THE ISOLATION OF ADENYLOSUCCINATE SYNTHETASE MUTANTS IN YEAST BY SELECTION FOR CONSTITUTIVE BEHAVIOR IN PIGMENTED STRAINS*

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ROMAN's (1956) selective system for the isolation of yeast mutants blocked in early steps of purine biosynthesis (for review see MAGASANIK 1962) was based on the red color of aerobic *ade1* and *ade2* cells. His method led to the identification of the genetic loci *ade3* through *ade8* and stimulated a number of investigations which (1) exploit the availability of many independent alleles at specific loci (DORFMAN 1964a; JONES 1964; ESPOSITO 1968), (2) are concerned with mutagenesis (MARQUARDT, VON LAER, and ZIMMERMANN 1966), or (3) depend on a system that signals rare recombinational events (FOGEL and HURST 1963). Recent studies explained the nature of the simultaneous requirement for both adenine and histidine imparted by the *ade3* mutation (JONES and MAGASANIK 1967; MAZLEN 1968) and identified the pathway steps associated with several known adenineless loci (SILVER 1968).

The red *ade1* and *ade2* cells can serve as a starting point toward the genetic analysis of purine pathway regulation. Since the presence of adenine in the medium ordinarily inhibits the formation of red pigment, purine constitutive mutants should be distinguishable by their differential red color on adenine-rich medium. In haploid yeasts, colony selection by this criterion, after mutagenic treatment with EMS, has systematically yielded a class of mutants, designated *ade12*, which are blocked at the penultimate step of AMP[1] biosynthesis, specifying the enzyme adenylosuccinate synthetase. The *ade12* locus is homologous in biosynthetic function to the *ad-8* locus of *Neurospora crassa* (PARTRIDGE and GILES 1957), but the yeast mutants differ in at least two respects. They are involved in the regulation of purine pathway activity, the property by which they were identified, and their capacity to utilize exogenously supplied adenine is relatively reduced. Previous work on the regulation of purine biosynthesis in yeast has established that feedback inhibition *in vivo* is responsive to intracellular levels of IMP (BURNS 1964). Moreover, purine nucleosides have been shown to repress several specific pathway enzymes in *Bacillus subtilis* (NISHIKAWA,

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[1] *Abbreviations Used:* AMP, adenosine monophosphate; AIR, 5-amino-4-imidazole ribonucleotide; EMS, ethyl methanesulfonate; IMP, inosine monophosphate; IDP, inosine diphosphate; ITP, inosine triphosphate; SAICAR, 5-amino-4-imidazole (N-succinylo-carboxamide) ribotide; AICAR, 5-amino-4-imidazole-carboxamide ribotide; AMPS, adenylosuccinic acid; FGAR, formylglycinamide ribotide.

MOMOSE, and SHIIO 1967). The study of mutations to constitutive activity should contribute to an understanding of the genetic mechanism controlling purine regulation. In the present case, the occurrence of constitutivity with the simultaneous loss of a biosynthetic pathway step presents the interesting possibility that the protein product of the affected locus functions both as repressor and as an enzyme.

The genetic analysis of adenine specific mutations was extended in this study to include the final reaction step in the biosynthetic sequence by the auxotrophic isolation of a mutant, *ade13*, lacking adenylosuccinase activity.

MATERIALS AND METHODS

Stocks and media: Mutations at the loci *ade12* and *ade13* were generated in several strains of *Saccharomyces cerevisiae*. Those described in this paper originated in M41, α, *ade2–1*, obtained from Dr. T. R. MANNEY as XT300 3A, and in a strain received from Dr. M. ESPOSITO as I-17A, a, *lys2*, *try5*, *ura1*, and serially converted by single mutagenic steps to E1813, a, *lys2*, *ura1*, *ade1*, *can*. All other loci are described in the report of the Carbondale Yeast Genetics Conference (1963). The symbols a and α, *ade*, *his*, *leu*, *lys*, *try*, and *ura*, respectively, denote mating type and recessive requirements for adenine, histidine, leucine, lysine, tryptophan, and uracil; *can* denotes recessive resistance to canavanine. Synthetic minimal medium (MM) was prepared from amino acid-free Difco yeast nitrogen base, 6.7 g/l, and glucose, 20 g/l. Complete medium (SC) contained the following supplements to MM, in μg/ml: adenine sulfate (referred to hereafter as adenine), 20; L-histidine HCl, 10; L-leucine, 60; L-lysine HCl, 60; L-tryptophan, 10; uracil, 10. In addition to the drop out series (SAD, adenineless, etc.) for testing individual requirements, adenine of SC was replaced by hypoxanthine, 20 μg/ml (SHX), to distinguish blocks preceding IMP formation from those which follow. Canavanine resistance was tested by adding 60 μg/ml L-canavanine sulfate to SC, as required (SCAN). In tests for color discrimination supplements to MM were provided in an empirically determined mixture (GBHA) containing glucose, 50 g/l and the following, in μg/ml: adenine, 75; L-histidine HCl, 5; L-leucine, 60; L-isoleucine, 60; L-lysine HCl, 60; L-tryptophan, 30; uracil, 20; L-arginine HCl, 10; L-methionine, 30; L-aspartic acid, 10; glycine, 10; L-proline, 40; L-serine, 20; and L-phenylalanine, 50. When only the presence or absence of purine was at issue, MM was enriched with 5 g/l Difco casein hydrolysate, either without adenine (MC), with 10 μg/ml adenine (MCJ), or with 50 μg/ml adenine (MCA). Ingredients of other media, per liter: YEP, yeast extract 10 g, peptone 20 g, glucose 20 g; for pre-sporulation: GNA, Difco nutrient broth 8 g, yeast extract 3 g, glucose 100 g; for sporulation: AYDAX, potassium acetate 9.8 g, yeast extract 2.5 g, glucose 1 g, adenine 50 mg, L-histidine HCl 20 mg, L-lysine HCl 60 mg, and xanthine 20 mg. Solid media contained Difco agar, 15 g/l and Tween 20, 0.5 ml/l.

Growth Studies: Cell densities were estimated at 600 nm with a Coleman, Jr. spectrophotometer in disposable 16 $\times$ 125 mm glass test tubes selected for optical equivalence to $\pm$ 0.002 O.D. For each genotype populations were grown in MM + 20 μg/ml adenine for 2 days at 30°C in a New Brunswick G25 incubator-shaker. Aliquots of cells were collected, washed by centrifugation with the respective media in which they were to be incubated, and distributed into 10 ml volumes in pairs of duplicate tubes of MM supplemented with adenine at 0, 2, 5, 10, 20, 40, 80, and 120 μg/ml. Inoculum densities ranged from 0.8×10^6 to 9.8×10^6 cells/ml. The tubes were rotated at 50 rpm in a near-horizontal position, at 28°C. In a few cases reversions were detected at the loci *ade1*, *ade12*, or *ade13*, in the routine tests performed for this purpose on samples of the growth-tube populations. The data from the affected tubes were discarded.

Enzyme assay: Adenylosuccinase activity was determined by the decrease in optical density at 280 nm when extracts were incubated at 37°C with AMPS by the method of FOLEY, GILES, and ROBERTS (1965). The AMPS used was a gift of Dr. C. W. H. PARTRIDGE. Cells were grown in YEP for 48 hrs, transferred for 4 hrs to adenineless SC (in which L-arginine and L-methionine were replaced by L-aspartic acid and L-glutamic acid, 10 μg/ml each), harvested by centrifugation,

washed with 0.005 M potassium phosphate buffer, pH 7.2, and rapidly frozen. The pellets were ground in a mortar at 0°C with 0.2 mm glass beads. After the debris was removed by centrifugation, the supernatant extracts were made 1% in streptomycin sulfate and cleared by centrifugation at 2,000 × *g* for 15 min. The recovery of soluble protein, by biuret determination (GORNALL, BARDAWILL, and DAVID 1949), was between 5.5 and 8.4 mg protein/g wet weight. The assay mixture contained 50 μmoles Tris (hydroxymethyl) aminomethane buffer, pH 8.0, 48 nmoles AMPS, and 0.1 ml cell extract in a volume of 1 ml.

Chromatography: Details are given in footnotes to Table 1. Protein-free yeast extracts were prepared by adding to the cells an amount of chloroform-methanol (2:1 v/v) equal to 1.5 times the volume of the washed cell pellets. The mixture was stirred vigorously on a Vortex mixer, allowed to stand at room temperature for 5 min, then centrifuged at 500 × *g* for 10 min. The clear supernatant water-methanol layer was decanted, and samples applied to paper, either directly, or after concentration by lyophilization.

Identification of arylamines: AIR was assayed by the BRATTON-MARSHALL procedure of LEVENBERG and BUCHANAN (1957), and SAICAR according to LUKENS and BUCHANAN (1959).

RESULTS

Isolation of mutants: Color formation in cultures of *ade1* or *ade2* yeasts is directly affected by environmental factors, such as aeration, glucose concentration and the variety and amount of amino acids and purines available in the medium. Anaerobic cultures and most petites ordinarily remain white for the first several days, or show only a slight color after long incubation. Aerobic cultures supplied with enough adenine or hypoxanthine (10–20 μg/ml) to approach rapid growth rates turn red earlier and become darker in proportion to the glucose content in the range between 1% and 8%. When the adenine concentration was raised to 75 μg/ml in synthetic media, such as GBHA, or 100 μg/ml in YEP, the amount of the purine intermediate, AIR, available as the starting point for pigment formation was sufficiently low so that *ade1* or *ade2* colonies were only faintly colored, if at all, after two days. The conversion of AIR to pigment (SMIRNOV, SMIRNOV, BUDOWSKY, INGE-VECHTOMOV, and SEREBRJAKOV 1967) is presumably dependent on protein metabolism, and hence is sensitive to the amino acid composition of the medium. For example, if arginine, or both serine and proline were omitted from GBHA medium, color formation in most genetically red strains was enhanced. In the other direction, leucineless or tryptophanless auxotrophs produced less pigment when leucine or tryptophan, respectively, were provided at growth-limiting levels. Similar observations in homozygous diploid red strains enabled FOGEL (personal communication) to distinguish the darker post-mitotic conversion or crossover heterozygotes for arginine, leucine, threonine, or tryptophan auxotrophy from the lighter color homozygous recessive parentals, or to specify by color which one of a given heteroallelic pair of *his1* mutants had undergone conversion to prototrophy.

Red cells are not uniformly colored. The pigment was microscopically observed in single cells from unusually dark cultures only. The red or pinkish color was seen to be localized in the large vacuoles of the individual cells, while the surrounding cytoplasm had the usual colorless to greenish tint. Internal secretion of excess metabolites serves here as a substitute for excretion from the cell, and suggests the possibility that the pigment may be a non-enzymatic oxidation

product (W. S. CHILTON, personal communication) of imidazoles combined with protein fragments in the vacuole.

The sensitivity of color development in *ade1* or *ade2* yeasts to exogenous concentrations of adenine is consistent with a mechanism of genetic regulation that is responsive to intracellular concentrations of purine nucleotides. The mutational loss of normal regulatory function should yield constitutive mutants that would be distinguishable by their early red color in the presence of high adenine concentration at a time when normal *ade1* or *ade2* colonies remain white.

Haploid cells, *a ade1* or *α ade2*, were treated for mutagenesis with 3% EMS for 45 min and plated on YEP medium containing 5% glucose and 100 μg/ml each of adenine and guanine. Colonies which turned red before most of the others, "precocious reds," were removed for further tests. The remaining colonies were replica plated to synthetic media for the isolation of additional classes of purine mutations. Adenine-specific mutants grew on adenine but not on hypoxanthine, while guanine auxotrophs (unpublished data) failed to grow in the absence of guanine.

Several mutants which showed precocious development of color in the presence of adenine were outcrossed for genetic analysis. Routine tests of the progeny for growth response to purines revealed that the factor conferring precocious color also imposed an adenine requirement that was not satisfied by hypoxanthine. In complementation tests against all the known adenineless loci, *ade1* through *ade9*, which do utilize hypoxanthine for growth, the heterozygous diploids uniformly grew on minimal medium, indicating that with respect to its adenine requirement the precocity factor constituted a new recessive locus in the purine biosynthetic pathway. The symbol *ade12* was assigned to this locus. Subsequent phenotypic assay for the *ade12* mutation in progeny from crosses was based on either adenine-specific auxotrophy, the expression of red color on a high glucose-high adenine medium, such as GBHA (whenever *ade1* or *ade2* was also present), or the failure to complement appropriately chosen *ade12* tester strains after transfer to adenineless medium. Initial attempts to segregate *ade12* mutations from the background *ade1* or *ade2* markers by genetic recombination were frustrated by the fact that *ade12*$^+$ recombinant spores rarely germinated, and then often produced weak, perishable colonies. In the course of many dissections, however, some *ade12*$^+$ ascospore cultures of a few independent *ade12* alleles did survive, and the growth characteristics of a relatively vigorous one of these is described in the following section.

Effect of adenine concentration on cell yield: In the course of growing yeast populations in liquid media, it became apparent that *ade12* adversely affected the cells' growth potential. Growth rates and final yields are both lower for *ade12* cells than for cells blocked at other steps in the purine pathway. In pilot kinetic studies, populations of *ade1*, *ade5/7*, and *ade8* cells showed a linear increase with time at limiting concentrations of adenine, such as 2–5 μg/ml, while growth was logarithmic at higher concentrations of adenine. In contrast, the *ade12* strain, AW217/2A, retained the linear pattern of increase at supplements as high as 20–40 μg/ml of adenine, attaining logarithmic increase for part of the time span

under observation only at 80–120 μg/ml. Since the heterogeneous growth rate patterns are not straightforwardly reducible to exact doubling times, the genotypic distinctions are illustrated by analysis of the cell yields obtained after a fixed interval, 22 hrs of incubation at 28°C, with various levels of adenine. The respective multiples of increment above inoculum size for each of several genotypes are plotted in Figure 1 as fractions of the expected wild-type yield at each specific inoculum size employed. The wild-type standard, A2Y124/18C, has no auxotrophic markers, and its growth rate and yield are unaffected by adenine supplement in minimal medium within the range tested. Its net increase varied from 43.1 fold at an inoculum of 1.7×10^6 cells/ml to 9.4 fold at 8.7×10^6 cells/ml. Yeasts blocked early in adenine biosynthesis, *ade2*, *ade5/7*, and *ade8* have a pattern of yield ratios in response to available adenine similar to that of the late-blocked adenylosuccinase mutant, *ade13* (see below). The corresponding curve for *ade12* parallels the others but is shifted toward the higher concentrations. Adenine seems to be approximately one-eighth as efficient in promoting the growth of *ade12* as that of the other adenineless genotypes. A limit to the absolute yield of *ade12* at 65% of wild type is also suggested in the range of 180–200 μg/ml, although the present data go no higher than 120 μg/ml.

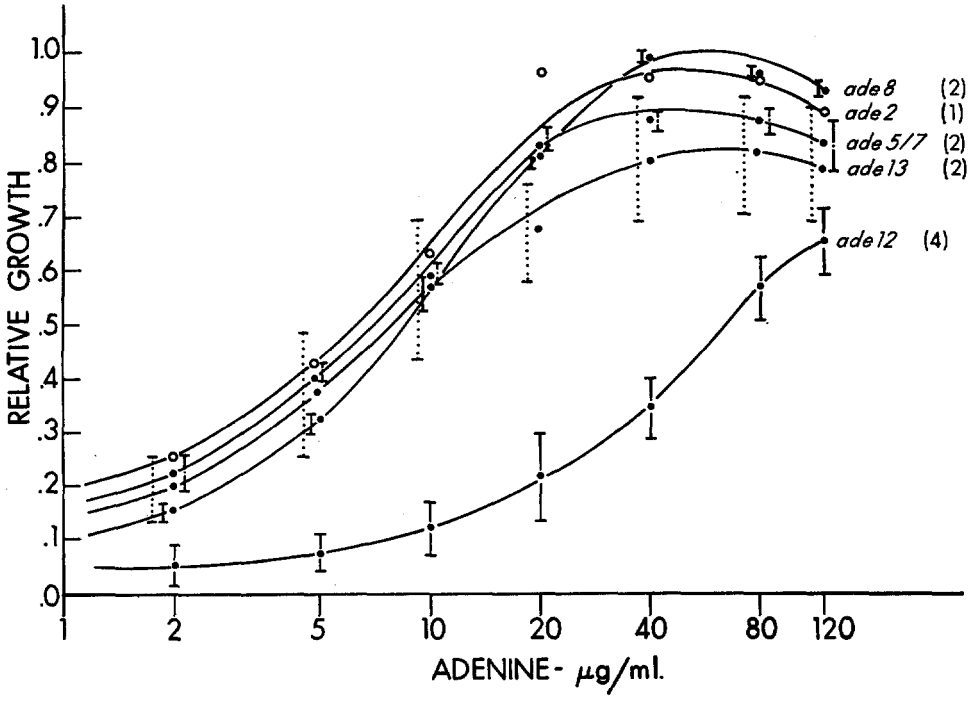

FIGURE 1.—Growth of adenineless yeast in MM as a function of exogenous adenine. Relative yield-ratios are shown as $(N_f - N_0/N_0)_{experimental}/(N_f - N_0/N_0)_{wild\ type}$, where N_0 = inoculum density and N_f = density after growth at 28° for 22 hrs. Wild-type yields were averaged for all adenine sulfate concentrations, and plotted to produce an inoculum *vs.* yield-ratio curve to which experimental values were normalized with respect to inoculum size. The number of independent tests of each genotype is shown in brackets.

Genetic analysis: The *ade12* mutation segregates in heterozygous diploids as a single recessive gene, 2 *ade12* : 2+ in each ascus. The two independent properties associated with the locus, i.e., 1) precocious color in genetically red cultures and 2) adenine-specific auxotrophy, have remained completely associated in the spores of over 535 asci dissected from 45 crosses. For most of the *ade12* alleles available, color intensity on synthetic media 5% in glucose and 75 μg/ml in adenine is a sufficient criterion by which to distinguish red *ade1 ade12* or *ade2 ade12* colonies from the corresponding pink *ade1*$^+$ or *ade2*$^+$ clones grown from random plated spores. This selection technique has been applied, in addition to ascus dissection, to the transfer of *ade12* alleles through appropriate crosses from *ade1* parental genotypes to progeny carrying *ade2* as the only red marker, and vice versa. No specific attempt was made to map the *ade12* locus, but the tetrad types obtained from the dissection of various crosses including several *ade12* alleles have been summarized in Table 1. In order to avoid any bias that might result from *ade12* spore lethality, *ade2* was made homozygous in crosses testing linkage of *ade12* to *ade1*. Conversely, *ade1* was homozygous in *ade12 ade2* linkage tests. Since both *ade1* and *ade2* were present in the crosses to other *ade* loci, homozygosity for any one was unnecessary. There is no apparent linkage of *ade12* to any other *ade* locus, nor to any of the markers listed in Table 1. It can also be concluded that *ade12* is not centromere-linked.

TABLE 1

Pooled ascus-type data from crosses with ade12 *or* ade13 *alleles*

	ade12			*ade13*		
	PD*	NPD	T	PD	NPD	T
ade1	13	8	49	0	2	3
ade2	20	21	80	3	1	3
ade4	2	3	8	..	..	..
ade6	1	3	5	..	..	..
ade7	2	6	8	..	..	..
ade8	0	3	6	..	..	..
ade12	..	..	..	1	1	5
ade13	1	1	5	..	..	..
α	38	47	153	2	1	8
arg6	..	..	..	5	3	13
can	4	5	14	3	1	3
his1	11	13	37	10	2	7
his3	15	10	51	1	0	4
leu1	11	14	58	1	2	4
lys2	4	3	12	1	1	3
lys5	15	17	66	..	..	..
try1	16	12	64	..	..	..
try2	11	17	56	0	4	5
try5	2	2	10	..	..	..
ura1	12	14	67	1	1	3
ura3	..	..	..	1	1	18

* PD = parental ditype ascus; NPD = nonparental ditype; T = tetratype.

Identification of the function controlled by the ade12 *locus:* Although it was initially isolated on the basis of a phenotype expected to be characteristic of regulatory constitutive mutants, the *ade12* mutation itself imparts a requirement for adenine. Auxanographic tests showed that other purines such as hypoxanthine, xanthine, and guanine did not support growth, nor did the respective ribosides or ribotides, including adenosine and adenylic acid. The nutritional pattern suggests that the locus specifies one of the two enzymes responsible for the conversion of IMP to AMP, adenylosuccinate synthetase or adenylosuccinase. However, evidence is presented below that adenylosuccinase is specified by another locus, *ade13*, which complements *ade12* mutants and is unlinked to them.

The accumulation product characteristic of the *ade12* block was identified by chromatography of the water layer from chloroform-methanol extracts of AW217/2A, grown for two days in MCA followed by transfer to MC for 4 hrs. The R_f values of the pertinent UV absorbing spots are shown in Table 2. The bands identified as inosine in the extracts were eluted with 0.1 N HCl and cochromatographed with inosine, migrating without separation in each of the solvents of Table 2. It is clear, therefore, that inosine accumulates in *ade12*-blocked cells. Although purine biosynthesis proceeds at the ribotide level *in vivo*, IMP was not identified in the extracts, presumably because of phosphatase activity. A similar cleavage of ribotides to ribosides has been observed repeatedly to occur with imidazole compounds accumulated in adenineless strains of Neurospora (BERNSTEIN 1961), yeast (DORFMAN 1964b; SILVER 1968), and *B. subtilis* (DEMAIN and HENDLIN 1967).

The typical failure of recombinant *ade12*$^+$ spores to germinate suggests that a high endogenous concentration of IMP or its derivatives is toxic to yeast cells, at least in the spore condition. This may explain why adenylosuccinate synthetase mutants have generally not been recovered in mutagenesis studies with haploid *ade*$^+$ yeasts. Part of the excess inosine is excreted from *ade12* cells as hypoxanthine (R. A. WOODS, personal communication). The presence of hypoxanthine as a specific excretion product of *ade12* mutants, is illustrated by an experiment shown in Figures 2 and 3. Wild-type cells were grown in MC, and

TABLE 2

R_f of UV-absorbing spots from extracts of ad12 *cells*

Strain or substance	Whatman paper	A*	B	BuA	W
A1816D *ade12–1*	1	.40	.61	. .	.75
Inosine	1	.43	.65	. .	.75
Hypoxanthine	1	.44	.66	. .	.67
AW217/2A *ade12–217*	3 MM	.32 (4)†	.57 (4)	.34 (7)	. .
Inosine	3 MM	.37 (6)	.63 (10)	.33 (6)	. .

* Developing Solvents: A and B, isopropanol, water, conc. aq. ammonia (sp. gr. 0.880). A, 70:40:10; B, 70:20:10 (BERNSTEIN 1961). BuA, n-butanol, glacial acetic acid, water, 120:30:50. W, distilled water. 40 × 28 cm sheets were stapled into cylinders and developed in glass-covered 6 × 12 inch chromatography jars.

† The number of independent tests which were averaged is shown in brackets.

ade12 cells in MC + 10 μg/ml adenine, for two days. The respective media were filter sterilized and diluted with equal volumes of fresh MC, supplemented with hypoxanthine at final concentrations of 0, 2, 4, 6, and 8 μg/ml. Growth of *ade8* cells in medium depleted by wild-type cells (Figure 2), is approximately proportional to the added hypoxanthine. This growth is exceeded by the same culture of *ade8* regardless of hypoxanthine supplement, in medium depleted by *ade12* cells, indicating that during their growth the *ade12* cells had excreted hypoxanthine in excess of 8 μg/ml. Cells dependent specifically on adenine, *ade13*, do not grow on *ade12*-depleted medium (Figure 3, curve A), indicating that the adenine initially supplied was effectively exhausted by the *ade12* cells during growth. The possibility that metabolic inhibitors were present is excluded by the growth pattern shown in Figure 3, curves B. These compare the growth of wild-type yeasts in parallel samples of fresh medium diluted respectively with an equal volume of water or with media depleted by growth of wild-type or *ade12* cells. No differences are evident.

The auxotrophic specificity and the observed accumulation and excretion products consistently associate the *ade12* locus with control of the enzyme adenyl-

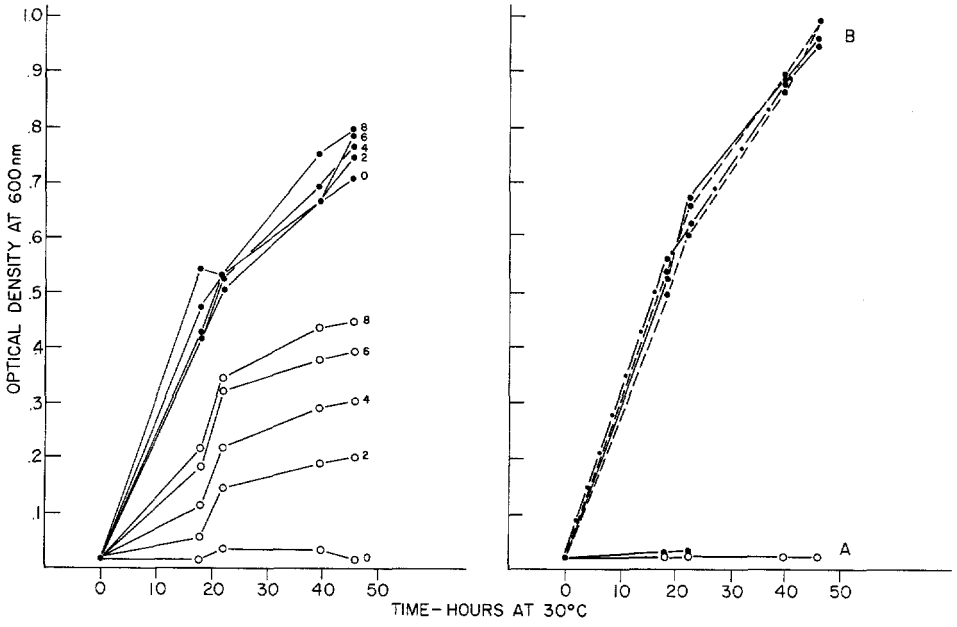

FIGURE 2.—*Left.* Growth of *ade8* cells in MCJ medium depleted by previous growth of wild type (open circles) and *ade12* (closed circles). The depleted media were filter sterilized and mixed with an equal volume of fresh MC. Numerals show final concentrations, in μg/ml, of hypoxanthine added with the *ade8* cells.

FIGURE 3.—*Right. A.*: Growth of *ade13* cells in MCJ depleted by previous growth of wild type (open circles) and *ade12* (closed circles) filter sterilized and mixed with equal volumes of fresh MC. *B.*: Growth of wild-type cells in MC diluted with an equal volume of water (– – –), MCJ depleted by WT cells (–.–.–), MCJ depleted by *ade12* cells (——).

osuccinate synthetase. We may enquire why such mutants should diminish the restraints normally exerted by exogeneous adenine on purine biosynthesis. If the imidazole molecules channeled into pigment formation in *ade1 ade12* cells had their origin in the degradation of excess IMP (presumably derived from GMP deamination and/or salvage of AICAR released in histidine biosynthesis) via the catabolic pathway known in Clostridium (RADINOWITZ 1956), while *de novo* purine synthesis were limited by normal regulation, intracellular levels of AIR, and pigment development, should be independent of the presence or absence of genetic blocks at loci preceding AIR synthetase. Triply blocked adenineless strains, marked with *ade1 ade12* and also at one of *ade4*, *ade5*, *ade6*, *ade7*, or *ade8* were constructed by recombination. When these were tested for intracellular AIR after incubation, with or without adenine, the results were uniformly negative. The cells, moreover, were white in all culture conditions. Thus, as no purely catabolic AIR was detectable, AIR and pigment measured in this and other experiments must have been produced by the *de novo* pathway, exclusively. Inosine accumulation via salvage pathways in *ade1 ade12* double mutants would not be extensive in any case. Experimentally, neither inosine nor IMP was detectable by chromatography of *ade1 ade12* extracts.

Identification of the function controlled by the ade13 *locus:* In chicken liver and yeast (MILLER, LUKENS, and BUCHANAN 1959), and in Neurospora (GILES, PARTRIDGE, and NELSON 1957), adenylosuccinase catalyzes two distinct but parallel reactions in which fumarate is cleaved from SAICAR and AMPS. A mutation at the relevant locus in yeast would result in an adenine-specific auxotrophy accompanied by accumulation of the earlier intermediate, SAICAR. Several adenine-specific mutants obtained in the mutant search described above were crossed to remove the *ade1* marker. The progeny asci of mutant E2322 regularly segregated an adenine-specific gene independent of the *ade1* marker in the parental haploid. Clonal populations from a white adenineless progeny spore, A473/2A, yielded cell extracts which gave a positive reaction in the specific Bratton-Marshall procedure for SAICAR (LUKENS and BUCHANAN 1959), producing a chromophore with a spectral peak at 570 nm. Finally, crude protein extracts of *ade13* cells were compared with those of wild type and *ade12* for their ability to convert AMPS to AMP. Specific activity, measured as loss of O. D. at 280 nm per mg protein per hr, was 0.200 for wild type and 0.157 for *ade12*, but 0.0 for extracts of *ade13*. Thus the *ade13* locus may be referred to the control of adenylosuccinase by the criteria of nutritional pattern, accumulation product and lack of enzymatic activity.

Progeny tetrad types from several crosses containing *ade13* are shown in Table 1. Although the total number of asci dissected is small the possibility that *ade13* is located on linkage group V is not excluded, and justifies further attention for verification and estimate of map distances.

DISCUSSION

In analyzing the regulation of purine biosynthesis in *S. cerevisiae* the isolation and characterization of mutants at the loci controlling the conversion of IMP to

AMP allow several comparisons to be drawn. The *ade13* locus, specifying adenylosuccinase, resembles the homologous loci, *ad-4* of *N. crassa* (GILES, PARTRIDGE, and NELSON 1957) and *adA* of *Aspergillus nidulans* (FOLEY, GILES, and ROBERTS 1965). Mutation to *ade13* imposes approximately the same quantitative requirement for adenine in support of yeast growth as mutations at earlier steps in IMP biosynthesis, and shows no discernible effects on the regulation of pathway activity.

On the other hand, the *ade12* adenylosuccinate synthetase mutations cause more drastic consequences in yeast than their counterparts at the *ad-8* locus in Neurospora (PARTRIDGE and GILES 1957). As noted above, *ade12* yeast spores seldom germinate unless another step in adenine synthesis is also deficient. One can apply what is presumably the same physiological stress to the zygotes formed in haploid mating mixtures such as *a ade1 lys2 ade12* × *α ade2 try5 ade12*. Both the recombinational and the conjugational events act to remove the pre-existing genetic obstructions to the synthesis of IMP, which then accumulates and breaks down in *ade12*—blocked cells. Although zygotic cells are formed in the heteroallelic *ade12* matings, they yield no diploid colonies on MM + 180 μg/ml adenine, unless histidine is added to the medium. This dependence on histidine contrasts with the situation in Neurospora where histidine stimulates mycelial growth rates and yield at adenine concentrations up to 100 μg/ml (ISHIKAWA 1961) by sparing the adenine otherwise required for histidine biosynthesis, but growth can proceed in the absence of histidine. Relatively high concentrations of IMP are known to inhibit the first step of the histidine pathway in *Schizosaccharomyces pombe* (WHITEHEAD, NAGY, and HESLOT 1966) but whether this is the mechanism of histidine dependence of *ade12* homozygotes in *S. cerevisiae* remains to be determined.

The most interesting property of the *ade12* mutation is its influence on the regulation of pathway activity, a property that is perhaps relevant to its metabolic control of IMP, at the branch point in the biosynthesis of AMP and GMP. The effect is manifested by constitutive activity in the presence of adenine, either on the secondary level (pigment from purine precursors) or on the primary level of AIR biosynthesis in *ade1 ade12* cells (unpublished observations). Experiments with triply-blocked adenineless strains confirm the *de novo* origin of the accumulated AIR. The rapid production of inosine in *ade12* cells grown with a high concentration of adenine likewise indicates a reduction of the normal pathway regulation. In contrast, the accumulation of FGAR, typical of cells blocked at *ade6*, is almost entirely abolished by growth in 250 μg/ml adenine (SILVER 1968).

The constitutive behavior imposed by the *ade12* mutation may be thought of in terms of an imbalance in the intracellular purine pool or as the consequence of mutational changes in the protein product of the locus. Metabolite induction by IMP is readily excluded by its known property of inhibiting purine biosynthesis (BURNS 1964; WYNGAARDEN and ASHTON 1959). Another possible mechanism concerns adenine uptake. If it be assumed that adenine uptake can be inhibited by elevated levels of IMP, then it follows that the resulting intracellular adenine deficiency could account for the poor growth pattern of *ade12*

cells and their high rate of purine biosynthetic activity. Such an hypothesis, however, implies a delicate balance of IMP levels which could retard uptake without inhibiting the activity of phosphoribosylpyrophosphate amidotransferase at the same time. Although it is plausible to expect some increase in intracellular IMP in *ade1 ade12* cells relative to wild type, despite the precedent blockage, the increase, in fact, must be quite small since neither IMP nor inosine were detected by paper chromatography of such cell extracts.

Hence, the alternative possibility must be considered that the constitutive nature of the mutant results from the mutational modification of the normal *ade12* protein product. This would be the case if that product also functioned as an aporepressor in the synthesis of one or more early enzymes in the pathway. On the basis of the present observations, therefore, it is a reasonable hypothesis that the *ade12* locus codes for a bifunctional protein possessing both enzymatic and regulatory properties. For comparison it is worth recalling the regulatory function ascribed by SELS, FUKUHARA, PÉRÉ, and SLONIMSKI (1965) to the isozyme iso-2-cytochrome c molecules of *S. cerevisiae* in controlling the synthesis of iso-1-cytochrome c. The conclusion in that case was based on the kinetics of the respective synthesis of each of the isozymes as anaerobic cultures were shifted to aerobic conditions.

The hypothesis that the *ade12* locus specifies a bifunctional protein implies several testable genetic and biochemical predictions that are currently under investigation in terms of complementation mapping, and mechanisms of enzyme repression. The method by which the *ade12* mutants were found is also applicable to the isolation of dominant constitutive mutants from diploid stocks, and such studies are in progress.

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SUMMARY

Purine constitutive mutants were selected in red *ade1* or *ade2* haploid yeasts by the criterion of precocious color formation in the presence of adenine. A high proportion of the mutants so obtained are found to be functionally blocked for adenine biosynthesis at a single locus, *ade12*, unlinked to other adenineless loci. Since the mutants specifically require adenine, accumulate inosine, and excrete hypoxanthine, the *ade12* locus is believed to code for the synthesis of the enzyme adenylosuccinate synthetase. It is suggested that the locus governs the synthesis of a protein with both enzymatic and regulatory functions. Upon genetic isolation the *ade12* mutation is typically lethal, but alleles which permit survival nevertheless impair the cells' ability to utilize the required adenine.—Adenylosuccinase mutants, *ade13*, were also isolated, and identified by specific requirement for adenine, accumulation of 5-amino-4-imidazole (N-succinylo-carboxamide) ribotide (SAICAR), and lack of ability to cleave adenylosuccinic acid (AMPS) *in vitro*.

LITERATURE CITED

BERNSTEIN, H., 1961 Imidazole compounds accumulated by purine mutants of *Neurospora crassa*. J. Gen. Microbiol. **25:** 41–46.

BURNS, V. W., 1964 Regulation and coordination of purine and pyrimidine biosynthesis in yeast. I. Regulation of purine biosynthesis and its relation to transient changes in intracellular nucleotide levels. Biophysical J. **4:** 151–166.

Carbondale Yeast Genetics Conference, 1963 Microbial Genet. Bull. **19:** (Suppl.).

DEMAIN, A. L., and D. HENDLIN, 1967 Phosphohydrolases of a *Bacillus subtilis* mutant accumulating inosine and hypoxanthine. J. Bacteriol. **94:** 66–74.

DORFMAN, B., 1964a Allelic complementation at the $ad_{5/7}$ locus in yeast. Genetics **50:** 1231–1243. —— 1964b Complementation and meiotic recombination at the $ad_{5/7}$ locus in *Saccharomyces cerevisiae*. Ph.D. thesis, Yale University, New Haven.

ESPOSITO, M. S., 1968 X-ray and meiotic fine structure mapping of the adenine-8 locus in *Saccharomyces cerevisiae*. Genetics **58:** 507–527.

FOGEL, S., and D. D. HURST, 1963 Coincidence relations between gene conversion and mitotic recombination in Saccharomyces. Genetics **48:** 321–328.

FOLEY, J. M., N. H. GILES, and C. F. ROBERTS, 1965 Complementation at the adenylosuccinase locus in *Aspergillus nidulans*. Genetics **52:** 1247–1263.

GILES, N. H., C. W. H. PARTRIDGE, and N. J. NELSON, 1957 The genetic control of adenylosuccinase. Proc. Natl. Acad. Sci. U.S. **43:** 305–317.

GORNALL, A. G., C. J. BARDAWILL, and M. M. DAVID, 1949 Determinations of serum proteins by means of the biuret reaction. J. Biol. Chem. **177:** 751–766.

ISHIKAWA, T., 1961 Genetic and biochemical studies at the ad_8 locus in *Neurospora crassa*. Ph.D. thesis, Yale University, New Haven.

JONES, E. W., 1964 A comparative study of two adenine loci in *Saccharomyces cerevisiae*. Ph.D. thesis, University of Washington, Seattle.

JONES, E. W., and B. MAGASANIK, 1967 Phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase activity in the adenine-histidine auxotroph ad_3 of *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Comm. **29:** 600–604.

LEVENBERG, B., and J. M. BUCHANAN, 1957 Biosynthesis of the purines. XII. Structure, enzymatic synthesis, and metabolism of 5-aminoimidazole ribotide. J. Biol. Chem. **224:** 1005–1018.

LUKENS, L. N., and J. M. BUCHANAN, 1959 Biosynthesis of the purines. XXIII. The enzymatic synthesis of N-(5-amino-1-ribosyl-4-imidazolylcarbonyl)-L-aspartic acid 5′-phosphate. J. Biol. Chem. **234:** 1791–1798.

MAGASANIK, B., 1962 Biosynthesis of purine and pyrimidine nucelotides. pp. 295–334: In: *The Bacteria*. Edited by I. C. GUNSALUS and R. Y. STANIER. Academic Press, New York.

MARQUART, H., U. VON LAER, and F. K. ZIMMERMANN, 1966 Das spontane, nitrosamid- und nitrit-induzierte Mutationsmuster von 6 Adenin-Genloci der Hefe. Z. Vererb. **98:** 1–9.

MAZLEN, A. S., 1968 A biochemical study of the adenine and histidine requiring mutant adenine-3 of *Saccharomyces cerevisiae*. Ph.D. thesis, Brooklyn College, The City University of New York.

MILLER, R. W., L. N. LUKENS, and J. M. BUCHANAN, 1959 Biosynthesis of the purines. XXV. The enzymatic cleavage of N-(5-amino-1-ribosyl-4-imidazolylcarbonyl)-L-aspartic acid 5′-phosphate. J. Biol. Chem. **234:** 1806–1811.

NISHIKAWA, H., H. MOMOSE, and I. SHIIO, 1967 Regulation of purine nucleotide synthesis in *Bacillus subtilis*. II. Specificity of purine derivatives for enzyme repression. J. Biochem. **62:** 92–98.

PARTRIDGE, C. W. H., and N. H. GILES, 1957 Identification of major accumulation products of adenine-specific mutants of Neurospora. Arch. Biochem. Biophys. **67:** 237–238.

RABINOWITZ, J. C., 1956 Purine fermentation by *Clostridium cylindrosporum*. III. 4-amino-5-imidazolecarboxylic acid and 4-aminoimidazole. J. Biol. Chem. **218:** 175–187.

ROMAN, H., 1956 A system selective for mutations affecting the synthesis of adenine in yeast. Compt. Rend. Trav. Lab. Carlsberg, Ser. physiol. **26:** 299–314.

SELS, A. A., H. FUKUHARA, G. PÉRÉ, and P. P. SLONIMSKI, 1965 Cinétique de la biosynthèse induite de l'iso-1-cytochrome c et de l'iso-2-cytochrome c au cours de l'adaptation à l'oxygène. Biochim. Biophys. Acta **95:** 486–502.

SILVER, J. M., 1968 Biosynthetic blocks of eight non-allelic adenine requiring mutants of *Saccharomyces cerevisiae*. Ph.D. thesis, Brooklyn College, The City University of New York.

SMIRNOV, M. N., V. N. SMIRNOV, E. I. BUDOWSKY, S. G. INGE-VECHTOMOV, and N. G. SEREBRJAKOV, 1967 Red pigment of adenine-deficient yeast, *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Comm. **27:** 299–304.

WHITEHEAD, E., M. NAGY, and H. HESLOT, 1966 Interactions entre la biosynthèse des purines nucleotides et celle de l'histidine chez le *Schizosaccharomyces pombe*. Compt. Rend., Ser. D. **263:** 819–821.

WYNGAARDEN, J. B., and D. M. ASHTON, 1959 The regulation of activity of phosphoribosylpyrophosphate amidotransferase by purine ribonucleotides: a potential feedback control of purine biosynthesis. J. Biol. Chem. **234:** 1492–1496.