate denatured protein and the absorbance of the supernatant was read at 355 nm on a Zeiss PMQII spectrophotometer against a blank of distilled water. Data are reported as absorbance at 355 nm of the supernatant solution.

MethyleneTHFA dehydrogenase: To a tube on ice were added 2 μmoles formaldehyde, 0.18 μmoles d,l-L-THFA, 10 μmoles 2-mercaptoethanol, 50 μmoles potassium phosphate buffer pH 7.5, 0.6 μmoles NADP in a volume of 0.2 ml. After addition of 0.3 ml of column fraction the mixture was incubated 10 minutes at 37° after which the reaction was stopped by addition of 1.0 ml of 35% PCA. After standing at least 10 minutes on ice, the tubes were centrifuged and the absorbance of the supernatant at 355 nm was read. Data are reported as absorbance at 355 nm of this supernantant solution.

MethenylTHFA cyclohydrolase: A cuvette containing 30 μmoles 2-mercaptoethanol and 40 μmoles potassium malate buffer pH 7.0 was pre-incubated at 30° at least one minute. 0.05 μmoles 5,10-methenylTHFA was added and the absorbance at 355 nm was followed on a Gilford absorbance recorder attached to a Beckman DU spectrophotometer. Then 0.05 ml of the column fraction was added to the reaction mixture (1 ml final volume) and the absorbance was again monitored. The rate of reaction was calculated as the difference between the initial rates with and without addition of the column fraction. Results are reported as the change in absorbance at 355 nm per 2 minutes.

TEAE-cellulose chromatography: TEAE-cellulose was washed and prepared according to Peterson and Sober (1962). It was equilibrated in 0.01 M potassium phosphate buffer pH 7.35. Six ml of extract (22–35 mg. protein/ml) of the strain to be submitted to chromatography was dialyzed against 400 volumes of 0.01 M potassium phosphate pH 7.35. Six ml of the extract were incubated with 4.2 mg phenylmethylsulfonyl fluoride (PMSF) dissolved in 0.6 ml ethanol for 20 minutes on ice. Four ml of the PMSF treated extract was applied to a 1.5 × 30 cm TEAE cellulose column equilibrated with 0.01 M phosphate pH 7.35. Elution was achieved with a linear gradient produced by 300 ml of potassium phosphate buffer pH 7.35, 0.01 M in phosphate and 0.01 M KCl in the mixing chamber and 300 ml buffer 0.01 M in phosphate and 0.3 M in KCl in the reservoir. Three ml fractions were collected with a flow rate of approximately 100 ml/hr.

Chemicals: *Adenosine triphosphate* (ATP), d,l-L-tetrahydrofolic acid (THFA), barium 3-phosphoglycerate, nicotinamide-adenine dinucleotide phosphate (NADP) and TEAE-cellulose were obtained from Sigma Chemical Company. Calcium 5-formyltetrahydrofolic acid was obtained from American Cyanamid Company. 5,10-methenylTHFA was prepared from calcium 5-formylTHFA according to Rabinowitz and Pricer (1956).

RESULTS

Isolation of mutants: Following exposure to EMS, approximately 24,000 colonies were screened for loss of ability to use formate and low levels of glycine to fill their serine requirement. 108 colonies had this phenotype. Strains showing this phenotype were assayed for levels of formylTHFA synthetase and meth-

yleneTHFA dehydrogenase with procedures employing permeabilized cells. Fifteen strains (14% of the strains with the indicator phenotype) were deficient in formylTHFA synthetase or methylene-THFA dehydrogenase activity, or both. Since a permeabilized cell assay was not available for screening for methenyl-THFA cyclohydrolase activity, mutants deficient only for cyclohydrolase activity could not be detected.

Of the 15 strains deficient for synthetase or dehydrogenase, two proved to grow sufficiently well on low gly-formate medium to preclude genetic mapping and were discarded. Two of the thirteen remaining strains proved to be double mutants. One carried an independently segregating methionine requirement; the second carried an independently segregating suppressor, not further characterized, which permitted the mutant to grow slowly on the restrictive medium. Through outcrossing the mutations preventing formate utilization, *1014* and *1006*, respectively, were isolated free of the second mutations. In all further studies the additional mutations were absent.

All strains were outcrossed to M16-14D, of genotype α *ser1 ura1*, to recover the mutations in the opposite mating type and with the *ura1* allele. Diploid strains homozygous for *ser1* and for the *ade3* allele in question were constructed. These diploid strains were used for analyses of enzyme levels and for growth studies. Diploids were used since some of the original mutant isolates were vegetative petites. Diploids heterozygous for the mutations were also constructed. All thirteen mutations proved to be recessive.

Enzyme studies: Specific activities in cell free extracts for the dehydrogenase, cyclohydrolase, and synthetase for the diploid homozygous for the wild-type allele at the *ade3* locus (Table 1) are somewhat lower than levels previously re-

TABLE 1

Specific activities of tetrahydrofolate interconversion enzymes in S. cerevisiae *strains (nmoles/min/mg protein)*

Genotype at *ade3* locus	Dehydrogenase	Cyclohydrolase	Synthetase
+/+	6.4	8.9	45.2
1006/1006	5.2	6.4	4.8
1009/1009	4.4	7.2	6.4
1052/1052	6.4	6.0	8.0
1004/1004	4.4	7.2	5.2
1010/1010	6.0	6.4	4.4
1012/1012	4.0	4.8	4.8
1050/1050	6.0	8.4	4.0
1020/1020	0.8	3.2	38.0
1151/1151	1.2	5.6	46.0
1003/1003	1.1	0.2	38.2
1005/1005	2.0	0.2	38.8
1014/1014	2.0	0	38.0
1001/1001	1.6	0	5.2

ported (LAM and JONES 1973). The values previously reported are 7.1, 12.1 and 53.4 nmoles/min/mg respectively.

The mutants have been entered in Table 1 in four groups, according to the enzyme deficiencies apparent in cell free extracts. The first group comprises mutants whose primary deficiency seems to be in synthetase activity. Mutants in the second group lack dehydrogenase activity, in the third group both dehydrogenase and cyclohydrolase activity. The mutant in the fourth group lacks all three activities. Justification for categorizing these mutants as alleles at the *ade3* locus will be given below in the section on complementation.

The seven mutants in the first group have been classed as deficient in synthetase activity. Specific activities for synthetase range from 4.0 to 8.0 nmoles/min/mg. These values are quite comparable to values previously reported for mutations in the *ade3* locus which result in adenine auxotrophy, where values ranged from 2.81 to 7.4 (LAM and JONES 1973; JONES 1977). Specific activities for cyclohydrolase among these synthetase mutants in all cases are somewhat lower than levels found in the parent strain (a range of 4.8 to 8.4 among mutants as compared to 8.9 in the parent strain). These cyclohydrolase levels, however, are much higher than those found in strains truly deficient for this enzyme. Values for mutants deficient in cyclohydrolase range from 0.21 to 0.79 (LAM and JONES 1973; JONES 1977). Inspection of the entries for the third group of mutants in Table 1 again reveals levels of 0 to 0.2 rather than the relatively high levels found in the synthetase mutants.

The levels of dehydrogenase in the synthetase mutants range from 4.0 to 6.4 nmoles/min/mg, clearly lower than the parent diploid, but substantially higher than levels for mutants deficient in dehydrogenase. Levels ranging from 0.27–1.91 have been reported for *ade3* auxotrophic mutants (LAM and JONES 1973; JONES 1977). Mutants classified as dehydrogenase negative in Table 1 have values ranging from 0.8–2.0, comparable to other dehydrogenase negatives. Further justification for classing these synthetase negatives as dehydrogenase positives emerges from complementation analyses and will be presented below.

The synthetase mutants presented in Table 1 have been grouped according to map position. Alleles *1006*, *1009*, and *1052* map in one group; alleles *1004*, *1010*, *1012*, and *1050* map in a second group (manuscript in preparation). No correlation between map position and enzyme levels is discernible.

The second group of mutants have been classified as deficient primarily in dehydrogenase activity. The synthetase levels are high and comparable to the wild-type diploid. Mutant *1151* has substantial levels of cyclohydrolase. The levels of dehydrogenase are well within the range typical of mutants (0.27–1.91). Mutant *1020* has been classed as cyclohydrolase positive. The specific activity of 3.2 observed is lower than typical cyclohydrolase positives (4.8–6.4) but is substantially higher than typical mutants (0–0.79).

Mutants deficient in cyclohydrolase and dehydrogenase are reported in the third group in Table 1. They have levels of synthetase at least 85% of the wild type. They have the low levels of cyclohydrolase typical of mutants, and have levels of dehydrogenase within the mutant range. That mutant *1005* indeed lacks

the dehydrogenase isoenzyme normally absent in *ade3* mutants (LAZOWSKA and LUZZATTI 1970b) will be shown below.

The final mutant lacks activity for all three enzymes. This is the pattern previously reported for *ade3* auxotrophs (JONES and MAGASANIK 1967a; LAZOWSKA and LUZZATTI 1970a; LOMAX, GROSS and WOODS 1971; LAM and JONES 1973; JONES 1977). As will be reported below, the diploid homozygous for *1001* is able to grow in the absence of adenine and histidine and hence probably possesses the interconversion enzymes at some level *in vivo*. Possibly the enzymes are unstable *in vitro*. As will be shown, however, the growth rate of this mutant strain is considerably enhanced if adenine and histinine are added.

The results presented in Table 1 indicate that one can obtain mutants which are deficient in only one of the three interconversion enzymes. However, inspection of the data in Table 1 reveals additional consequences of these mutations. It is worth noting that the average level of synthetase in mutants that possess substantial synthetase activity is somewhat lower than the levels found in wild-type strains. Only one mutant (*1151*) has levels of synthetase higher than the wild type.

Similarly, of all the synthetase mutants, only one (*1052*) has levels of dehydrogenase comparable to the wild type. In all other mutants the levels are somewhat lower. The same finding holds true when cyclohydrolase levels are considered.

These observations can be restated in the following way. Any mutation which substantially reduces the level of one of three enzymes almost invariably reduces the levels of the other interconversion enzyme(s).

Previous studies (LAZOWSKA and LUZZATTI 1970b) revealed that methylene-THFA dehydrogenase was present in two forms in wild-type *Saccharomyces cerevisiae* and that one of these forms was absent in *ade3* strains. Cell free extracts of our parent strain and its mutant derivatives were subjected to TEAE-cellulose chromatography to determine whether the *ade3* associated dehydrogenase activity was altered in the mutants and whether the three enzyme activities cochromatographed.

The chromatographic profile for the wild-type diploid is presented in Figure 2. The first sharp peak of dehydrogenase, synthetase and cyclohydrolase activity eluting prior to initiation of the salt gradient is due to overloading the column and can be avoided. This peak will not be considered further. MethyleneTHFA dehydrogenase elutes in two peaks; peak I around 0.11–0.12 M KCl and peak II around 0.19 M KCl. A large peak of cyclohydrolase activity and a peak of synthetase activity elute around 0.12 M KCl in the region of the profile where peak I of dehydrogenase elutes. Ratios of the three activities were not calculated since the blanks were fairly high and variable.

There is a suggestion that there might be a second peak of synthetase activity in the region of the profile corresponding to fractions 110–130, for the absorbance values for these fractions are of the order of 0.2 to 0.25, as compared to values of 0.15 at the beginning of the profile and in the vicinity of tubes 20 to 40. This suggestion of a second peak of synthetase activity is confirmed if one chro-

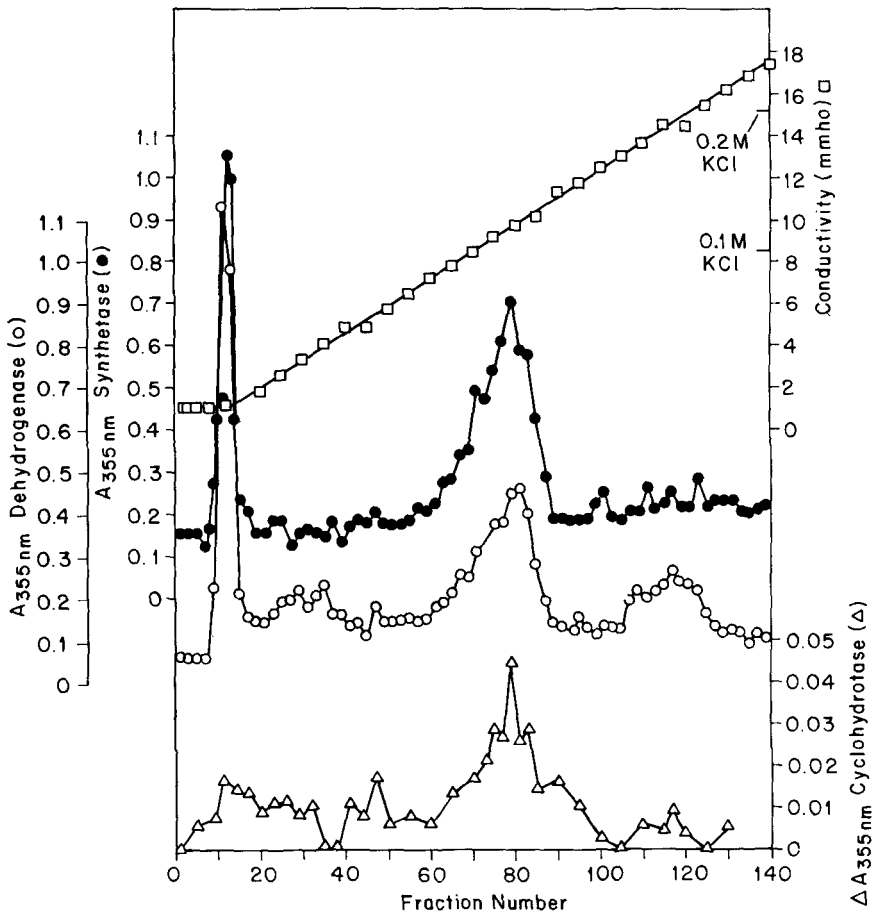

FIGURE 2.—TEAE-cellulose chromatography. Cell free extract of the wild-type diploid was applied (see MATERIALS AND METHODS for details). Activities are: synthetase (●) A_{355nm}/0.04 ml/10 min; dehydrogenase (○) A_{355nm}/0.3 ml/10 min, and cyclohydrolase (△) ΔA_{355nm}/0.05 ml/2 min.

matographs fractions from ammonium sulfate fractional precipitation. Fractions enriched in this fashion for peak II of dehydrogenase show a substantial peak of synthetase activity eluting around 0.20 M KCl (unpublished observations). The question of the second synthetase peak is raised, for although it does not relate directly to analysis of *ade3* mutations, it may provide an explanation of the finding that all *ade3* mutants have levels of synthetase approximately 10% of the wild-type levels even if the mutations present in the strains are deletions or nonsense mutations in the *ade3* locus.

In summary, there are two peaks of dehydrogenase in the wild type as has been reported (LAZOWSKA and LUZZATTI 1970b). It is apparent that the major synthetase peak and the cyclohydrolase peak elute from the column at a salt concentration comparable to that at which peak I of dehydrogenase elutes.

If a cell free extract of a diploid homozygous for *ade3-130*, a deletion for a large portion of the *ade3* locus (JONES 1977), is subjected to chromatography, peak I of dehydrogenase and peak I of synthetase are absent from the profile (data not presented). Cyclohydrolase was not assayed.

The chromatographic profile for the diploid homozygous for *1005*, a mutation which results in a deficiency for dehydrogenase and cyclohydrolase, is presented in Figure 3. As can be seen in Figure 3, there is little evidence for cyclohydrolase activity throughout the profile, certainly nothing comparable to the peak of activity eluting around 0.12 M KCl in the wild type.

There is a very large peak of synthetase activity eluting in the vicinity of 0.12 M KCl. It is unfortunate that an irregularity in production of the salt gradient occurred in the vicinity of this peak. There is again a suggestion of a second peak of synthetase activity in the region of fractions 105–120.

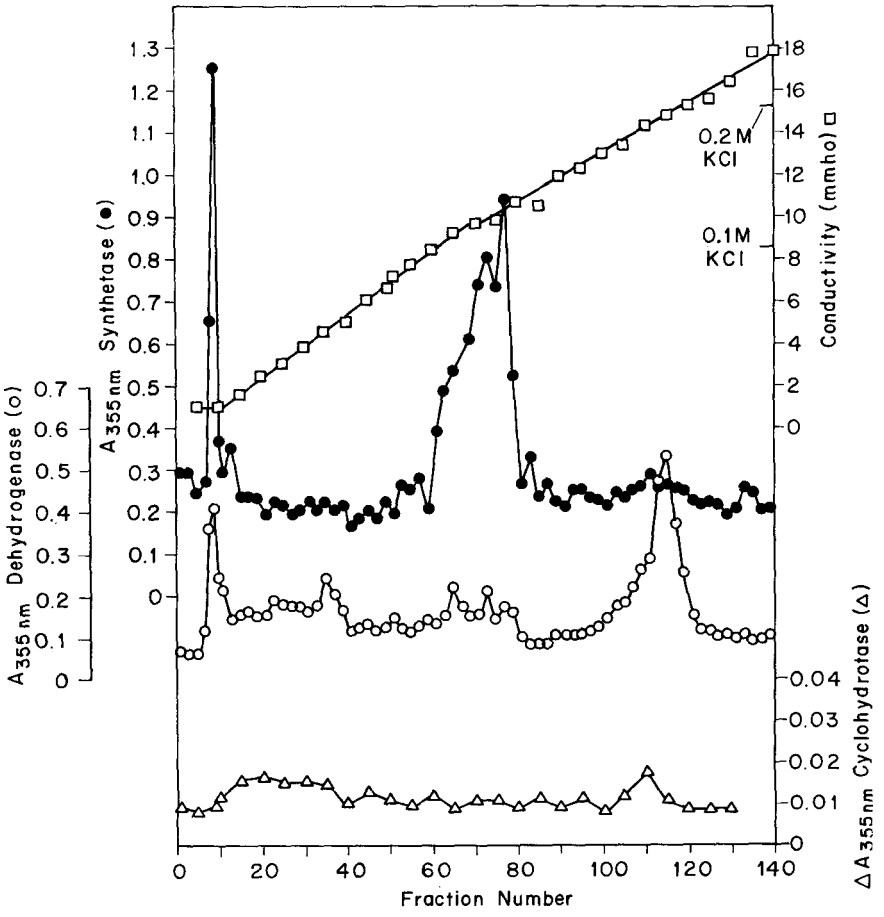

FIGURE 3.—TEAE-cellulose chromatography. Cell free extract of the diploid homozygous for *1005*, a mutation causing a deficiency for dehydrogenase and cyclohydrolase activities. Activities as in legend to Figure 2.

A large peak II of dehydrogenase activity is present and elutes around 0.19 M KCl as usual. There is some evidence for dehydrogenase activity in the region of the profile where peak I would normally elute (fractions 60–80), but the peak is greatly diminished. Residual peak I activity or high peak II activity could account for the observation that dehydrogenase levels in this mutant are on the high side of the range for dehydrogenase mutants (see Table 1).

If an extract of mutant *1151*, a dehydrogenase negative mutant, is chromatographed, peak I of dehydrogenase is absent, peak I of synthetase and cyclohydrolase elute around 0.12 M KCl (data not shown).

When the profile for mutant *1050*, a synthetase deficient mutant, is examined (Figure 4) one finds, as expected, two peaks of dehydrogenase activity, a large peak of cyclohydrolase activity eluting in the region of the profile corresponding to peak I of dehydrogenase, and an absence of the large peak of synthetase. Peak II of dehydrogenase elutes around 0.19 M KCl as is typical for this activity.

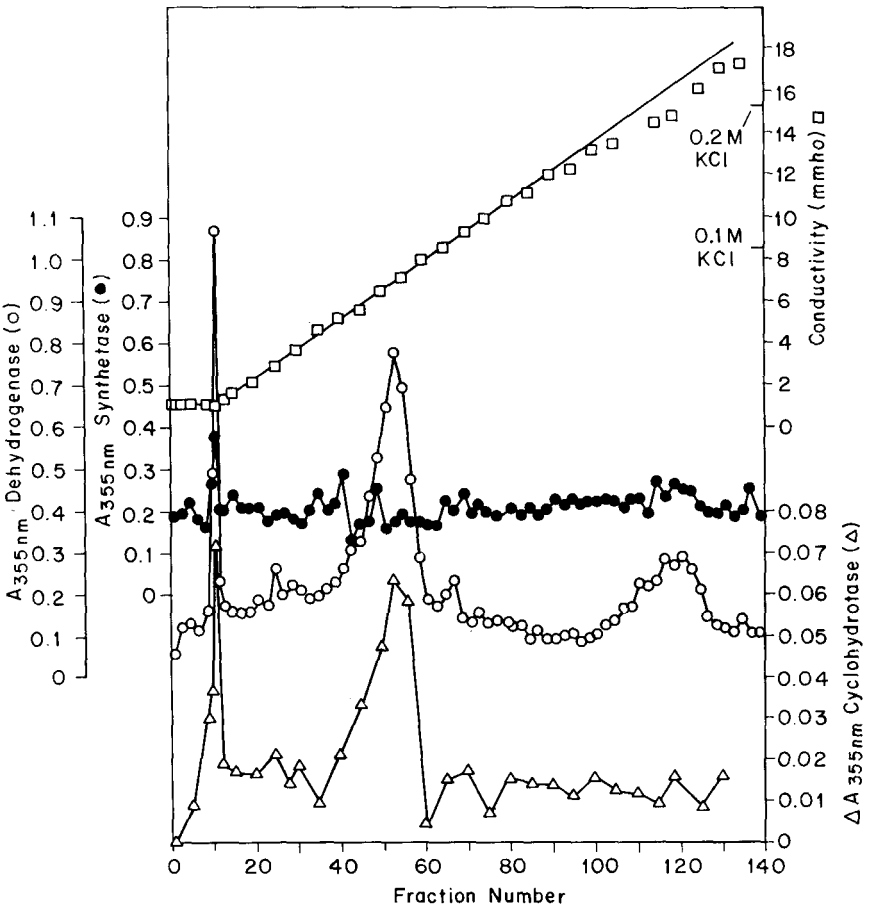

FIGURE 4.—TEAE-cellulose chromatography. Cell free extract of the diploid homozygous for *1050*, a mutation causing a deficiency for synthetase. Activities as in legend to Figure 2.

There is one feature of this profile which differentiates it from other profiles. As mentioned above, peak I of dehydrogenase activity and cyclohydrolase elute together. However, instead of eluting around 0.12 M KCl, they eluted between 0.07 and 0.08 M KCl. Presumably this change in the elution properties of these two activities is an additional consequence of the mutation which eliminated peak I of synthetase activity. Peak II of dehydrogenase provides an internal control for this column profile. It eluted at its normal position around 0.19 M KCl. 60 fractions separate the two peaks of dehydrogenase activity in *1050*, whereas about 40 fractions separate the two peaks in the wild type.

In summary, it is clear that these new mutants, isolated because of their inability to mobilize formate, lack the peaks of the interconversion enzymes associated with the *ade3* locus. When dehydrogenase activity is reduced, it is peak I of dehydrogenase which is absent or present in diminished quantities. When synthetase is reduced, it is peak I of synthetase which is absent. In addition, the three activities elute at comparable salt concentrations in the profiles. And finally, when the dehydrogenase elutes at an atypical position in the profile, cyclohydrolase elutes with it in the atypical position.

Genetic complementation: The *ade3* mutants which require adenine and histidine and which lack the three interconversion enzymes would be expected to be unable to mobilize formate. The doubly mutant strain *ade3–41 ser1* and *ade3–*

TABLE 2

Ability of diploids heterozygous for two ade3 *alleles to use formate as a source of single carbons**

	3–41	*3–118*	*1001*	*1004*	*1006*	*1009*	*1010*	*1012*	*1050*	*1052*	*1020*	*1151*	*1003*	*1005*	*1014*
3–41	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
3–118		0	0	0	0	0	0	0	0	0	0	0	0	0	0
1001			0	0	0	0	0	0	0	0	0	0	0	0	0
1004				0	0	0	0	0	0	0	+	+	+	+	+
1006					0	0	0	0	0	0	+	+	+	+	+
1009						0	0	0	0	0	+	+	+	+	+
1010							0	0	0	0	+	+	+	+	+
1012								0	0	0	+	+	+	+	+
1050									0	0	+	+	+	+	+
1052										0	+	+	+	+	+
1020											0	0	0	0	0
1151												0	0	0	0
1003													0	0	0
1005														0	0
1014															0

* The prefix 3 indicates that the strain has an absolute requirement for adenine and histidine and lacks the three interconversion enzyme activities *in vitro*; absence of the prefix 3 indicates that the strain does not require adenine and histidine or that the requirement is not absolute. *1001* grows slowly in the absence of adenine and histidine and better in their presence and lacks all three enzyme activities *in vitro*. Mutants *1004* through *1052* (reading across the top of the Table) lack synthetase activity *in vitro*, mutants *1020* and *1151* lack dehydrogenase activity, and mutants *1003*, *1005* and *1014* lack dehydrogenase and cyclohydrolase activities. All diploids are homozygous for *ser1*. + signifies that the diploid will grow within 48 hrs on a minimal medium containing, as a source of serine, 20 mg/l glycine and 500 mg/l Na formate; 0 signifies no growth on this medium in 48 hrs.

118 ser1 were constructed. As expected they require adenine, histidine and serine. Formate and low levels of glycine will not satisfy the serine requirement.

The mutants deficient in formate utilization, including *ade3-41* and *ade3-118*, have been crossed in all pairwise combinations. All diploids so constructed are homozygous for *ser1*. The ability of the diploids to utilize formate and glycine to satisfy the serine requirement was determined and is reported in Table 2.

The three mutations which eliminate all three enzyme activities *in vitro*, namely *3–41*, *3–118*, *and 1001*, fail to complement any of the other mutations. Mutations which eliminate only synthetase complement all mutations which do not eliminate synthetase. For example, *1004*(S^-) complements *1020*(D^-) and *1003*(D^-C^-). All mutations which reduce synthetase activity fail to complement all other mutations which eliminate synthetase activity. This is true whether the second mutation eliminates only synthetase (*1004* × *1006*, for example) or reduces all three enzymes (*1004* × *3–41*, for example). All mutations classified as causing dehydrogenase deficiency fail to complement any other mutation classified as causing dehydrogenase deficiency, be the second mutation D^- (*1020* × *1151*) or D^-C^-(*1020* × *1003*) or $S^-D^-C^-$(*1020* × *3–41*).

These data justify the classification of the new mutations as alleles at the *ade3* locus despite the fact that the new mutants do not require adenine and/or histidine. (Our screening procedure precluded isolation of mutants which required

TABLE 3

Growth rates of various diploid strains of S. cerevisiae*

Genotype at *ade3* locus†	Additions to minimal medium: Glycine	Glycine formate	Serine	Minimum doubling time‡	Growth condition (Additions to MV + serine)
+/+	>24.0	3.20	1.80		
1006/1006	>24.0	8.70	1.90	1.80	
1009/1009	>24.0	8.10	1.95	1.80	
1052/1052	>24.0	>24.0	2.90	2.30	ade
1004/1004	>24.0	>24.0	2.60	1.90	his
1010/1010	>24.0	>24.0	2.50	2.00	ade + his
1012/1012	>24.0	>24.0	3.00	2.60	ade
1050/1050	>24.0	>24.0	5.20	2.80	ade + his or met
1020/1020	>24.0	>24.0	2.10	2.0	
1151/1151	>24.0	>24.0	1.90	1.90	
1003/1003	>24.0	17.0	3.20	2.00	ade
1005/1005	>24.0	>24.0	2.30	1.50	ade
1014/1014	>24.0	>24.0	2.90	1.70	ade
1001/1001	>24.0	>24.0	9.60	3.60	ade + his

* Growth rates are given as mass doubling time in hours. Concentrations of added nutrients in mg/l are glycine(20), Naformate(1000), serine(50), adenine(20), histidine(20) and methionine (40).

† All diploids are homozygous for *ser1*.

‡ Minimum doubling time and growth condition are that medium which gives the maximum growth rate and the growth rate found for that medium. Where the growth condition is not indicated, the maximum growth rate is achieved with addition of serine only to the medium.

adenine and/or histidine.) In addition, the data establish two complementation groups within the *ade3* locus.

Growth studies: In Table 3 are reported results which establish that the mutants described in this paper are indeed unable to mobilize formate as a source of single carbons. The diploid homozygous for *ser1* and for the wild-type allele at *ade3* grows at a normal rate (1.90 hr doubling time) if supplied with serine. If glycine and formate are supplied as a source of serine, the wild-type diploid grows more slowly than when serine is supplied. If formate is omitted, leaving glycine as the sole source of glycine, serine, and single carbons, the wild-type strain does not grow.

Similar data for the various mutants are presented in the bottom portion of the table. It is apparent that none of the mutants can grow if only glycine is supplied and that all can grow if serine is supplied. There is, however, evidence of differences in the growth rates of the diploids when serine only is supplied. Some diploids grow well (*1006*, *1009*, *1020*, *1151*) while others grow poorly (*1050* and *1001*). With the exception of diploids *1006* and *1009*, none of the diploids show measurable growth when glycine and formate are presented as the serine source. *1006* and *1009* grow about 20% as rapidly when glycine and formate are supplied as when serine is supplied. Inspection of the data reveals, however, that *1006* and *1009* grow more rapidly on glycine-formate medium than *1001* does on serine-containing medium.

As the mutations which eliminate formate utilization are allelic to *ade3* mutations which cause adenine and histidine requirements, we were interested in determining whether addition of adenine and/or hisidine would increase the growth rates of the diploids homozygous for these mutations. We also obtained data on the effects of methionine on these mutants, as LOMAX, GROSS and WOODS (1971) have reported inhibitory effects of methionine on *ade3* mutant strains that were isolated as adenine auxotrophs.

In the final two columns of Table 3 are reported the maximum growth rates achieved by our strains and the supplementations required to achieve these growth rates. Complete data are presented in Table 4.

Consideration of Table 3 reveals that supplementation with adenine or histidine or methionine or a combination of these can significantly enhance the growth rates of some strains. Synthetase negative mutants can attain growth rates approaching, but in some cases not equaling, those achieved by the wild-type strain. However, the metabolite(s) which allow achievement of these maximum rates are diverse. Two of the synthetase negatives grow best when adenine is supplied, one when histidine is supplied and two others when adenine and histidine are supplied. For *1050*, methionine can substitute for adenine and histidine.

The dehydrogenaseless, cyclohydrolaseless strains grow best when adenine is supplied and achieve normal growth rates. *1001* never achieves a very good rate of growth but its growth rate is enhanced if adenine and histidine are supplied. The results for *1001* lead one to suspect that if it has significant levels of the three interconversion enzymes *in vivo* (as its ability to grow at all in the absence of adenine and histidine would indicate) then these levels are probably quite low

TABLE 4

Growth rates of various diploid strains of S. cerevisiae*

Genotype at *ade3* locus	Additions to MV + serine medium					
	None	Histidine	Adenine	Histidine Adenine	Methionine	Histidine Adenine Methionine
+/+	1.80	1.80	1.80	1.70	1.70	1.50
1006/1006	1.90	2.15	2.15	2.15	2.0	1.80
1009/1009	1.95	1.80	1.80	1.90	1.80	1.80
1052/1052	2.90	4.10	2.30	3.00	3.10	2.20
1004/1004	2.60	1.90	2.20	1.90	1.90	2.20
1010/1010	2.50	3.00	2.50	2.20	2.40	2.00
1012/1012	3.00	2.90	2.60	2.80	2.80	2.80
1050/1050	5.20	7.50	5.70	2.80	2.80	2.80
1020/1020	2.10	2.00	2.00	2.10	2.10	2.10
1151/1151	1.90	3.90	2.50	3.35	1.90	2.20
1003/1003	3.20	2.40	2.00	2.10	3.00	2.00
1005/1005	2.30	2.00	1.50	1.60	2.00	1.50
1014/1014	2.90	5.10	1.70	2.40	3.60	1.50
1001/1001	9.60	14.00	4.00	3.60	>24.0	4.60

* Growth rates are given as mass doubling time in hours. Concentrations of nutrients added to minimal medium are (in mg/l) serine(50), adenine(20), histidine(20), and methionine(40).

indeed. Its failure to complement any other mutation also accords with these observations.

When one considers the effects of nutrients added singly or in combination, (Table 4) no pattern emerges. Methionine is inhibitory only to *1001* and then only if other supplements are omitted.

DISCUSSION

A review of the pathway of interconversion of tetrahydrofolate coenzymes at the formate level of oxidation suggests that any strain which possesses activity for any two of the three interconversion enzymes should not require adenine so long as endogenous supplies of formate and serine are adequate. This expectation is realized for dehydrogenase negative mutants, for they growth quite well without purine supplementation. Some of the synthetase negative mutants also grow well without supplementation, as expected. However, other mutant strains grow more rapidly if adenine is supplied. There is no apparent correlation in these synthetase negatives between the levels of dehydrogenase and cyclohydrolase in these mutants and the ability of the strains to grow without supplementation. A conspicuous example is mutant *1050* which has levels of dehydrogenase and cyclohydrolase that appear nearly normal, yet supplementation of the medium with adenine and histidine nearly doubles the growth rate. It is worth recalling that the cyclohydrolase and dehydrogenase activities in this mutant show altered chromatographic properties. Whether these activities respond in an aberrant

fashion to the intracellular environment is, of course, unknown. The peculiar growth characteristics of some of the synthetase negatives might well reflect these more subtle differences in enzyme properties, not readily discerned when levels of the enzymes in crude extracts are determined.

A quite unexpected finding is that mutants which lack dehydrogenase and cyclohydrolase grow quite well without supplementation. Our possible explanation of this finding is provided by the chromatographic profile of mutant *1005*. The major dehydrogenase peak is greatly reduced and the cyclohydrolase peak is absent. However, residual activity for peak I dehydrogenase is evident in the profile, eluting at its normal position. Possibly, sufficient activity is retained to supply the single carbons needed for synthesis of adenylic acid.

Several lines of evidence suggest that the three interconversion activities may reside in an enzyme complex. The first, and weakest, emerges from the chromatographic profile. Dehydrogenase I, the major peak of synthetase, and the peak of cyclohydrolase activity elute at comparable salt concentrations from TEAE-cellulose. As the enzymes were purified further, the three activities remained together (JONES, unpublished observations). This line of evidence is strengthened, however, when the profile for mutant *1050*, a synthetase negative, is considered. The cyclohydrolase activity and dehydrogenase activity of peak I elute at a salt concentration considerably lower than that found for these activities in the wild type or in other mutants. Moreover, these two activities elute at the same new salt concentration. It would appear that the mutation which eliminates synthetase activity has affected the chromatographic behavior of the other two enzymatic activities.

A second line of evidence is the properties of these mutants, deficient for one or two of the interconversion activities. It is a feature of the enzymatic analyses that any mutation which eliminates one of the three enzyme activities almost invariably results in reduced activities for the remaining interconversion enzyme(s). This is most easily interpretable if the three activities are in an enzyme complex.

Additional support for this hypothesis emerges from fine-structure mapping of these mutations (JONES and MCKENZIE, in preparation). Mutations causing a synthetase deficiency map at the left end of the *ade3* locus, interspersed among nonsense mutations which eliminate all three activities. The dehydrogenase negatives and those lacking dehydrogenase and cyclohydrolase map at the right end of the locus, amongst nonsense mutations which again eliminate all three activities. The locus appears to be divided into two regions, one concerned with synthetase activity and a second concerned with dehydrogenase and cyclohydrolase activity. Nonsense mutations anywhere in the region appear to eliminate all three activities. These observations are most easily explicable if an enzyme complex exists and are not explicable on grounds of polarity, for polar mutations at one end of the locus or the other (which end will depend upon the direction of translation) should permit synthesis of the enzyme activity encoded proximal to that mutation.

The pattern of complementation is compatible with the existence of a complex.

Given that a complex exists, however, complementation will not provide an answer to whether such a complex is comprised of one or several polypeptides.

If the three activities are carried in a single polypeptide chain, complementation might occur by formation of hybrid proteins (SCHLESINGER and LEVINTHAL 1963) or by internal cross feeding of intermediates between two different aggregates (CASE and GILES 1971; FINK 1971). If the three activities are encoded in separate polypeptide chains as part of an operon, then complementation could occur by formation of wild-type enzyme by random aggregation of wild-type subunits (hemoglobin is the obvious example) or again by internal cross feeding.

In bacteria, formyltetrahydrofolate synthetase and methylenetetrahydrofolate dehydrogenase exist as separate enzymes. (RABINOWITZ and PRICER 1962; UYEDA and RABINOWITZ 1967; LUNGDAHL *et al.* 1970; O'BRIEN, BREWER and LUNGDAHL 1973; MOORE, O'BRIEN and LUNGDAHL 1974.)

Methylenetetrahydrofolate dehydrogenase has been partially purified from yeast (RAMASASTRI and BLAKLEY 1962). Cyclohydrolase and synthetase activities were not assayed during this purification. The enzymes from other eukaryotes have been partially purified or have been purified to homogeneity. ROWE and LEWIS (1973) reported that formyltetrahydrofolate synthetase from beef can be separated from the dehydrogenase and cyclohydrolase. However, all three enzymes are reported to be extremely labile, so this finding is difficult to assess. Partially purified synthetase preparations from human erythrocytes (BERTINO, SIMMONS and DONOHUE 1962) and from spinach leaves (IWAI, SUZUKI and MIZOGUCHI 1967) are said to be free of cyclohydrolase activity, but no experimental details are supplied. Partially purified dehydrogenase from chicken liver is reported to be "substantially free" of the cyclohydrolase (OSBORN and HUENNEKENS 1957). MCKENZIE (1973) reported that dehydrogenase, synthetase, and cyclohydrolase from pig liver could not be separated from one another. Moreover, NADP, a cofactor for the dehydrogenase, protected all three enzyme activities against heat denaturation. PAUKERT, STRAUSS and RABINOWITZ (1976) have purified to homogeneity a protein which possesses dehydrogenase, cyclohydrolase and synthetase activities. The enzyme apparently contains two identical subunits. Hence in two eukaryotic organisms, sheep and pig, the evidence is that the three activities comprise an enzyme complex.

E. W. J. expresses gratitude to BORIS MAGASANIK, in whose laboratory the work on interconversion enzymes began, for discussions, tutelage and unflagging enthusiasm.

This work was supported by Public Health Service Research Grants AM14254 and AM18090, Research Career Development Awards AM36710 and AM00056 (to E. W. J.) and a National Science Foundation predoctoral fellowship (to K. Q. M.).

NOTE ADDED IN PROOF

A protein isolated from yeast, containing two identical polypeptides, combines the functions of methyleneTHFA dehydrogenase, methenylTHFA cyclohydrolase and formylTHFA synthetase (PAUKERT, WILLIAMS and RABINOWITZ 1976; RABINOWITZ 1976).

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Corresponding editor: F. Sherman