GENETIC AND BIOCHEMICAL ANALYSIS OF ISOLEUCINE-VALINE MUTANTS OF YEAST[1]

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IN the organisms so far investigated the biosynthesis of valine and isoleucine seems always to proceed from pyruvic acid by a series of four steps to the amino acids. The pathway shown in Figure 1 is supported by the results of a

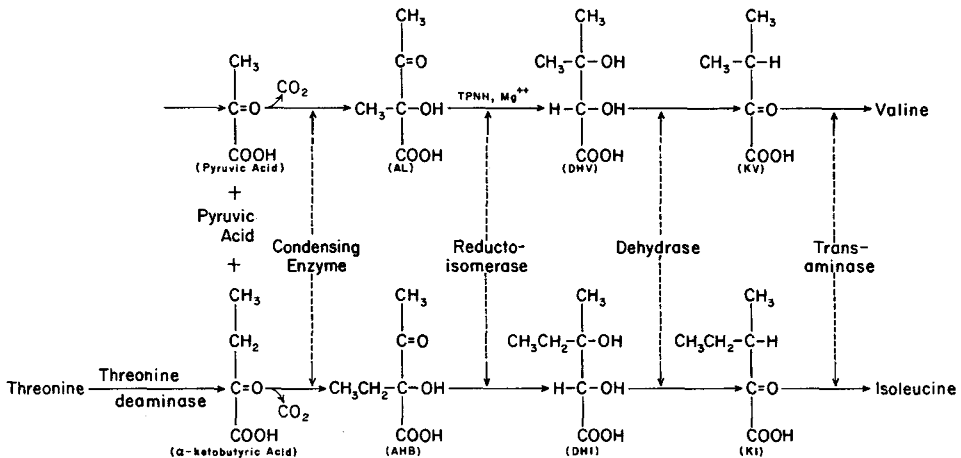

FIGURE 1.—The biosynthetic pathway leading to isoleucine and valine. AL = α-acetolactate, AHB = α-aceto-α-hydroxybutyrate, DHV = α,β-dihydroxyisovalerate, DHI = α,β-dihydroxy-β-methylvalerate, KV = α-ketoisovalerate, KI = α-keto-β-methylvalerate.

number of workers using yeast as the experimental organism, (STRASSMAN, THOMAS and WEINHOUSE 1955; STRASSMAN, THOMAS, LOCKE and WEINHOUSE 1956; STRASSMAN, SHATTON, CORSEY and WEINHOUSE 1958; STRASSMAN, SHATTON and WEINHOUSE 1960; LEWIS and WEINHOUSE 1958; WIXOM, SHATTON and STRASSMAN 1960). The present study was undertaken to analyze the basic biochemical characteristics of a group of mutants of the yeast, *Saccharomyces cerevisiae*, requiring both isoleucine and valine, and to correlate these characteristics with such genetic information as could be obtained.

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MATERIALS AND METHODS

The basic media used and the general techniques employed for genetic analysis have been described previously by KAKAR (1963). The minimal medium referred to in the present work contained glucose, mineral salts and vitamins (ROMAN 1956), but no amino acids, purines or pyrimidines, unless otherwise stated. The "complete" medium contained in addition DL-isoleucine (60 μg per ml), DL-valine (60 μg per ml), L-histidine (10 μg per ml) and L-tryptophan (10 μg per mil). Solid medium contained 1.5 percent Bacto-agar.

The diploid cells were sporulated for dissection as described by FOGEL and HURST (1963).

The isoleucine-valine requiring mutants were isolated from haploid strains 7029C, A429C and A468A obtained from the yeast stock collection at the University of Washington, Seattle. Strain 7029C is mating type *a* and requires tryptophan for growth while A429C and A468A are mating type α and require histidine.

Mutants were obtained by inoculating approximately 400 cells of the parent strains on the surface of plates containing the complete medium described above, and irradiating with a dose of ultraviolet light sufficient to produce approximately 50 percent survivors. After three to four days incubation at 30°C the colonies were tested for an isoleucine and valine requirement by the replica plating method with a velvet stamp. A total of 19 isoleucine-valine requiring mutants was obtained, 11 from 7029C, four from A429C and four from A468A. In addition seven mutants were obtained which required isoleucine only. Two more isoleucine-valine mutants, EU-38 and Nr-211-1aM were obtained from DR. R. K. MORTIMER of the University of California, Berkeley, and DR. W. LASKOWSKY of the Freie Universität, Berlin, respectively. Both of these mutants are of mating type α.

Complementation between the various isoleucine and isoleucine-valine mutants was studied by crossing mutant cells of opposite mating type on yeast extract-peptone medium containing 1 percent yeast extract, 2 percent bactopeptone, 2 percent glucose and 1.5 percent agar. After 18 to 24 hours incubation to permit mating to take place, the diploid zygotes were tested on isoleucine free, valine free and isoleucine-valine free media. Those diploids with complementing genes grew on the test media.

To determine whether mutation to a simultaneous requirement for isoleucine and valine involved the mutation of one or more than one gene, the mutant strains were crossed to one of the parent wild-type strains of opposite mating type. The resulting diploids were then analyzed after sporulation. Also, tests for allelism between the mutant genes of various complementation groups were made by crossing representative mutants from each of the groups and analyzing the resulting diploids.

For enzyme studies a small inoculum was grown on a shaker in 5 ml of liquid yeast extract-peptone medium for two days. The cells were washed by centrifugation and suspended in 2 ml of water. Two flasks, each containing 500 ml of complete liquid medium, were then inoculated with 1 ml of the yeast suspension, and incubated 20 to 24 hours at 30°C with continuous shaking. One mutant, M-22, grew very slowly on this medium and it was necessary to grow it four to five days to obtain a sufficient number of cells. The cells were harvested by centrifugation, and the medium analyzed for the accumulation of carbinols, dihydroxy acids and keto acids by the methods described by WAGNER and BERGQUIST (1960).

The harvested cells were washed twice in distilled water and suspended in cold 0.1 M phosphate buffer at pH 7.5. They were then broken in a press by the method and apparatus described by EATON (1962). The broken cells in suspension were centrifuged 20 min at 56,000 $\times$ *g* or 70,000 $\times$ *g* in a Spinco Model L centrifuge. The pellets were discarded and the supernatants used for enzyme analyses. Protein concentrations of the supernatants were determined by the method of LOWRY, ROSENBROUGH, FARR and RANDALL (1951). Enzyme assays with the supernatants were carried out as follows.

Threonine deaminase activity was assayed by adding 20 micromoles of L-threonine to supernatant extract containing 1 to 2 mg of protein in sufficient 0.1 M phosphate buffer at pH 8.0 to make 2.0 ml, and 40 μg of pyridoxal phosphate. This reaction mixture was incubated at 37°C for 10, 20 and 30 minutes, and the reaction stopped by the addition of 0.5 ml 10 percent tri-

chloracetic acid. The α-ketobutyric acid produced was determined by the method of FRIEDMANN and HAUGEN (1943).

The condensing enzyme was assayed in a mixture containing 500 μg sodium pyruvate, 100 μg thiamine pyrophosphate, 10 μmoles $MgSO_4$, 1 to 2 mg of protein and sufficient 0.1 M phosphate buffer at pH 6.25 to make a total volume of 4.5 ml. The reaction mixture was incubated 30 min at 37°C and stopped by adding 0.5 ml of 6N sulphuric acid. After the addition of the acid the tubes were incubated 30 min at 60° to decarboxylate the remaining α-acetolactate. The carbinol remaining was then determined quantitatively by the WESTERFELD (1945) test.

Reductoisomerase activity was measured by determining the rate of oxidation of $NADPH_2$ (reduced nicotinamide-adenine dinucleotide phosphate) spectrophotometrically in the presence of α-aceto-α-hydroxybutyrate or α-acetolactate. The assay mixture contained 100 μmoles of one of the α-aceto acid substrates, 10 μmoles of $NADPH_2$, 100 μmoles of $MgSO_4$, and about 0.1 mg of extract protein in sufficient 0.1 M phosphate buffer at pH 7.5 to make a total volume of 0.9 ml. In practice the cuvette containing all of the reactants save the $NADPH_2$ was incubated first for 30 min at 37°C and then the $NADPH_2$ added and the change in optical density recorded at 340 mμ with a Beckman or Cary recording spectrophotometer.

The determination of dehydrase enzyme activity was accomplished by incubating, for 30 min at 37°C, a mixture containing 20 μmoles of α,β-dihydroxy-β-methylvalerate or α,β-dihydroxy-isovalerate, 20 μmoles $MgSO_4$, and 1 to 2 mg of extract protein in 0.1 M Tris buffer at pH 7.4 to make 2.0 ml. The reaction was stopped by the addition of 0.5 ml of 10 percent trichloracetic acid. The mixture was then assayed for α-keto acids by the method of FRIEDMANN and HAUGEN (1943).

Transaminase enzyme activity was determined chromatographically. A mixture containing 40 μmoles of α-keto-β-methylvalerate or α-ketoisovalerate, 40 μmoles of donor amino acid, 20 μg of pyridoxal phosphate, 3 to 4 mg of extract protein and sufficient 0.1 M phosphate buffer at pH 8.0 to make 1.5 ml was incubated 3 hr at 37°C. The reaction was stopped by heating the incubated mixture in boiling water for 5 minutes. After cooling, 0.5 ml of glacial acetic acid was added and the resulting precipitate removed by centrifugation. A small sample of the supernatant was then applied to Whatman No. 1 filter paper and ascending chromatography carried out with a solvent system consisting of n-butanol, glacial acetic acid and water in volume proportions of 40:10:50. Isoleucine or valine were determined qualitatively by comparison with known standards.

The substrates α-acetolactate and α-aceto-α-hydroxybutyrate were prepared in this laboratory using the methods described by KRAMPITZ (1948) and RADHAKRISHNAN and SNELL (1960). The α,β-dihydroxy acids were prepared by the method of SJOLANDER, FOLKERS, ADELBERG and TATUM (1954). $NADPH_2$, pyridoxal phosphate and α-ketoisovalerate were obtained from the Sigma Chemical Company.

RESULTS

Genetic analysis: If the requirement for both isoleucine and valine is the result of a single gene mutation, then among the products of meiosis of a mutant × non-mutant cross, the two requirements would be expected to segregate together. Otherwise, some of the segregants would require either isoleucine or valine alone. Mutants M-12, M-22, M-3, M-4, six mutants from Group III (see complementation results below) and six mutants from Group IV have been analyzed in this way. From ten to 40 asci were dissected in each case. In general, poor viability of the spores has been a problem thus producing a very low proportion of four spored asci. The extreme cases have been the crosses involving EU-38 (25 asci were dissected) and M-28 (ten asci were dissected) where only 13 and four spores grew respectively. However, in all the cases, among the segregants that did grow, the two requirements always segregated together.

The results of the crosses are given in Table 1. From them it can be tentatively concluded that the double requirement for isoleucine and valine arises as the result of a single gene mutation.

Crosses were also made to test for allelism and linkage among the mutants. Isoleucine-valine independent segregants were readily obtained from crosses involving mutants of the following type: Group I × Group III, Group I × IV, Group II × Group III, Group II × Group IV and Group III × Group IV. The results suggested that the Group III and IV mutant genes are not linked to one another nor to the Group I and II mutants. A cross between the Group I mutant M-12 and the Group II mutant M-22 gave results indicating close linkage, however. From this cross two isoleucine-valine independent spores were found from among 40 asci analyzed. Thus M-12 and M-22 appear to be approximately 2.5 units apart.

Complementation tests: Table 2 presents the results from the complementation tests. It is clear from these data that most of the mutants fall into two main complementation groups: Group III with ten, and Group IV with eight mutants. Mutants M-12 and M-22 are designated as Group I and II respectively, since they complement with all others as well as one another. The isoleucine mutants do not complement with one another, but do complement with all the isoleucine-valine mutants, and are hence designated as Group V.

TABLE 1

Segregation for isoleucine and valine requirement

Cross	Number of asci dissected	Number of spores growing	Number of I^+V^+ spores	Number of I^-V^- spores	Number of 4-spored asci (2:2 segregation)
Group I					
M-12 × WT	37	119	73	46	14
Group II					
M-22 × WT	26	43	35	8	1
Group III					
M-7 × WT	6	17	10	7	2
M-13 × WT	11	30	20	10	0
M-14 × WT	40	90	62	28	4
M-16 × WT	24	62	38	24	5
M-18 × WT	7	23	14	9	2
M-28 × WT	10	4	4	0	0
Group IV					
M-1 × WT	16	42	32	10	0
M-2 × WT	10	27	17	10	0
M-11 × WT	26	63	40	23	6
M-15 × WT	16	45	33	12	2
EU-38 × WT	25	13	10	3	0
Nr-211-1aM × WT	6	22	11	11	5
Others					
M-3 × WT	16	28	18	10	1
M-4 × WT	16	51	30	21	5

TABLE 2

Complementation between various isoleucine and isoleucine-valine mutants

Group ↓	α ↓ / a →	I: M-12 is$^-$	M-12 v$^-$	III: M-7 is$^-$	M-7 v$^-$	M-13 is$^-$	M-13 v$^-$	M-26 is$^-$	M-26 v$^-$	M-28 is$^-$	M-28 v$^-$	M-4 is$^-$	M-4 v$^-$	IV: M-2 is$^-$	M-2 v$^-$	M-5 is$^-$	M-5 v$^-$	M-11 is$^-$	M-11 v$^-$	M-1 is$^-$	M-1 v$^-$	M-3 is$^-$	M-3 v$^-$	V: M-6 is$^-$	M-6 v$^-$	M-8 is$^-$	M-8 v$^-$	M-9 is$^-$	M-9 v$^-$	M-10 is$^-$	M-10 v$^-$	M-27 is$^-$	M-27 v$^-$	Control is$^-$	Control v$^-$
II	M-22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
III	M-14	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	M-16	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	M-18	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	M-23	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	M-24	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
	M-25	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+s	+s	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	-
IV	EU-38	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
	M-15	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+s	+s	+	+	+	+	+	+	+	+	+	+	-	-
	Nr-211-1aM	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	+s	+s	+	+	+	+	+	+	+	+	+	+	-	-
	M-3	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+	+	+	+	+	-	-
V	M-20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+
	M-21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	-	+	-	+
	Control	-	-	-	-	-	-	-	-	-	-	-	+s	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	+		

(+) and (−) represent whether or not the diploids grow on isoleucine-free (is$^-$) or valine-free (v$^-$) media.
(s) represents that the diploids grew slowly on the plates.

Enzymatic activities: Enzymatic activities for the centrifuged crude extracts of the various strains are given in Table 3. All the isoleucine-valine mutants were tested as well as the three parent strains. Where a range of values is given in the table it indicates the range found for a number of assays for a single mutant which is designated in the table, or for the entire group of mutants.

When compared to the wild-type strains, it is seen that mutants M-12 and M-22 showed comparable ranges of enzyme activity except for reductoisomerase activity in the presence of Mg^{++}. This is true for both substrates, α-aceto-α-hydroxybutyrate and α-acetolactate. A significant point, furthermore, is the fact that the activity was found to be the same for the mutants in the presence and absence of added Mg^{++}. When, however, Mg^{++} is omitted from the assay mixtures of the wild types or the other mutant strains a considerable depression of activity is noted. The Mg^{++} dependence of the reductoisomerases of *Escherichia coli, Salmonella typhimurium* and *Neurospora crassa* has been well established by the work of RADHAKRISHNAN, WAGNER and SNELL (1960) and ARMSTRONG and WAGNER (1961). The activity remaining in the absence of Mg^{++} in crude extracts such as exhibited here in yeast has been called the "residual activity" (ARMSTRONG and WAGNER 1961) and presumably reflects the activity of another enzyme which converts the α-aceto acids to some unknown compound in the presence of $NADPH_2$ rather than to the α, β-dihydroxy acids.

The low threonine deaminase activity of M-22 may be the result of prolonged growth. It is this mutant which must be grown for several days to obtain sufficient cells for preparing enzyme extracts. We have found that if we allowed the isoleucine-valine independent strain, 7029C, to grow on the shaker for four to five days, the threonine deaminase activity was reduced to the level observed for M-22.

The Group III mutants are deficient in dehydrase activity as determined by the ability of their extracts to convert α, β-dihydroxy-β-methylvalerate to the

TABLE 3

Specific activity of crude extracts of various yeast strains
(μmoles product formed/mg protein/hour)

	Enzymes and substrates						
	Threonine deaminase with L-threonine	Condensing enzyme with pyruvate	Reductoisomerase with α-aceto-α-hydroxybutyrate		Dehydrase with α,β-dihydroxy-β-methylvalerate	Transaminase with	
Strains			plus Mg^{++}	no Mg^{++}		α-keto-isovalerate	α-keto-β-methyl-valerate
Parent strains	1.3–6.8	1.0–4.0	2.7–6.3	1.1–3.7	0.8–1.6	+	+
Group I (M-12)	0.9–1.8	2.0–3.2	1.2–1.8	1.2–1.9	1.8–3.5	+	+
Group II (M-22)	0.2–0.8	0.7–1.9	0.8–0.9	0.6–1.0	5.4–7.8	+	+
Group III	3.3–4.2	0.8–2.2	3.8–11.7	0.8–1.8	0–0.1	+	+
Group IV	3.2–7.3	0.6–3.0	2.8–5.3	0.8–2.1	1.4–2.9	+	+
Group V							
M-6	0	2.5	2.8	1.6	1.8	+	+
M-27	0	..	..	..	..	..	..

α-keto acid precursor of isoleucine. Two of the mutants, M-14 and M-4, were also tested for activity with α, β-dihydroxyisovalerate, the precursor of valine, and found to be correspondingly inactive with this substrate too.

It can be seen from Table 3 that the Group IV mutants have the wild-type range of activity for all enzymes. The reason for their requirement for isoleucine and valine is difficult to understand, but it should be pointed out that in this respect they are quite similar to the Group II isoleucine-valine mutants of *Neurospora crassa* described by WAGNER, KIRITANI and BERGQUIST (1962).

The isoleucine mutants M-6 and M-27 are both deficient in threonine deaminase activity. The other isoleucine mutants were not tested.

Crude extracts of *Neurospora crassa* are active in the transamination of α-ketoisovalerate and α-keto-β-methylvalerate in the presence of L-phenylalanine (WAGNER and IFLAND 1956; SEECOF and WAGNER 1959). L-phenylalanine is, however, a poor donor for these keto acids in yeast, and its conversion to phenylpyruvate cannot be measured spectrophotometrically in the wild-type strain 7029C using the method of CAMMARATA and COHEN 1951. Table 4 presents the results of testing 17 different amino acids as donors for the α-keto analogues of isoleucine and valine by the method described above, and using chromatography to identify the end products. The three parent strains and all of the isoleucine-valine mutants were tested and all gave the same pattern of results. Leucine seems to be the best donor for both keto acids while valine transaminates readily with α-keto-β-methylvalerate and isoleucine with α-ketoisovalerate. None of the mutants tested gave any indication of being transaminase deficient by the relatively crude assay procedure used here.

TABLE 4

Transaminase activity of the crude extracts of yeast

Donor amino acid	With α-ketoisovalerate	With α-keto-β-methylvalerate
ornithine	W	W
leucine	S	*
alanine	M	W
norvaline	.	M
methionine	*	M
valine	. .	S
isoleucine	S	. .
phenylalanine	W	W
norleucine	M	•
glutamate	M	M
lysine	—	—
α-aminobutyrate	—	—
glycine	—	—
tyrosine	—	—
tryptophan	—	—
aspartic acid	—	—
threonine	—	—

S=strong spots, M=medium spots, W=weak spots.
* Valine or isoleucine spots were not distinguishable from the donor amino acid spots in the solvent system used.

UMBARGER and BROWN (1958a) have shown that the threonine deaminase of *Escherichia coli* is inhibited by isoleucine, and its condensing enzyme by valine. In Neurospora and Salmonella, however, evidence has been presented that such feedback inhibition is not found for the condensing enzymes or the enzymes which follow it in the isoleucine-valine pathway. It was, therefore, of interest to determine whether isoleucine or valine had any effect on these enzymatic activities in yeast (RADHAKRISHNAN and SNELL 1960; ARMSTRONG, GORDON and WAGNER 1963). The results given in Table 5 show that the activity of threonine deaminase was completely inhibited by the addition of isoleucine to the assay mixture. No effect on the other enzymes was noted with this amino acid or valine.

DISCUSSION

It should be noted that all the enzyme activity analyses reported were done with crude extracts after centrifugation of broken cells. Therefore, when an extract is found to be deficient in an enzyme, it may not be for the reason that the enzyme is missing, but because it is inhibited by some other substance or substances present in the crude extract of the mutant, but not the wild type, in sufficient concentration to cause complete inhibition. This should be borne in mind in the following discussion.

The isoleucine-valine mutants described here may be assigned to four complementation groups. However, on the basis of their enzyme activities they may be assigned to only three groups since M-12 (Group I) and M-22 (Group II) both appear to be deficient in the reductoisomerase. The fact that these two strains, deficient in the same enzyme, complement readily in diploids is of some interest. Furthermore, the evidence is that they have mutations at separate loci, although this is still inconclusive, since it is based on finding two prototrophs from among 40 asci examined. They are both comparable to the Group I mutants of *Neurospora crassa* (WAGNER, KIRITANI and BERGQUIST 1962) and the *ilvaA* mutants of *Salmonella typhimurium* (WAGNER and BERGQUIST 1960).

TABLE 5

Effect of isoleucine and valine on the activity of the various isoleucine-valine enzymes in crude extracts of strain 7029C

Additions into the assay system	Enzymes and substrates						
	Threonine deaminase with L-threonine	Condensing enzyme with pyruvate	Reductoisomerase with α-aceto-α-hydroxybutyrate	Dehydrase with α, β-dihydroxyvalerate	Dehydrase with α, β-dihydroxy-β-methylvalerate	Transaminase with α-keto isovalerate*	Transaminase with α-keto-β-methylvalerate*
None	6.8	1.8	3.4	0.98	1.3	+	+
0.01 M isoleucine	0	2.0	3.3	1.04	1.2	+	+
0.01 M valine	8.0	1.8	3.4	0.87	0.87	+	+
0.01 M glycine	6.4	1.7	3.9	1.1	1.1	+	+
0.01 M isoleucine + 0.01 M valine	0	1.95	3.6	0.87	1.04	+	+

* With L-leucine as donor.

The Group III mutants are comparable to the Group III mutants of *Neurospora crassa* since they too show a deficiency of dehydrase activity. Similar deficient dehydrase mutants have also been found in Salmonella (WAGNER and BERGQUIST 1960) and *Escherichia coli* (MYERS and ADELBERG 1954).

The data are quite clear in showing that the crude supernatant extracts of the Group IV mutants have all the enzyme activities. In this characteristic they are quite similar to the Group II isoleucine-valine mutants of Neurospora (WAGNER, KIRITANI and BERGQUIST 1962). Recently WAGNER and BERGQUIST (1963) have presented evidence that valine and isoleucine are synthesized from pyruvate in a particulate fraction associated with the mitochondria in Neurospora. Wild-type strains have active particles, but the Group II Neurospora mutants do not. Unpublished results from this laboratory indicate that these mutants have particles which are unable to convert the dihydroxy acids to the amino acids although they do have active dehydrase in the supernatant. The suggestion has been made that the isoleucine-valine system is organized in Neurospora and the Group II mutants have a disorganization of this system. The Group IV mutants of yeast may be similar in this respect.

In Neurospora and Salmonella the loci controlling the production of the reductoisomerase and the dehydrase are closely linked (WAGNER, KIRITANI and BERGQUIST 1962; GLANVILLE and DEMEREC 1960). This does not appear to be true in yeast, since the Group I and II mutants recombine readily with the Group III mutants to produce isoleucine-valine independent prototrophs.

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SUMMARY

Twenty-one mutants of *Saccharomyces cerevisiae* requiring isoleucine plus valine have been partially characterized genetically and biochemically. They fall into four complementing groups with linkage indicated between only two of the groups. Enzymatic analysis reveals that two mutants which complement readily are both deficient in the reductoisomerase enzyme. A third group designated as complementation Group III has all of its members deficient in the dihydroxy acid dehydrase, while the Group IV noncomplementing mutants show no deficiency in any of their enzymes required for isoleucine-valine synthesis.

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