Isolation and Properties of Yeast Mutants with Highly Efficient Thymidylate Utilization

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Thymidylate Low Requirement, Thymidylate Sensitivity, Thymidylate Uptake

A screening procedure is presented which allows the isolation of yeast mutants (*typ tlr*) with highly efficient utilization of exogenous deoxythymidine-5′-monophosphate (5′-dTMP) (>50%). Data are given concerning the phenomenon of 5′-dTMP utilization in general: (i) The ability of *S. cerevisiae* to incorporate exogenous 5′-dTMP was found to already be a wild type feature of this yeast, *i. e.* apparently not to be due to any mutation such as *typ*, *tup*, *tmp per* or *tum*. Consequently these mutations are interpreted as amplifiers of a pre-given wild type potency. So far eight stages of 5′-dTMP utilization were detected as classified by the optimal 5′-dTMP requirement, with 5′-dTMP biosynthesis blocked, of the corresponding mutant strains isolated. All of them fit well into a mathematical series of the type "$2^n \times 1.5$" ($n = 0, 1, 2, \ldots, 11$), where the product term for $n=11$ represents the 5′-dTMP requirement (μg/ml) of the best 5′-dTMP utilizing wild type strain found. (ii) Amplification of the 5′-dTMP utilizing potency obviously is due to any genetically determined alteration of the yeast 5′-dTMP uptaking principle itself or of physiological processes accompanying the monophosphate's uptake. (iii) The functioning of 5′-dTMP uptake requires acidic ($\leq$ pH 6) conditions in the yeast cell's outer environment. (iv) Some yeast *typ* and *typ tlr* mutants were found to exhibit a more or less pronounced sensitivity towards exogenously offered 5′-dTMP. The response of a sensitive strain towards inhibitory concentrations of the nucleotide apparently is co-conditioned by the presence or absence of thymidylate biosynthesis. With 5′-dTMP biosynthesis blocked the 5′-dTMP mediated inhibition is a permanent one and finally leads to the death of a cell. With a functioning thymidylate biosynthesis, in contrast, the inhibition is only temporary. (v) Yeast *typ* or *typ tlr* strains were observed to dephosphorylate exogenous 5′-dTMP to thymidine due to a phosphatase activity which cannot be eliminated at pH 7 + 70 mM inorganic phosphate conditions in the growth medium. This 5′-dTMP cleavage obviously occurs outside the cell and does not seem to be correlated both to the monophosphate's uptake and to the phenomenon of 5′-dTMP sensitivity. The destruction of 5′-dTMP does not disturb (5′-dTMP) DNA-specific labelling.

Introduction

In two recent papers [1, 2] we reported the assay of thymine containing dimers UV-induced in the yeast *S. cerevisiae* after highly efficient (5′-dTMP) DNA-specific labelling. Here we wish to present some detailed data concerning the isolation and properties of yeast strains which exhibit such highly efficient utilization of exogenous 5′-dTMP.

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Abbreviations: 5′-dTMP, deoxythymidine-5′-monophosphate; Ade, adenine; Thy, thymine; Thd, thymidine; APT, aminopterin; SAA, sulfanilamide; P_i, inorganic phosphate; *typ*, genetic symbol for thymidylate uptaking mutant; *tmp*, genetic symbol for thymidylate auxotrophy; *tlr*, when used as a genetic symbol for yeast strains stands for 5′-dTMP low requirement with thymidylate biosynthesis blocked. The *tlr* mutation is thought to be recessive in analogy to the *typ* mutation.

Materials and Methods

Strains

The strains used in the experiments are compiled in Table I.

Media, growth conditions, monitoring of cell growth

Medium N, I, R and "bracket nomenclature": [3, 4]; medium 4×N: 4-fold concentrated medium N. For standard growth conditions see Fäth and Brendel [3]. Cell titer determinations were done *via* cell counting in a chamber.

Labelling of cells, chromatography

Labelling and monitoring of label incorporation into cells were essentially performed as previously described [3, 4], the labelled compound employed being $(NH_4)_2$·[methyl-^{3}H]-5′-dTMP (Amersham; specific activity 25.6 Ci/mmol). Chromatography for separation of 5′-dTMP, Thy and Thd was performed on sheets of PEI cellulose (20×20; Macherey & Nagel; 2× washed with H_2O dest.), the solvent employed being H_2O dest. Running up to 18 cm (start-

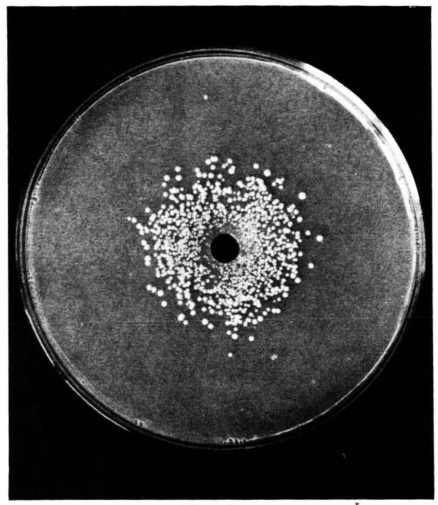

Fig. 1 a.

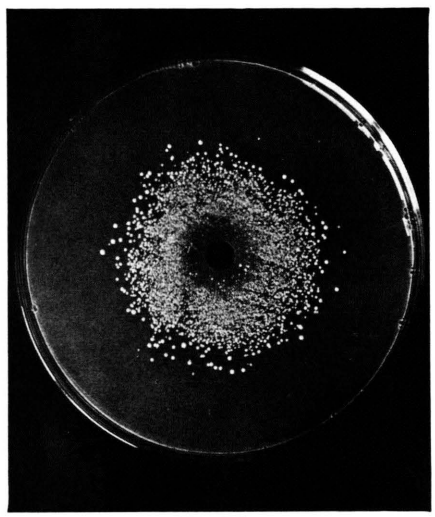

Fig. 1 b.

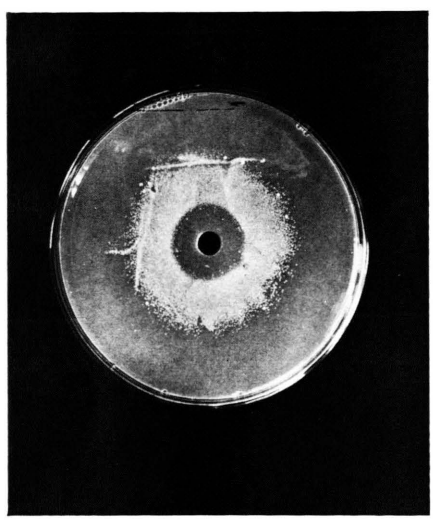

Fig. 1 c.

Fig. 1. Screening for mutants of *S. cerevisiae* with highly efficient 5′-dTMP utilization potency. a. and b. resp., medium I (50 μg Ade/ml) 5′-dTMP gradient plates seeded with 5×10^3 and 5×10^4 cells, resp., of strain T6-425 for $(typ1\ tlr)_{1.5}$ mutant screening. Macrocolonies at the periphery of the dense growth halo are putative $(typ1\ tlr)_{1.5}$ mutants. c. Medium *N* 5′-dTMP gradient plate seeded with 5×10^6 cells of strain T6-425 *tmp1-51* for $(typ1\ tlr)_{1.5}$ mutant screening. Macrocolonies at the periphery of the dense growth halo often are revertants to 5′-dTMP prototrophy; the clear halo around the central hole reflects the effect of (permanent) 5′-dTMP mediated inhibition. — For photographic purposes petri dishes of diameter 140 mm filled with 100 ml agar medium were used.

Table I. Strains of *S. cerevisiae* employed in experiments.

Strains	Genotype	Ref.
211-1aM	*α ilv2 rad2 rho⁺*	3
211-1aMT2	*α ilv2 typ1 rho⁰*	4
211-1aMT2	*α ilv2 typ1 tmp1-1 rho⁰*	4
211-1aMT4	*α ilv2 typ2 rho⁰*	4
211-1aMT6	*α ilv2 typ1 rho⁺*	4
211-1aMT11	*α ilv2 typ3 rho⁰*	4
211-1aMT6-425 [a]	*(α) ilv2 typ1 rho⁰*	1
211-1aMT6-425 [b]	*(α) ilv2 typ1 tmp1-51 rho⁰*	1
3104-1C [c]	*a leu2 his2 his6 ilv3 met14 tyr17 ade2 rad1 can1 lys1 rho⁺*	
458-1A [d]	*α his1 rho⁺*	
ZIII-1D [e]	*α his5-2 lys1-1 leu1-12 rho⁺*	
MB1052 [f]	*α ilv2 typ1 tmp1-3* + + + + + *a ilv2 typ1 tmp1-3 ade arg4-17 his5-2 lys1-1* *rho⁰*	1

a. Strain T6-425 in the text; b. strain T6-425 *tmp1-51* in the text; c. obtained from the Yeast Genetic Stock Center, Berkeley (Calif.), USA; d. obtained from Dr. P. Slonimski *via* Dr. J. Marmur; e. constructed by Dr. N. A. Khan; f. derived *via tlr* screening from a zygote constructed by the second author and shown to be diploid by G. Höltz, Ph. D. Thesis, J. W. Goethe-Universität, Frankfurt (Main), 1976.

ing line at 1 cm) gave clear separation of 5′-dTMP (R_F = 0.07), Thy (R_F = 0.77) and Thd (R_F = 0.86) as determined by the location of the UV spots of these compounds. The amount of medium supernatant radioactivity employed per single run was approx. 5000 cpm.

Screening for yeast mutants with highly efficient utilization of exogenous 5′-dTMP

Previously [4] we employed the double layer medium I (Ade)/medium R gradient plate technique for yeast *typ tlr* mutant screening. This technique did not prove to be adequate to allow screening for *typ tlr* mutants with an optimal 5′-dTMP requirement <12 μg/ml in medium R. As an alternative we chose the center hole gradient plate technique (Fig. 1) * which is reported to do good service in *thy tlr* screening of bacterial *thy* mutants [5]. For screening of very low 5′-dTMP requiring yeast *typ tlr* mutants the following procedure proved successful: A medium N inoculum of the *typ* strain to be submitted to *tlr* screening is grown just to the stationary phase ($N_{0\,h} = 10^6$/ml $- N_{24\,h} = 1 - 2 \times 10^8$/ml). An aliquot of the freshly prepared suspension is 3× washed in phosphate buffer (0.067 M, pH 7) and then diluted to a titer of 5×10^3 to 10^4/ml. 200 μl of this dilution are plated onto plates (ϕ = 85 mm) of medium I agar additionally supplemented with 50 μg Ade/ml. After drying a cylindric hole is stamped into the center of a plate, the cylinder volume being sufficient to take up an amount of aqueous 5′-dTMP solution which contains an amount of 5′-dTMP (μg) equivalent to the product "agar volume per plate (ml) × 5′-dTMP requirement in medium R of the strain to be screened (μg/ml)". After a 3 – 5 days' incubation at 30 °C a halo of colonies will have grown around the plate's center. At its periphery one will find approx. 20 macrocolonies (putative *typ tlr* mutants with a lower 5′-dTMP requirement in medium R as compared to that of the parental strain). These colonies are streaked onto medium N plates and the streaks incubated for 2 – 3 days at 30 °C. To check their *tlr* quality in brief medium R inocula are prepared with an initial titer of 10^6/ml – the medium R having 1/3 of the 5′-dTMP concentration known to be the optimal requirement of the parental strain. The inocula are incubated for 2 – 3 days and then microscopically compared to that of control inocula of the parental strain in the same medium R (= CI) and in a medium R where the parent is optimally supplemented with 5′-dTMP (= CII). Mutants with lowered 5′-dTMP requirement will disclose their identity as follows: The cell size is approx. that of the cells in CII, *i. e.* no or little cell swelling or chain formation tendency should be observed as will be typical for CI; the cell titer is approx. 2 – 3× that in CI and close to that in CII.

It is not recommendable to employ more than 10^4 cells/plate in a *tlr* screening assay as otherwise growth at the halo periphery is too poor with respect to colony size. Furthermore most of the cells of the putative *tlr* mutant colonies will be subject to 5′-TMP less death under such conditions [6, 7]. For the isolation of any *typ tlr* mutant quality it is well sufficient to prepare 5 – 10 plates in the way described above, and this procedure is applicable both for haploid (*typ*) and diploid (*typ/typ*) yeast strains. If desired the *typ tlr* mutant isolated may be made auxotrophic for 5′-dTMP according to Brendel and Fäth [8]. The 5′-dTMP auxotrophy should not be introduced at the beginning of *tlr* mutant screening. Doing so, *i. e.* performing screening on medium N 5′-dTMP gradient plates, one will be confronted with the fact that at least some of the putative *tlr* mutants have totally or partially reverted to 5′-dTMP prototrophy (Fig. 1 c).

Results and Discussion

Isolation of typ tlr mutants with highly efficient 5′-dTMP utilization

In Fäth, Brendel, Laskowski and Lehmann-Brauns [4] we reported the isolation of yeast strains

* Fig. 1a – c see Plate on page 468 b.

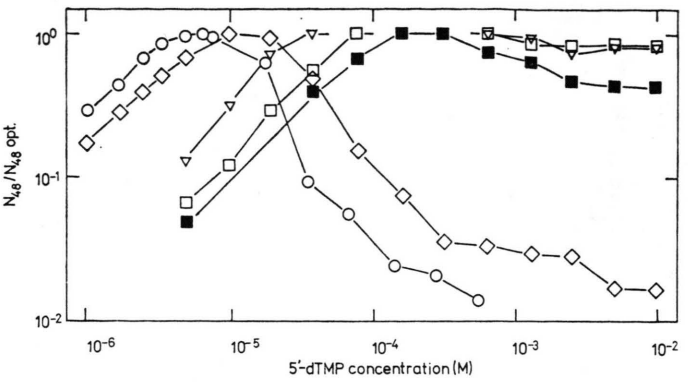

Fig. 2. Growth behaviour in medium N/R (5′-dTMP) of yeast strains exhibiting different 5′-dTMP requirement with 5′-dTMP biosynthesis blocked. ○—○, strain T6-425 *tmp1-51* (*typ1 tlr*)$_{1.5}$; ◇—◇, strain T6-425 *tmp1-51* (*typ1 tlr*)$_3$ or T6-425 (*typ1 tlr*)$_3$; ▽—▽, strain 211-1aMT2 *tmp1-1* (*typ1 tlr*)$_{12}$; □—□, strain 211-1aMT2 (*typ1*)$_{25}$; ■—■, strain 211-1aM.

with "low" requirement of exogenous 5′-dTMP (*typ tlr* strains in medium R and *typ tlr tmp* strains in medium N, resp.). These mutants were found to exhibit an efficiency of 5′-dTMP utilization of approx. 12%. Such an efficiency may be well sufficient for a lot of studies in yeast after (5′-dTMP) DNA-specific labelling. However, it is still unsatisfactory in experiments where the specific activity of the labelled DNA should be as high as possible to allow quantitative working with a minimum of cellular material.

Bacterial (*e. g. E. coli*) *thy tlr* mutants are known to have a Thy requirement of 2 μg/ml [5, 19]. Assuming a standard stationary titer of $1-2\times10^9$ at this Thy concentration for an *E. coli thy tlr* mutant an efficiency of Thy utilization between 50 and 100% can be calculated. Demanding such a magnitude of order (*e. g.* 75%) for 5′-dTMP utilization of yeast *tlr* mutants one can make an estimate about the optimal 5′-dTMP requirement (μg/ml) for a haploid (diploid) yeast *tlr* mutant of this quality by making use of the following formula: 5′-dTMP optimum (μg/ml) = DNA contents of a cell (= A, (μg)) × DNA Thy contents (= B, (%)) × stationary titer (= C, (1/ml)) × 1/0.75. With $A=2.2\times10^{-8}$ (4.4×10^{-8}) [9–11], B = 30.5 [12] and $C=2\times10^8$ one can predict the 5′-dTMP optimum of the hypothetical haploid (diploid) *tlr* mutant to be 1.8 (3.6) μg/ml.

To realize such yeast *tlr* mutants we chose the *typ1* mutant 211-1aMT6 [4] and the *typ1/typ1* zygote MB1052 **, resp., as parental strains employing the screening procedure described in Materials and Methods. In fact we were successful in isolating *tlr* mutants exhibiting a 5′-dTMP requirement of 1.5 (haploid) and 3 (diploid) μg 5′-dTMP/ml in medium R. However, the isolation of these mutants made necessary several successive screening steps – in contrast to what is reported for *thy tlr* screening of bacterial *thy* strains [5]. For haploid *S. cerevisiae* this may be symbolized as follows: strain 211-1aMT6 = (*typ1*)$_{25}$ → (*typ1 tlr*)$_{12}$ → (*typ1 tlr*)$_6$ → (*typ1 tlr*)$_3$ → (*typ1 tlr*)$_{1.5}$ – the indices standing for the optimal 5′-dTMP requirement (μg/ml) in medium R. It is obvious that the 5′-dTMP optimum of any series member is half that of its direct parent. This led us to the idea that strain 211-1aM as the direct parent of strain 211-1aMT6 [4] might equally well fit into this scheme. The *typ* mutant screening is described in ref. 13. As a rationale for their screening procedure these authors stated strain 211-1aM not to be able to grow on medium R (10 μg 5′-dTMP/ml) interpreting this as its failure to utilize exogenous 5′-dTMP in the presence of aminopterin (APT) + sulfanilamide (SAA). This is in contradiction to our previous finding [14]: Though it is true that strain 211-1aM cannot overcome APT + SAA inhibition in a medium R (10 μg 5′-dTMP/ml) it could unequivocally be shown to DNA-specifically incorporate [^{3}H]- or [^{32}P]-5′-dTMP under these conditions. As a consequence growth inhibition of strain 211-1aM in that medium R was speculated to rather result from a suboptimal 5′-dTMP supplementation. Fig. 2 shows that this is in fact the case, the 5′-dTMP optimum being 50 – 60 μg/ml in medium R. In view of this finding mutations such as *typ* [4, 13] or *tmp per* [7] surely can no longer be interpreted as being responsible to allow 5′-dTMP utilization in the presence of APT + SAA [13]; they rather must be regarded as mutations *enhancing* 5′-dTMP utilization potency and hence should be-

** Constructed from the haploid strains 211-1aM *a tmp1-3* and MB1051-13C *a tmp1-3* [33].

Table II. Restoration of growth by exogenous 5′-dTMP after APT+SAA inhibition in strains of *S. cerevisiae* that represent "wild type" character with respect to 5′-dTMP utilization.

Growth medium	Titer N_{48h} (1/ml) strain ZIII-1D	strain 458-1A	strain 3104-1C
R (3200 μg 5′-dTMP/ml)	$(1.1 \pm 0.1) \times 10^8$	$(3.4 \pm 0.4) \times 10^7$	$(9.5 \pm 0.9) \times 10^6$
R (1600 μg 5′-dTMP/ml)	$(8.0 \pm 0.8) \times 10^7$	$(2.4 \pm 0.3) \times 10^7$	$(7.0 \pm 0.7) \times 10^6$
R (800 μg 5′-dTMP/ml)	$(3.7 \pm 0.4) \times 10^7$	$(2.2 \pm 0.2) \times 10^7$	$(5.2 \pm 0.5) \times 10^6$
R (400 μg 5′-TMP/ml)	$(1.1 \pm 0.2) \times 10^7$	$(9.7 \pm 1.0) \times 10^6$	$(2.5 \pm 0.3) \times 10^6$
I	$(5.3 \pm 0.5) \times 10^6$	$(3.9 \pm 0.4) \times 10^6$	$(2.1 \pm 0.2) \times 10^6$
N	$(2.0 \pm 0.2) \times 10^8$	$(2.1 \pm 0.2) \times 10^8$	$(5.8 \pm 0.6) \times 10^7$

All media were supplemented with 50 μg adenine/ml; the initial titers of the inocula were 10^6/ml each.

long to that class of mutations which we named *tlr*.

Wild type of S. cerevisiae with respect to 5′-dTMP utilization

Utilization of exogenous 5′-dTMP by yeast was first reported for strain 211-1aM of *S. cerevisiae* [15]. This phenomenon – referred to as "*211-1aM character*" in the literature – has long been thought to be a curiosity. Meanwhile Wickner [16, 17], Brendel [18] and Little (pers. commun.) could isolate "5′-dTMP uptaking mutants" of *S. cerevisiae* derived from strains totally different from strain 211-1aM with respect to their origin. These mutants (*tup* [16, 17], *tum* [18]) were obtained by essentially the same screening procedure as that employed for *typ* or *typ tlr* mutant screening [4, 13] save that *tup* or *tum* mutant screening proceeded from real wild types with respect to 5′-dTMP utilization potency. The designations "*tup*" (obviously chosen as an abbreviation for 5′-d*T*MP *up*take) and "*tum*" (abbreviation for 5′-d*T*MP *u*ptaking *m*utant) suggest the impression that such mutations enable a yeast cell to utilize exogenous 5′-dTMP, *i. e.* introduce a feature which is absent in a wild type yeast. Alternatively, however, one could hypothesize that mutations like *tup*, *tum* or "*211-1aM character*" might as well as *typ*, *tmp per* and *tlr* not introduce but only enhance the ability of "wild type" *S. cerevisiae* to utilize exogenous 5′-dTMP. If so, any strain of this yeast should be able to incorporate the nucleoside monophosphate. To check this we chose three different "wild types" of *S. cerevisiae* (sensitive towards APT + SAA) and submitted them to liquid medium R conditions, the range of concentration of the 5′-dTMP supplement being $0 - 10^{-2}$ M. The results of this test are summarized in Table II: It is obvious that even in a "wild type" yeast an APT + SAA mediated inhibition of thymidylate biosynthesis can be overcome by the presence of 5′-dTMP in the inhibitory medium. For strain ZIII-1D a 5′-dTMP supplementation near 10^{-2} M is sufficient to totally restore growth; for the strains 458-1A and 3104-1C this should be expected at concentrations of exogenous 5′-dTMP $> 10^{-2}$ M.

These findings should support the above hypothesis. So far seven discrete stages of 5′-dTMP utilization were detected as classified by the optimal 5′-dTMP requirement, with thymidylate biosynthesis blocked, of the corresponding yeast strains (Table III). All of them apparently fit well into a mathematical series of the type "$2^n \times 1.5$" (n = 0, 1, 2, ..., 11) where the product term for n = 0 stands for the 5′-dTMP requirement (μg/ml) of the best 5′-dTMP utilizing *typ tlr* mutant and that for n = 11 represents the 5′-dTMP optimum in medium R of strain ZIII-1D. Consequences of the formula:

Table III. Stages of 5′-dTMP requirement in haploid *S. cerevisiae* with 5′-dTMP biosynthesis blocked.

Optimal 5′dTMP requirement with thymidylate biosynthesis blocked [μg/ml]	Series number "n" in "$2^n \times 1.5$"	Strain
>3200	>11	3104-1C; 458-1A
3200	11	ZIII-1D
1600	10	?
800	9	MB1093-1C; MB1093-6B$^+$
400	8	MB1093-1C; MB1093-6B$^+$
200	7	?
100	6	?
55	5	211-1aM
25	4	211-1aMT2, -T4, -T6
12	3	211-1aMT2 *tmp1-1*; 211-1aMT6 (*typ1 tlr*)$_{12}$
6	2	211-1aMT11; 211-1aMT6 (*typ1 tlr*)$_6$
3	1	T6-425 (*typ1 tlr*)$_3$; T6-425 *tmp1-51* (*typ1 tlr*)$_3$
1.5	0	T6-425 *tmp1-51* (*typ1 tlr*)$_{1.5}$

(+), Constructed by the second author [18]; the optimal 5′-dTMP requirement of the strains should be 400 or 800 μg/ml.

(i) With the correlation "$2^n \times 1.5$" valid one should expect five stages of 5′-dTMP utilization to be realizable corresponding to the product terms for $n = 6-10$. At least one such "missing link" has meanwhile been identified [18] though its exact position in the series remains to be determined (Table III). (ii) Wild type character, with respect to 5′-dTMP utilization, of the yeast *S. cerevisiae* obviously does not correspond to one single stage of thymidylate utilization potency as given by the term "$2^n \times 1.5$". The discrimination between wild type and mutant rather is a matter of definition.

Genetical data concerning this subject are sparse up to now [4, 16, 17] and the understanding of the phenomenon surely requires a more detailed genetical study than so far performed. For such an analysis the "211-1aM system" will not be an adequate basis: Unfortunately strain 211-1aM, its *typ* and *typ tlr* mutants either are rather poor maters or even have totally lost their mating type (as judged from unsuccessful forced matings). In addition genetical studies in this "system" would have been questionable anyway when strain 211-1aM itself cannot be referred to as a wild type without scruples. Hence such an analysis was started in a strain totally different from strain 211-1aM. Some preliminary data are presented in a parallel paper [18].

Optimization of 5′-dTMP utilization

As a first condition for such an optimization it was necessary to isolate mutants where, with 5′-dTMP biosynthesis blocked, the optimum of 5′-dTMP requirement is a minimum or at least near the minimum. This is *e.g.* realized in our *(typ1 tlr)*$_3$ and *(typ1 tlr)*$_{1.5}$ mutants. A second parameter of interest is the size of the stationary titer at the 5′-dTMP optimum. By experience optimal stationary cell titers of the yeast *S. cerevisiae* are known to reach values between $2-3 \times 10^8$/ml. As compared to this the stationary titers in media N/R (5′-dTMP) listed in Table IV are clearly suboptimal. Hence we looked for modified medium N/R (5′-dTMP) conditions where this parameter is at its optimum. It was found that a medium $4 \times$ N or a medium R based on medium $4 \times$ N meets these requirements. As a rule here the stationary titers are $2-3$ times those measured for comparable medium N/R (5′-dTMP) conditions. The efficiencies of 5′-dTMP utilization thus obtained for the *(typ1 tlr)*$_{1.5}$ or *(typ1 tlr)*$_3$ mutants are well comparable to the efficiencies of Thy(Thd) utilization found for *thy tlr* mutants of bacteria (Table IV).

The amount of exogenous 5′-dTMP utilized by a yeast cell for DNA synthesis should be indirectly proportional to the 5′-dTMP offer required for optimal growth at conditions where thymidylate biosynthesis is blocked. This correlation is well affirmed by the data given in Table IV, column 3 (a, b). It is remarkable, however, that such a correlation obviously also is valid for the amount of exogenous 5′-dTMP incorporated into a cell as a whole (Table IV, column 3 (c); strain 211-1aM *vs.*

Table IV. Optimization of 5′-dTMP utilization in the yeast *S. cerevisiae*.

Strain	Assay in medium	Radioactivity/10^8 cells [cpm] (a) TCA precipitable (b) TCA precipitable after alkali (c) untreated cells	Titer after 48 h [1/ml]	Efficiency of 5′-dTMP utilization [%] (a) in medium N/R (b) in medium $4\times$N
211-1aM	R^+ (60 µg 5′-dTMP/ml)	(a) $(8.5 \pm 0.9) \times 10^3$ (b) $(9.1 \pm 0.9) \times 10^3$ (c) $(9.0 \pm 0.9) \times 10^3$	$(8.5 \pm 0.8) \times 10^7$	(a) 1.0
211-1aMT2 *tmp1-1* *(typ tlr)*$_{12}$	N^+ (12 µg 5′-dTMP/ml)	(a) $(3.2 \pm 0.3) \times 10^4$ (b) $(3.5 \pm 0.4) \times 10^4$	$(1.6 \pm 0.1) \times 10^8$	(a) 9.0
T6-425 *tmp1-51* *(typ1 tlr)*$_3$	N^+ (3 µg 5′-dTMP/ml)	(a) $(1.1 \pm 0.1) \times 10^5$ (b) $(1.2 \pm 0.1) \times 10^5$ (c) $(1.2 \pm 0.1) \times 10^5$	$(1.5 \pm 0.1) \times 10^8$	(a) 34 (b) 58^{++}
T6-425 *tmp1-51* *(typ1 tlr)*$_{1.5}$	N^+ (1.5 µg 5′-dTMP/ml)	(a) $(2.0 \pm 0.2) \times 10^5$ (b) $(2.3 \pm 0.2) \times 10^5$	$(1.2 \pm 0.1) \times 10^8$	(a) 54 (b) 90^{+++}

+, Supplementation with 2.5 µCi [^{3}H]-5′-dTMP/ml; ++, a stationary titer of 2.6×10^8/ml is taken as a basis for calculation; +++, a stationary titer in medium $4\times$N of 2×10^8/ml is assumed. For calculation of the efficiencies of 5′-dTMP utilization the formula given in the text was employed.

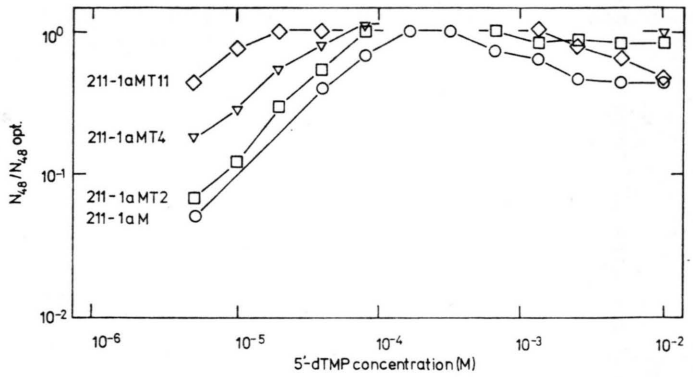

Fig. 3. Growth behaviour in medium R of strain 211-1aM and one representative each of its three classes of *typ* mutants.

strain T6-425 *tmp1-51* (*typ1 tlr*)$_3$). This may indicate that the mutative enhancement of 5′-dTMP utilization potency is due to either a genetically determined alteration of the yeast 5′-dTMP uptaking principle itself or/and of physiological processes directly associated to the monophosphate's uptake.

Special features of yeast strains with highly efficient utilization of exogenous 5′-dTMP

(A) Inhibition of growth by superoptimal offer of exogenous 5′-dTMP. When the (*typ1 tlr*)$_3$ mutant T6-425 was checked for its optimal 5′-dTMP requirement in media R ($0-10^{-2}$ M 5′-dTMP) the stationary levels of cell yield were found to be clearly reduced at 5′-dTMP concentrations $>10^{-5}$ M (= 5′-dTMP optimum) (Fig. 2). This phenomenon has meanwhile been chosen as the subject of another paper [21]. Here some further details may be given and briefly discussed: (i) The observed inhibition in medium R is a permanent one as no significant increase of cell yield beyond the 24 h titer is found even after a week's incubation – the initial cell titers being 10^6/ml. (ii) This permanent inhibition of growth should not be the result of any sensitivity of the strain against the antimetabolites APT + SAA expressed in the presence of superoptimal 5′-dTMP offer in the growth medium. It rather should be due to the presence of excess 5′-dTMP itself as the *ts* (thymidylate synthetase) mutant T6-425 *tmp1-51* [1, 22] of strain T6-425 is similarly inhibited in media N ($>10^{-5}$ M 5′-dTMP). (iii) The permanent nature of excess 5′-dTMP mediated inhibition obviously is correlated to the total blockage of thymidylate biosynthesis by either the synergistic action of APT + SAA [4] or the *ts* mutation: When the 5′-dTMP prototrophic strain T6-425 is incubated in media N ($>10^{-5}$ M 5′-dTMP) – where endogenous 5′-dTMP synthesis *a priori* is possible – an inhibition of growth is observed, too, as judged from the cell titer values after a 24 h′ incubation. However, with prolonged incubation the titers of all medium N (5′-dTMP) inocula approach that of the control in medium N (Fig. 3). (iv) Neither a marked permanent nor a temporary inhibition by superoptimal 5′-dTMP offer so far was observed for any haploid yeast strain exhibiting an optimal 5′-dTMP requirement $>10^{-5}$ M with thymidylate biosynthesis blocked (Figs 2, 4). On the other hand a significant 5′-dTMP sensitivity also was found for the diploid (*typ tlr*)$_6$ mutant MB1052 and its (*typ tlr*)$_3$ derivative (Fäth, unpublished data) which can be regarded as the pendants of the haploid (*typ tlr*)$_3$ and (*typ tlr*)$_{1.5}$ strains, resp. Consequently one might infer the phenomenon of excess 5′-dTMP inhibition to be necessarily associated with the ability of a yeast cell to highly efficient utilize exogenous 5′-dTMP. However, the following arguments do not justify such a conclusion: Our present data are too sparse as to allow such a correlation to be postulated. Growth

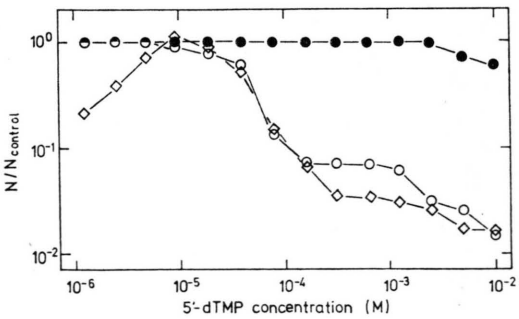

Fig. 4. Temporary and permanent inhibition of growth by exogenous 5′-dTMP, ◇—◇, strain T6-425 *tmp1-51* after a 48 h′ incubation; ○—○ (●—●), strain T6-425 after a 24 h′ and a 48 h′ incubation, resp.

inhibition by excess 5′-dTMP is at least indicated for some haploid strains with $>10^{-5}$ M 5′-dTMP requirement at conditions where thymidylate biosynthesis is blocked (Figs 2, 4). When cells of strain T6-425 *tmp1-51* were submitted to $(typ\ tlr)_{1.5}$ screening as described in the legend to Fig. 1 some 5′-dTMP auxotrophic clones were found within the halo of (permanent) excess 5′-dTMP inhibition that are 5′-dTMP resistant. Such clones could be *typ tlr* revertants with an increased 5′-dTMP optimum or they could have retained the parental 5′-dTMP utilization potency and have become insensitive towards superoptimal 5′-dTMP. The real nature of this 5′-dTMP resistance remains to be studied. It would not be surprising, however, if yeast strains could be isolated exhibiting highly efficient 5′-dTMP utilization potency plus resistance towards superoptimal 5′-dTMP. Though what we called a yeast *tlr* mutation should not be synonymous in essence with what was named *tlr* in bacteria it is at least worth mentioning that for these organisms two classes of *tlr* mutants are reported, one being sensitive and the second being resistant to Thd or other deoxyribonucleosides [20, 23, 24]. It may be noted here that neither strain T6-425 in medium N nor strain T6-425 *tmp1-51* in medium N (10^{-5} M 5′-dTMP) is affected in growth with any deoxyribo- or ribonucleoside offered up to 10^{-2} M.

Presently the cell physiological background of excess 5′-dTMP mediated inhibition is rather obscure to us. Nevertheless one consideration concerning that phenomenon may be added: Whatever the reason(s) are for the observed inhibition of the 5′-dTMP prototrophic strain T6-425 in medium N ($>10^{-5}$ M 5′-dTMP) – this sort of inhibition can finally be overcome. And it is strange that, with thymidylate biosynthesis blocked, the 5′-dTMP mediated inhibition should be a permanent one. This might suggest at least a second sort of 5′-dTMP conditioned inhibitory effect(s) to be operative when a yeast cell, which *a priori* exhibits sensitivity towards the exogenous monophosphate, is confronted with a situation where it depends on the supply with exogenous 5′-dTMP. Yeast strains auxotrophic for 5′-dTMP were reported to be subject to a more or less rapid "thymineless" death ("TLD") if they are not or insufficiently provided with the nucleotide [6]. A similar loss of viability could be observed for strain T6-425 *tmp1-51* submitted to medium N ($>10^{-5}$ M 5′-dTMP) conditions [21]. From this finding one might infer that in the presence of excess 5′-dTMP the utilization of the exogenous thymidylate might be disturbed. In principle such a disturbance can occur at two levels of utilization of exogenous 5′-dTMP: One is the process of the monophosphate's uptake, the second that of thymidylate anabolism and its incorporation into DNA. With a severe disturbance at the second level one should expect the 5′-dTMP prototrophic strain T6-425 to be as strongly inhibited in medium N ($>10^{-5}$ M 5′-dTMP) as is the

Table V. Dephosphorylation of exogenous 5′-dTMP by some *typ* or *typ tlr* mutants of *S. cerevisiae*.

Strain	Genotype with respect to 5′-dTMP utilization	5′-dTMP optimum with thymidylate biosynthesis blocked [μg/ml]	Labelling medium	Thd in medium after a week's incubation [%]	Inhibition by superoptimal 5′-dTMP offer
211-1aM	*"211-1aM"*	60	R^+ (60 μg 5′-dTMP/ml)	5.0 ± 0.5	weak
211-1aMT2	*typ1*	25	R^+ (25 μg 5′-dTMP/ml)	15.2 ± 0.7	not significant
211-1aMT4	*typ2*	25	R^+ (25 μg 5′-dTMP/ml)	22.2 ± 0.5	none
211-1aMT6	*typ1*	25	R^+ (25 μg 5′-dTMP/ml)	97.6 ± 0.3	none
211-1aMT11	*typ3*	6	R^+ (6 μg 5′-dTMP/ml)	99.4 ± 0.2	weak
211-1aMT2 *tmp1-1*	*typ1 tlr*	12	N^+ (12 μg 5′-dTMP/ml)	15.9 ± 1.3	not significant
T6-425 *tmp1-51*	*typ1 tlr*	3	N^+ (3 μg 5′-dTMP/ml)	94.9 ± 0.5	strong

+, Supplementation with 2 μCi [^{3}H]-5′-dTMP/ml; the data given in column 5 are the arithmetic means of three single determinations.

case for its derivative T6-425 *tmp1-51*. However, the inhibition of that strain was found to be only temporary which indicates that a possible 5′-dTMP mediated disturbance of this kind can be counterbalanced by the cell. Alternatively, with a permanent blockage of thymidylate uptake assumed – in addition to *a priori* compensable inhibitory effects, the different response of strain T6-425 and its *tmp* mutant towards excess 5′-dTMP could be explained: Whilst this would lead to cessation of division activity and to "TLD" in the 5′-dTMP auxotrophic strain it would leave unaffected strain T6-425.

(B) Destruction of exogenous 5′-dTMP to Thd. Medium N is known to be rich in inorganic phosphate (6 mM [3]). Hence, though this medium is slightly acidic by nature (pH 6) and though in medium N the pH value decreases to approx. 4.5 during growth of a yeast culture (Fig. 6 b) acidic phosphatase should not work [25, 26]. Consequently one will not expect any exogenously supplied 5′-dTMP to be cleaved to Thd + P_i by yeast grown in medium N (5′-dTMP). In contrast, with any significant phosphatase activity present in a medium N (5′-dTMP) yeast culture this would be traceable by employing [^{3}H]- or [^{32}P]-5′-dTMP: [^{3}H]- or [^{32}P]-radioactivity should be found intracellularly in material different from DNA or its precursors 5′-dTXP and/or [^{3}H]-Thd and $^{32}P_i$, resp., should appear in the medium. In fact no such indications for any acidic phosphatase mediated 5′-dTMP cleavage could be observed for strain 211-1aM and its *typ* mutant 211-1aMT2 – at least up to 24 h of incubation of an inoculum [3, 4, 14]. However, the situation is quite different for the highly efficient 5′-dTMP utilizing strains T6-425 and T6-425 *tmp1-51* (Fig. 5, Table V). Considerable amounts of [^{3}H]-Thd are accumulated in the medium during incubation of a medium N ([^{3}H]-5′-dTMP) inoculum. This phenomenon gave rise to the following questions:

Is the 5′-dTMP cleavage by the strains mentioned due to any acidic phosphatase activity not inhibited in medium N? If so, this effect should be reduced at an increased pH value and/or an increased P_i concentration in medium N – provided this phosphatase is identical with that characterized in [25, 26]. To check this strain T6-425 was incubated in a medium N (10^{-5} M 5′-dTMP; [^{3}H]-5′-dTMP) buffered to pH 7 by our standard phosphate buffer, *i. e.* at conditions pH 7 + 70 mM P_i. Fig. 6 c

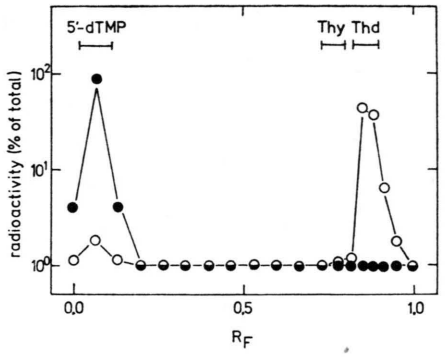

Fig. 5. Chromatographical analysis of medium N/R (5′-dTMP; [^{3}H]-5′-dTMP) for phosphatase mediated 5′-dTMP cleavage. ○—○, inoculum of strain T6-425 *tmp1-51* after a 5 d′ incubation; ●—●, sterile medium N (5′-dTMP) after a 0 and 5 d′ incubation at 30 °C.

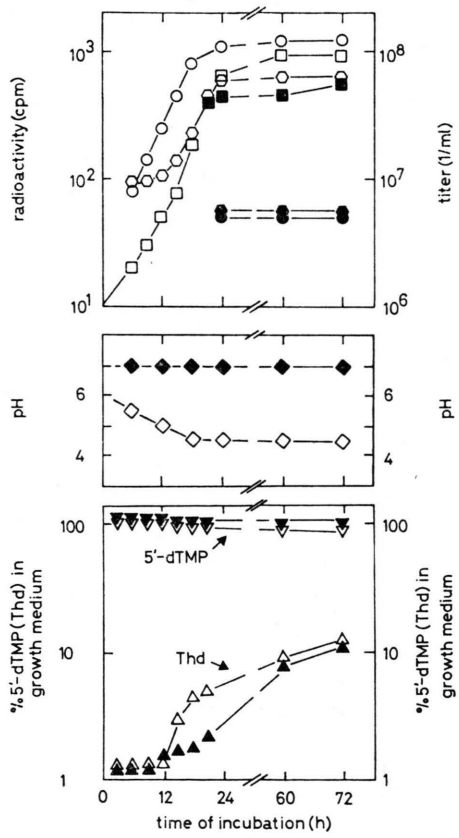

Fig. 6. 5′-dTMP utilization and dephosphorylation by strain T6-425 in medium N (10^{-5} M 5′-dTMP; 1 μCi [^{3}H]-5′-dTMP/ml) with and without buffering to pH 7. □—□ (■—■), growth kinetics; ◇—◇ (◆—◆) and ○—○ (●—●), resp., TCA precipitable label and label in untreated cells, resp. – the sample size being 100 μl each. Open symbols: medium N (5′-dTMP) inoculum; filled symbols: medium N (5′-dTMP; pH 7) inoculum.

demonstrates that here the 5′-dTMP cleavage is as intensive as in medium N. This suggests that for the monophosphate's dephosphorylation a phosphatase activity should be responsible which is different from that described in [25, 26].

Is the 5′-dTMP cleavage correlated to the 5′-dTMP uptake/utilization by highly efficient 5′-dTMP utilizing strains? As can be judged from Fig. 6 a the utilization of exogenous monophosphate is significantly reduced in a medium N (5′-dTMP) buffered to pH 7 as compared to medium N (5′-dTMP) conditions. Nevertheless in medium N (5′-dTMP; pH 7) the 5′-dTMP cleavage activity is as marked as in medium N (Fig. 6 c). Consequently both processes should be independent from each other or – if still correlated somehow – this interdependence presently is an obscure one.

Is the 5′-dTMP cleavage typical for highly efficient 5′-dTMP utilizing strains? To get information concerning this we compared strain T6-425 *tmp1-51* to its primordial parental strain 211-1aM and some *typ* or *typ tlr* mutants (Table V). It is clear from the Table that there obviously exists no correlation between 5′-dTMP cleavage activity and 5′-dTMP utilization potency: Strains with equal 5′-dTMP requirement can exhibit poor or strong 5′-dTMP cleaving activity (211-1aMT2, -T4, -T6); strains with a relatively high 5′-dTMP requirement are as good 5′-dTMP cleavers as such with very low 5′-dTMP requirement (211-1aMT6, T6-425 *tmp1-51*); strains with relatively low 5′-dTMP requirement can exhibit poor or strong 5′-dTMP cleaving (211-1aMT2 *tmp1-1*, T6-425 *tmp1-51*); 5′-dTMP cleaving potency even seems to vary for strains of the same *typ* genotype (211-1aMT2, -T6). It should be noted, however, that strain 211-1aM exhibits a very low 5′-dTMP cleaving activity as compared to all its *typ* or *typ tlr* derivatives listed in Table V. This might suggest that a weak *a priori* 5′-dTMP cleaving potency is (facultatively) enhanced in the course of *typ* or *typ tlr* screening, *i. e. via* the employment of APT + SAA in the screening procedure, and that the effect of enhanced 5′-dTMP cleavage potency is not causually connected to the 5′-dTMP uptake/utilization quality. Meanwhile a screening procedure for "*tlr*" mutants (*tum*) of *S. cerevisiae* is available which does not require the use of the antimetabolites APT + SAA but is based on thermo-conditional 5′-dTMP auxotrophy. A detailed analysis of such *tum* mutants with respect to 5′-dTMP cleavage potency and 5′-dTMP utilization should allow a more profound knowledge of this subject.

Is the phenomenon of 5′-dTMP cleaving correlated to that of inhibition by superoptimal 5′-dTMP offer? Here, too, no such correlation is obvious from Table V: Strong 5′-dTMP cleaving potency is observed in strains with strong and poor or no sensitivity towards superoptimal 5′-dTMP (211-1aMT2, -T6, -T11; T6-425 *tmp1-51*); poor 5′-dTMP cleaving is exhibited by strains showing a more or less marked inhibition by superoptimal 5′-dTMP (211-1aMT2, -T4, -T2 *tmp1-1*).

Does the 5′-dTMP cleavage disturb (5′-dTMP) DNA-specific labelling? The following arguments should disprove this: (i) 5′-dTMP cleavage to Thd is negligible up to a 24 h′ incubation of a 5′-dTMP labelling inoculum, *i. e.* during the period of time where the exogenous 5′-dTMP is taken up and incorporated into DNA (Fig. 6). (ii) 5′-dTMP cleavage obviously occurs outside the cells, *i. e.* does not require the monophosphate's uptake (Fig. 6 a). (iii) Thd cannot be utilized by yeast [4, 15, 27]. This was also found to be true for the strains T6-425 and T6-425 *tmp1-51*, resp.: When the strains were incubated in medium N ($10^{-6} - 10^{-2}$ M Thd; 1 μCi [^{3}H]-Thd/ml) and in medium N (10^{-5} M 5′-dTMP; $10^{-6} - 10^{-2}$ M Thd; 1 μCi [^{3}H]-Thd/ml), resp., for as long as 48 h – where normal stationary titers are obtained – no significant amounts of cell-bound TCA stable [^{3}H]-radioactivity were measurable. As compared to conditions where 1 μCi [^{3}H]-5′-dTMP/ml is employed instead of [^{3}H]-Thd the relative amount of cell-bound [^{3}H]-Thd *vs.* [^{3}H]-5′-dTMP radioactivity is $\leq 1\%$. (iv) After [^{3}H]-5′-dTMP labelling of strain T6-425 or strain T6-425 *tmp1-51* the ratios "TCA precipitable radioactivity with alkali treatment/TCA precipitable radioactivity without alkali treatment" are always significantly > 1. And this was reported as typical for [^{3}H]-5′-dTMP DNA-specific labelling in yeast [3]. (v) DNA-specificity of 5′-dTMP labelling in strain T6-425 and T6-425 *tmp1-51*, resp., is furthermore documented by the results presented in ref. 1.

Inhibition of utilization of exogenous 5′-dTMP under conditions of pH 7 + 70 mM P_i

It was found as a by-product of the above analyses that utilization of exogenous 5′-dTMP is not possible in a medium N (5′-dTMP; pH 7 + 70 mM P_i).

This is demonstrated by the failure of strain T6-425 to incorporate significant amounts of [^{3}H]-labelled thymidylate (Fig. 6 a) and by a permanent inhibition of growth of strain T6-425 *tmp1-51* under such conditions (Fäth, unpublished data). Majid (unpublished data) could meanwhile show that the inhibitory effect is due to the pH 7 rather than to the presence of 70 mM P_i in the growth medium. The observed blockage of label incorporation into strain T6-425 is not accompanied by a severe inhibition of growth with pH 7 ($+70$ mM P_i). This suggests that in a medium N buffered to pH 7 the utilization of exogenously offered 5′-dTMP is affected at the level of thymidylate uptake and not at that of cell interior anabolism of the monophosphate. At least three hypotheses could explain this: (i) The 5′-dTMP uptaking principle itself is somehow altered at neutral pH so that it cannot accept its substrate as it is able to do at acidic pH in the cell's outer environment and/or (ii) physiological processes accompanying the monophosphate's uptake are affected at pH 7 and/or (iii) the 5′-dTMP, at pH 7, is altered to a state where it is not recognized by the uptaking principle. The latter could *e. g.* mean that the weak acid 5′-dTMP is only accepted by the uptaking principle when it is in the non-dissociated form. In an acidic environment the non-dissociated state of the monophosphate should be favoured (which would explain an functioning 5′-dTMP uptake and the undisturbed growth of strain T6-425 *tmp1-51* in medium N (10^{-5} M 5′-dTMP)); at neutral or alkaline pH the dissociated form(s) of the nucleotide should prevail (which would explain the failure of [^{3}H]-5′-dTMP label incorporation into strain T6-425 and the permanent inhibition of growth of strain T6-425 *tmp1-51* in a medium N (10^{-5} M 5′-dTMP; pH 7)) (Fig. 7).

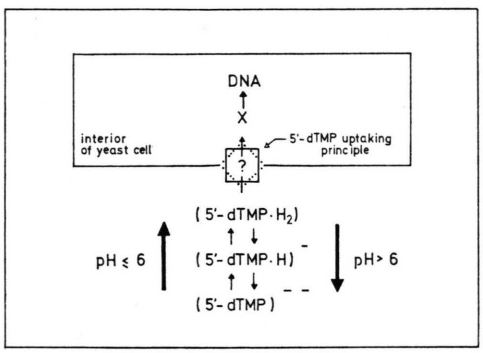

Fig. 7. Hypothetical interpretation for pH dependence of 5′-dTMP uptake in the yeast *S. cerevisiae*. 5′-dTMP uptaking principle figured as a square: state at acidic pH; dotted rhombus: represents inactivated 5′-dTMP uptaking principle plus possibly co-inhibited processes associated to thymidylate uptake, at pH 7; X, stands for thymidylate directly after uptake and is thought to be 5′-dTMP as judged from cell pool analyses after labelling with [^{3}H]-5′-dTMP (Fäth, unpublished data).

It may be extracted as an interesting result from this paper that the ability to utilize exogenous 5′-dTMP under appropriate conditions obviously is a general feature of at least the yeast *S. cerevisiae*. Hence it should be possible to more or less rapidly screen any strain of *S. cerevisiae* of choice for 5′-dTMP low requiring derivatives either by medium R screening or *via* the screening procedure described by Brendel [18]. Though thus a tool is at hand to DNA-specifically label any yeast with 5′-dTMP it would be somewhat unsatisfactory to know nothing more about the yeast 5′-dTMP uptaking principle than that it helps to substitute for the apparent lack of thymidine kinase [28] which would allow DNA-specific labelling with Thy or Thd as *e. g.* in bacteria.

So far information concerning utilization of exogenous 5′-dTMP in yeast is rather sparse: The 5′-dTMP utilizing potency obviously requires the exponential growth state of a cell [3, 4] and acidic conditions in the growth medium (present data). Highly efficient 5′-dTMP utilization as far as investigated is accompanied by the phenomenon of excess 5′-dTMP inhibition and accumulation of Thd in the growth medium (present data). Utilization of exogenous 5′-dTMP is found to occur in "*grande*" (*rho*$^+$) and in "*petite*" (*rho*$^-$; *rho*^{0}) strains of yeast [3, 4, 18] and it will seem that the efficiency of labelling does not depend on the rho factor [18].

To get a more detailed understanding of 5′-dTMP uptake in yeast our current study in this field is focussed onto the following problems: (i) Which are the primary biochemical effects in excess 5′-dTMP mediated (absolute or temporary) arrest of growth as observed for the strains T6-425 *tmp1-51* and T6-425, resp.? (ii) Is this phenomenon a fortuitous attribute of highly efficient 5′-dTMP utilizing potency or a necessary one in that it merely reflects extremely enhanced normal physiological events accompanying 5′-dTMP uptake and subsequent intracellular anabolism of the monophosphate? Among these might be the consumption of coenzymes especially of the nucleotide type and possible an ex-

cretion of nucleotides (*e. g.* nucleoside monophosphates) anticurrent to 5′-dTMP uptake. Such excretion is reported to occur in yeast [22, 29–31] though very little is known about the physiological reasons or its exact mechanism(s). (iii) Which is the rationale for the apparent cleavage of 5′-dTMP from exogenous source to Thd as found for 5′-dTMP utilizing yeast strains? (iv) Is the potency of yeast to utilize exogenous 5′-dTMP a mere speciality or is this organism able to utilize other nucleoside monophosphates, too? – Some preliminary data concerning these problems have already been published elsewhere [32].

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