EFFECT OF GENE DOSAGE ON TRYPTOPHAN SYNTHETASE ACTIVITY IN *SACCHAROMYCES CEREVISIAE*[1]

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A direct relation between gene dosage and amount of the corresponding protein is generally assumed to exist though it has been investigated in a limited number of cases. Yeasts seem to be an ideal material for this type of investigation as they can be easily obtained in different degrees of ploidy. Macromolecular components of yeast cells in polyploid series have been already determined (OGUR *et al.* 1952) and the relationships between gene dosage and enzyme production have been analysed for galactokinase (NELSON and DOUGLAS 1963) and iso-1-cytochrome (SLONIMSKI *et al.* 1965). Data reported here will deal with the construction of a polyploid series of *Saccharomyces cerevisiae* (haploid, diploids and tetraploids), the determination of DNA and soluble protein content on a per cell basis, and the relation of tryptophan synthetase[2] (TST) activity to ploidy and gene dosage.

MATERIALS AND METHODS

Culture media and cultural conditions: 1) YE medium: yeast extract, 10 g; peptone, 20 g; glucose, 20 g; agar, 30 g when needed; water, 1000 ml; pH 6.5–6.7. This medium was used in liquid form (without agar) for the preparation of large amounts of cells either in shake flasks (100 ml in 500 ml flasks) on an alternating shaker at 28°C, or in 5 to 20 liter fermentors at 28°C. For the biochemical determinations, cultures were harvested by centrifugation at the beginning of plateau. After washing with 0.001 M EDTA pH 6.8 containing 20 μg/ml of pyridoxal phosphate, the cells were used immediately or stored frozen at —12°C. 2) Minimal medium containing glucose, growth factors and inorganic salts: see MAGNI and VON BORSTEL (1962). 3) Sporulation medium 23 DHA: Na acetate, 2.5 g; dihydroxyacetone, 0.9 g; distilled water, 1000 ml.

Strains, crossing procedures, and genetic analysis: The strains prepared and used are reported under RESULTS. Crosses were always performed between strains carrying complementary biochemical requirements by mixing the two cultures in YE medium for 24 hrs and plating on selective medium (minimal or minimal partially supplemented). When crosses were made between strains of opposite mating types ($\alpha \times$ a)[3] the number of cells plated was very low (100–1000 cells per plate). On the other hand, when crosses were between strains not expected to have good mating capacity (a$\alpha \times \alpha$a) the inoculum was increased to 10^7–10^8 cells per plate.

Genetic analysis was performed by conventional methods, i.e. dissection of four spored asci and isolation of spores by means of a micromanipulator.

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[2] Due to the extensive usage in the literature, the term tryptophan synthetase is employed in this paper though a more correct terminology would be that of tryptophan synthase (L-serine hydro-lyase (adding indole), EC 4.2.1.20).

[3] The following abbreviations are used: a and α—mating type alleles, *ad*—adenine dependence, *ar*—arginine dependence, *hi*—histidine dependence, *le*—leucine dependence, *ly*—lysine dependence, *tr*—tryptophan dependence, TR_3—is the structural gene for tryptophan synthetase (MANNEY 1964), tr_{s-8}—is a super-suppressible mutation (HAWTHORNE and MORTIMER 1963) and most likely is a nonsense mutation (MANNEY 1964), TST—tryptophan synthetase.

Cell-free extracts: Frozen cells were thawed and suspended in 0.1 M Na-phosphate buffer pH 7.8 containing 0.05 M reduced glutathione (Sigma Chemical Co.) and 20 μg/ml of pyridoxal phosphate (Sigma Chemical Co.) (approximately 2–3 g wet weight of cells in 4.0 ml of buffer). The suspension was then subjected to sonication in an M.S.E. Ultrasonic Disintegrator for 5 min. Excess heating was avoided by immersing the cell container in an ice-acetone bath. Preliminary experiments had shown that sonication for this length of time resulted in the highest yields of tryptophan synthetase. Following ultrasonic treatment, the suspension was centrifuged at 5,000 × *g* for 10 min in a refrigerated centrifuge. The clear supernatant was used within a few minutes for the determination of enzyme activity.

Tryptophan synthetase activity: Enzyme activity was measured by the procedure of YANOFSKY (1955) modified as follows. The reaction mixture had the following composition: 0.05 M Na-phosphate buffer pH 7.8, 0.12 ml; 0.005 M indole, 0.08 ml; 0.1 M L-serine, 0.2 ml; pyridoxal phosphate, 200 μg/ml, 0.1 ml; 0.05 M reduced glutathione, 0.02 ml; cell-free preparation from 0.05 to 0.5 ml (0.3 to 12 μg of protein) and phosphate buffer-GSH-pyridoxal phosphate solution to a final volume of 1.02 ml. The cell-free preparation was added last and the reaction tubes incubated for 40 min in a water bath at 37°C. The reaction was terminated by adding 0.2 ml of 5% NaOH and cooling in the refrigerator for 10 min. Unreacted indole from the reaction mixture was extracted by shaking for 1 min with 4.0 ml of toluene. After separation of the phases, aliquots (0.5 and 1.0 ml) of the toluene solution were removed. To these were added 4.0 ml of 95% ethanol and 2.0 ml of a solution containing 36 g of *p*-dimethylaminobenzaldehyde (Carlo Erba, Italy) in 820 ml of 95% ethanol and 180 ml of concentrated HCl. The tubes were left at room temperature in the dark for 1 hr. The optical density at 540 mμ was then determined in a Coleman Spectrophotometer. Enzyme activity was expressed as units of tryptophan synthetase; one unit was defined as the amount of enzyme that converts 0.1 μmole of indole to tryptophan in 40 min at 37°C. Protein was determined according to the method of LOWRY *et al.* (1951) using as a standard crystalline egg albumin (Sigma Chemical Co.). Specific activity was expressed as units per mg of protein.

DNA content: A determined number of cells (from 0.5×10^9 to 1×10^{10}) was washed with 2.5 ml of 10% trichloroacetic acid (TCA) and suspended in 2.6 ml of 5% TCA. The suspension was heated under continuous shaking at 90°C for 15 min. After cooling, the suspension was centrifuged at 3,500 × *g* for 10 min. The supernatant was saved and the residue treated as above two times more. The combined supernatants were used for the colorimetric determination of DNA according to the method of SCHNEIDER (1957). Standard curves were prepared employing herring DNA (Fluka).

RESULTS

Preparation and genetic analysis of strains of different ploidy: The preparation of yeast strains of ploidy higher than two can be performed either by crossing clones hemi- or homozygous for their mating type alleles (e.g., $\alpha \times$ aa; $\alpha\alpha \times$ aa) and isolating the resulting polyploid zygotes with a micromanipulator or on a proper selection medium (ROMAN, PHILLIPS and SANDS 1955; LEUPOLD 1956; MORTIMER 1958).

A different technique developed by POMPER, DANIELS and MCKEE (1954) consists in culturing diploid cells heterozygous for mating types together with haploids or other diploids ($a\alpha \times \alpha$; $a\alpha \times a\alpha$) that possess complementary nutritional requirements. The rare polyploids arising in the mixed culture, by phenomena not yet clarified, can be selected on a proper medium. This procedure is much easier than the previous one and was used also for the production of pentaploid and hexaploid strains (MORTIMER 1958).

Polyploid strains produced by either of the above procedures require genetic

tests to confirm their ploidy and genotype. Other criteria such as those adopted by NELSON and DOUGLAS (1963) for the diagnosis of their tetraploids (cell size, formation of ascospores) may provide additional information but cannot substitute for genetic analysis.

The strains used in the present work were developed as follows: the haploid strain S 2684 D kindly supplied by DR. R. K. MORTIMER (*a* le_1 tr_{5-2} hi_8 ad_2 ar_7), was crossed to haploid 1475 of our collection (α *ly*) and a diploid 5253 heterozygous for all the markers was obtained.

From diploid 5253, 45 asci were dissected and analysed. The majority of asci showed 2:2 segregation for all seven markers with few exceptions probably due to gene conversion. For example, the gene tr_5 (tryptophan synthetase) segregated 2:2 in 44 tetrads and 3:1 in one tetrad. Haploid segregants with the proper genotype for further crosses were taken from tetrads where all the markers segregated 2:2. These haploid strains are listed in the first column of Table 1. The diploids listed in Table 1 were obtained from matings of two haploid strains of opposite mating type followed by selection of diploids on selective media.

The type of cross and selection used for the preparation of the above diploids left almost no doubt about their ploidy and genotypes. Nevertheless, a genetic analysis was performed on a limited number of asci (4 to 5 for each diploid) as a further check. Each diploid gave the expected segregations for every marker, i.e. $2a$:2α, 4:0 for +/+ genes, 2:2 for +/−, and 0:4 for −/− markers.

The tetraploid series was prepared by the procedure discussed above and described in MATERIALS AND METHODS. As expected, the frequency of tetraploid colonies on the selective media was very low, varying from 10^{-8} to 10^{-6}. Eleven tetraploids were isolated and used for further analysis. They are listed in Table 2. It may be noted that each cross was planned in order to allow the selection of the tetraploid and ensure the presence of at least one marker in duplex (++−−) con-

TABLE 1

Construction of diploid strains

Cross*	Code number	Diploid strains genotype						
		Mating type	*tr*	*le*	*hi*	*ad*	*ar*	*ly*
38A × 39A	3	a/α	−/−	+/−	−/−	−/+	−/−	+/−
40A × 5C	11	a/α	−/−	−/−	+/−	+/−	−/+	−/−
40A × 45B	18	a/α	−/+	−/+	+/−	+/−	−/−	−/−
40A × 23C	24	a/α	−/+	−/−	+/−	+/−	−/−	−/+
2C × 30B	23	a/α	−/+	−/+	−/−	+/−	−/−	+/+
10C × 41C	27	a/α	+/−	−/+	−/−	−/−	+/−	−/−
44A × 23C	30	a/α	+/+	+/−	−/−	−/−	−/−	−/+
10C × 1B	31	a/α	+/+	−/+	−/+	−/−	+/−	−/−

* For each haploid partner the number indicates the ascus of strain 5253 from which it has been derived and the letter one of the four spores of the ascus. The genotypes of haploid clones can be derived from that of diploids where letters (a or α) and signs (+ or −) to the left of virgules refer to the genotype of the first partner and the one to the right of virgules to the genotype of the second partner in each cross.

TABLE 2

Construction of tetraploid strains

Cross* diploid × diploid	Code number	Tetraploid strains—genotype						
		Mating type	*tr*	*le*	*hi*	*ad*	*ar*	*ly*
30 × 31	4A	*aaαα*	+ + + +	+ + — —	+ — — —	— — — —	+ — — —	+ — — —
30 × 18	4F	*aaαα*	+ + + —	+ + — —	+ — — —	+ — — —	— — — —	+ — — —
30 × 24	4G	*aaαα*	+ + + —	+ — — —	+ — — —	+ — — —	— — — —	+ + — —
31 × 24	4H	*aaαα*	+ + + —	+ — — —	+ + — —	+ — — —	+ — — —	+ — — —
3 × 31	4M	*aaαα*	+ + — —	+ + — —	+ — — —	+ — — —	+ — — —	+ — — —
30 × 11	4N	*aaαα*	+ + — —	+ — — —	+ — — —	+ — — —	+ — — —	+ — — —
18 × 27	40	*aaαα*	+ + — —	+ + — —	+ — — —	+ — — —	+ — — —	— — — —
23 × 14	4U	*aaαα*	+ — — —	+ — — —	+ — — —	+ + — —	+ — — —	+ + — —
3 × 24	4T	*aaαα*	+ — — —	+ — — —	+ — — —	+ + — —	— — — —	+ + — —
18 × 3	4S	*aaαα*	+ — — —	+ + — —	+ — — —	+ + — —	— — — —	+ — — —
3 × 11	4Z	*aaαα*	— — — —	+ — — —	+ — — —	+ + — —	+ — — —	+ — — —

* See Table 1.

dition. Genes in simplex (+———) or triplex (+++—) constitution do not permit diagnosis of ploidy because they segregate 2:2 and 4:0, respectively. Genetic markers in duplex constitution, on the other hand, give 4:0, 3:1 and 2:2 segregations. The relative proportions of these segregation classes vary with the distance of the gene from its centromere (ROMAN, PHILLIPS and SANDS 1955).

Since the scope of this phase of our analysis was only to confirm the ploidy of our strains, the ratio between the three types of segregation was not investigated in detail, but for each strain a number of asci was dissected in order to obtain for one ++—— marker at least one ascus of each type: 4:0, 3:1, and 2:2. This happened for all the tetraploids within a maximum of eleven asci.

The spore survival was always good thus excluding the possibility that our polyploids were triploids. The subsequent genetic analysis of locus TR_5 ruled out also the possibility of pentaploidy.

The TR_5 locus was more carefully analysed because the main purpose of the present work was to study the relationship between gene dosage and tryptophan synthetase activity which is controlled by this gene (MANNEY 1964). For every tetraploid except 4Z ($tr_5\ tr_5\ tr_5\ tr_5$) an ascus was chosen which gave four diploid segregants heterozygous for the mating type alleles (a/α). These four segregants were then analysed genetically in each case. The results are given in Table 3.

The expected genetic constitution at the TR_5 locus of all the tetraploids was confirmed.

Macromolecular components of yeast cells of different ploidy: The development of polyploid series of yeast has made it possible to correlate ploidy with different cellular parameters such as volume, dry weight, and content of macromolecular components (RNA and DNA). The volume of fully grown cells is strictly proportional to ploidy (MORTIMER 1958; LASKOWSKI 1960) in polyploid series from haploid to hexaploid. Dry weight and RNA content show a good correlation with ploidy (OGUR *et al.* 1952), in spite of some difficulties related to the estimation of cell number in a growing culture due to the presence of buds and of cells which

TABLE 3

Genetic analysis of diploid segregants from tetraploid strains

Tetraploid	Expected genotype for gene tr_5	Segregation for *tr*	Genotype of the four diploid segregants			
			A	B	C	D
4A	++++	4:0	++	++	++	++
4G	+++—	4:0	++	+—	+—	++
4H	+++—	4:0	+—	+—	++	++
4F	+++—	4:0	++	+—	+—	++
4M	++——	3:1	++	+—	+—	——
4O	++——	3:1	+—	——	++	+—
4P	++——	4:0	+—	+—	+—	+—
4U	+———	2:2	+—	——	+—	——
4T	+———	2:2	+—	+—	——	——
4S	+———	2:2	+—	+—	——	——

have not yet reached their maximum size. WILLIAMSON and SCOPE (1961) were able to demonstrate that "small" and "large" cells of the same culture have a dry mass, protein and RNA content proportional to their volumes. On the contrary, DNA is the most constant component; its content per cell is independent of cell size for a culture of a given ploidy (WILLIAMSON and SCOPE 1961), indicating that, at the time of appearance of a well distinguishable bud, DNA duplication has already taken place (OGUR *et al.* 1952; WILLIAMSON 1964). The amount of DNA per cell is proportional to ploidy (OGUR *et al.* 1952 and Table 4) and also the absolute estimates obtained by different methods are in very good agreement. The haploid complement of DNA calculated from DNA phosphorus data (OGUR *et al.* 1952) assuming the known A+T/G+C ratio of 1.80 (CHARGAFF 1955) is 2.06 $\mu g/10^8$ cells; the amount inferred from diploid cultures (WILLIAMSON and SCOPE 1961) is 2.30 $\mu g/10^8$ cells and our estimate (Table 4) is 2.52 $\mu g/10^8$ cells. We can therefore conclude that the DNA content of haploid cells of *S. cerevisiae* for cultures which have reached plateau is close to 2.30 $\mu g/10^8$ cells. In addition, the DNA content per cell increases in proportion to ploidy.

For studies on enzymatic activity another important parameter to be correlated with ploidy is the amount of soluble proteins present in cell-free extracts. The data reported in Table 4 show a strict proportionality between soluble protein content and ploidy. Of course, they do not give the absolute amount of soluble proteins present in the cell but only that fraction recovered with our methods of cell disruption which consistently gave the same percent breakage independently of ploidy. No significant variation was observed between strains of the same ploidy grown in identical media. It can therefore be concluded that titration of enzymatic activity can be done either on a per cell basis or on a per protein basis.

In our experiments determination of DNA and soluble protein was always performed on: a) an equal number of cells of different ploidy with the results shown in Table 4; and b) on a number of cells inversely proportional to ploidy i.e. 4:2:1 for haploid, diploid and tetraploid strains, respectively. In the second procedure, a constant amount of DNA and protein per sample was obtained.

Tryptophan synthetase activity: Before investigating the existence of a correlation between gene dosage and content in a specific enzyme, it was necessary to establish if the enzyme to be assayed was subjected to control mechanisms. If the synthesis of the enzyme were under regulatory control, variation in the enzyme

TABLE 4

DNA and soluble protein in strains of different ploidy of Saccharomyces cerevisiae

	Haploid*	Diploid**	Tetraploid***
DNA $\mu g/10^8$ cells	2.52 ± 0.12	4.62 ± 0.13	8.71 ± 0.29
Soluble protein mg/10^8 cells	.237 ± .019	.571 ± .087	1.250 ± .132

* Average of 6 haploid strains of Table 1.
** Average of 6 diploid strains of Table 2.
*** Average of 6 tetraploid strains of Table 3.
± Standard error.

TABLE 5

Effect of the composition of growth medium on tryptophan synthetase (TST) activity of cell-free extracts

Medium	Specific activity, units of TST/mg of protein
Minimal	2.33
Minimal + L-tryptophan (100 μg/ml)	2.39
Yeast extract (complete)	2.29

activity in the cell-free extracts should be observed when the cells were grown under different cultural conditions. Therefore, tryptophan synthetase activity was determined on cells grown in minimal, minimal plus tryptophan and complete (yeast extract) medium. As the data of Table 5 show, the specific activity of tryptophan synthetase is approximately the same in cell-free extracts prepared from cells grown in all the tested media. Similar results were recently reported for the tryptophan synthetase of the callus tissue of *Nicotiana tabacum* grown *in vitro* (DELMER and MILLS 1968). It may be added that, in our experiments, no significant difference was observed in the amount of tryptophan synthetase or soluble protein per unit of volume of the cell-free extracts from cells grown in different media. Therefore tryptophan synthetase activity was always determined on cells grown in yeast extract medium.

The first series of determinations was carried out on strains with full complement of TR_5 alleles, i.e. haploids 45B, 23C, 10C (TR_5), diploids 30 and 31 (TR_5 TR_5), and the tetraploid 4A (TR_5 TR_5 TR_5 TR_5). The enzyme activity was assayed on a per cell basis. In one set of experiments the extracts were derived from a constant number of cells (2.5×10^9); in a second set the number of cells extracted was inversely proportional to ploidy (1×10^{10} for haploids, 5×10^9 for diploids, and 2.5×10^9 for the tetraploid). The mean values of these assays as well as the specific activities are summarized in Figure 1. Enzyme activity shows a strict proportionality to ploidy. The activity per cell extrapolates to the origin and, as expected from our previous findings of soluble proteins per cell, specific activity is constant for all ploidies with an average value of 0.9 TST units per mg of protein.

Another set of experiments was performed on strains with different gene dosages. Strains 39A (tr_5), 11 (tr_5 tr_5) and 4Z (tr_5 tr_5 tr_5 tr_5) did not show any activity, at least within the limit of our method. Quantitative determinations were carried out on all the tetraploids strains described in Table 2 and on a selected number of heterozygous diploids (strains 18, 23 and 28). The mean specific activities for each gene dosage are plotted in Figure 2. Proportionality between specific activity and gene dosage is excellent.

DISCUSSION AND CONCLUSION

Two inducible enzymes have so far been studied in relation to ploidy and gene dosage. SLONIMSKI *et al.* (1965) analysed iso-1-cytochrome *c* in fully induced

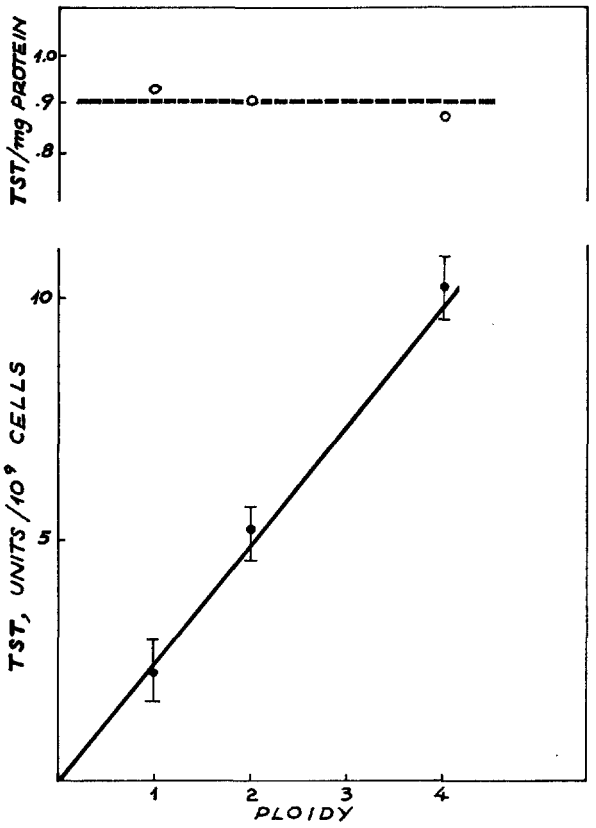

FIGURE 1.—Content and specific activity of tryptophan synthetase (TST) in haploid, diploid, and tetraploid strains of *S. cerevisiae*.

clones of yeast carrying different dosages of CY_1 and cy_1 alleles at the structural gene for iso-1-cytochrome. The specific activity of purified extracts showed a good proportionality to CY_1 alleles in diploid strains of composition CY_1 CY_1, CY_1 cy_1, cy_1 cy_1, in two triploids (CY_1 CY_1 cy_1 and CY_1 cy_1 cy_1) and in one tetraploid (CY_1 cy_1 cy_1 cy_1). On the other hand, iso-2-cytochrome *c* was not affected by CY_1 dosage.

The amount of galactokinase in cell-free extracts of a tetraploid series with different doses of the active GA_1 allele was determined by NELSON and DOUGLAS (1963). The specific activity of the enzyme increases with the number of GA_1 alleles but not in a strictly proportional way. The linear regression of the plot of enzyme activity against gene dosage does not extrapolate to zero for a zero GA_1 dose (ga_1 ga_1 ga_1 ga_1) but to a value approximately 2/3 of that obtained with one GA_1 allele.

To our knowledge so far no data have been presented on a gene dosage/protein amount relationship for enzymes which are not under conventional regulatory control. The data here reported for tryptophan synthetase, whose amount does

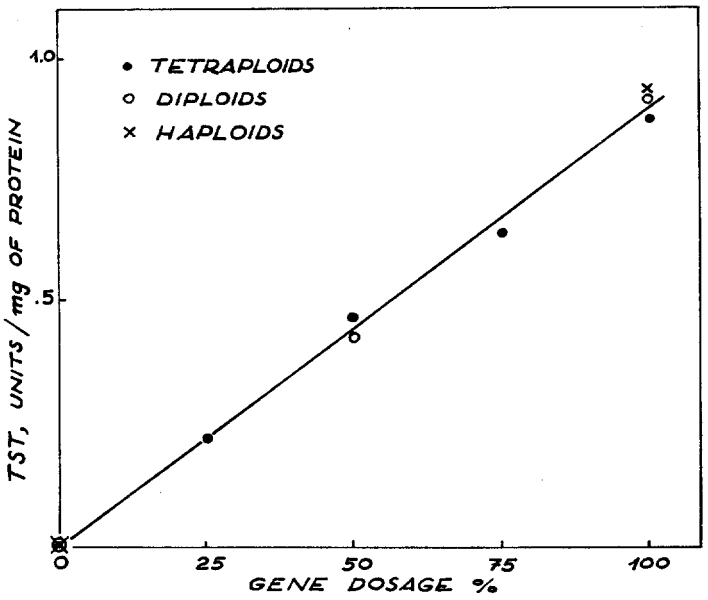

FIGURE 2.—Specific activity of tryptophan synthetase (TST) in haploid, diploid, and tetraploid strains of *S. cerevisiae* carrying different dosage of the TR_5 gene.

not vary under different cultural conditions and hence seems not to be under control, show that the proportionality between gene dosage and enzyme content on a per cell basis is strictly linear.

The determination of DNA content per cell performed with the aim of implementing the information on the macromolecular components of a polyploid series confirms OGUR's data (OGUR *et al.* 1962) and establishes unequivocally for this organism the direct proportionality between DNA content and ploidy.

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SUMMARY

Diploid and tetraploid strains of *Saccharomyces cerevisiae* carrying different dosages of the TR_5 gene (tryptophan synthetase TST) have been constructed. DNA, soluble protein and TST were determined on a per cell basis in all possible gene combinations in haploid, diploid and tetraploid strains. In all cases a strict proportionality between these macromolecular components and ploidy was observed. TST activity was also proportional to the dosage of the active gene.

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