GENETIC BLOCKS IN THE ISOLEUCINE-VALINE PATHWAY OF NEUROSPORA CRASSA[1]

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A group of mutants of *Neurospora crassa* which require both isoleucine and valine have been described in a series of previous communications from this laboratory (WAGNER, SOMERS and BERGQUIST 1960; WAGNER, KIRITANI and BERGQUIST 1962; and KIRITANI 1962). These mutants were all found to be closely linked between the linkage group V markers *lys-1* (lysine-1, 33933) and *sp* (spray) within a segment about four map units long. In part by genetic analysis and in part by considering their biochemical characteristics, the mutants were classified in three groups designated as 1, 2 and 3. (These were previously indicated with Roman numerals, but since this caused confusion with the linkage group numbers, Arabic numerals are substituted here.) However, the biochemical evidence presented in the earlier papers was not sufficient in all cases to establish with certainty the location of the genetic blocks causing the isoleucine plus valine requirement.

The chief difficulty met with in the earlier analyses was that the soluble enzyme extracts prepared from the homogenates of the mutants appeared in many cases to contain substantial activity for all four of the enzymes known to be involved in the synthesis of valine and isoleucine. These enzymes, the condensing enzyme (α-acetolactate-forming enzyme), the reductoisomerase, the dihydroxy acid dehydrase, and the transaminase are apparently active in the synthesis of both amino acids (RADHAKRISHNAN, WAGNER and SNELL 1960; RADHAKRISHNAN and SNELL 1960; SEECOF and WAGNER 1959), (Figure 1). Hence, the alteration or absence of any one of them should cause a requirement for one or both amino acids, whereas when all are present there should be no requirement. Mutations affecting steps prior to that at which the condensing enzyme acts should be expected to be either just isoleucine mutants, since only the isoleucine precursor, α-ketobutyrate, would be involved, or mutants unable to synthesize pyruvate or "active" acetaldehyde (see Figure 1). These latter mutants, assuming that they could be cultured, would have more than an isoleucine plus valine requirement.

The recent discovery that the isoleucine-valine system is apparently associated as an organized unit with the particulate fraction of the Neurospora homogenate

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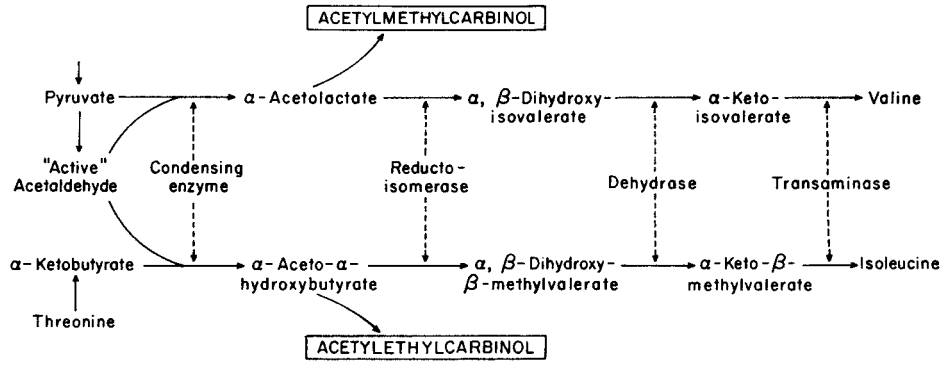

FIGURE 1.—Biosynthetic pathway for valine and isoleucine from pyruvate and threonine. The four enzymes discussed in the text are indicated at the steps they control. The carbinols, acetylmethylcarbinol and acetylethylcarbinol, are formed by decarboxylation of the respective indicated α-aceto acids in large quantities by certain of the isoleucine-valine mutants, but not by wild type.

(WAGNER and BERGQUIST 1963) centrifuged down at 39,000 × *g* made it possible to analyze the mutants requiring these amino acids by somewhat different approaches than had been used previously with them or other nutritional mutants.

The results of examining the particulate fractions of the mutants with the different precursors of valine are presented in the present communication together with additional evidence bearing on the occurrence or nonoccurrence of the reductoisomerase enzyme. In addition, it also describes a fourth group of mutants that require isoleucine and valine, but are located in another linkage group.

MATERIALS AND GENERAL METHODS

In addition to the 20 strains originally described by WAGNER, SOMERS and BERGQUIST (1960) 30 more isoleucine-valine mutants were obtained by the same method except that a linkage group V mutant, *lysine-1* (33933), was used as the parent strain, instead of the Em 5256A wild-type strain.

The wild-type strain used was KJT1960A. This was obtained from a cross between Em 5256A and Em 5297a. The mutant tester stocks used in the crosses to establish linkage group relations were: *leu-3* (47313)*a*, IL; *arg-5* (27947)*a*, IIR; *leu-1* (33757)*a*, IIIR; *pan-1* (5531)*a*, IVR; *cys-1* (84605)*a*, VIL; *me-7* (4894)*a*, VIIR obtained from the Fungal Genetics Stock Center at Dartmouth College.

All crosses were made in dilution bottles on solid WESTERGAARD medium (WESTERGAARD and MITCHELL 1947) supplemented appropriately with the required amino acids. The minimal medium used throughout the experiments was that described by VOGEL (1956).

The coenzymes and enzymes used in all experiments were obtained from the Sigma Chemical Company, and the amino acids and α-ketoisovaleric acid from the Nutritional Biochemical Company. The dihydroxy acids, α,β-dihydroxyisovaleric acid and α,β-dihydroxy-β-methylvaleric acid were prepared by the method described by SJOLANDER, FOLKERS, ADELBERG and TATUM (1954). The α-aceto acids, α-acetolactic acid and α-aceto-α-hydroxybutyric acid were synthesized by the method of RADHAKRISHNAN and SNELL (1960), and α-keto-β-methylvaleric acid was synthesized by the method of MEISTER (1952) from isoleucine.

Activities of individual soluble enzymes were determined by methods previously described as follows: condensing enzyme by RADHAKRISHNAN and SNELL (1960), dehydrase by WAGNER, RADHAKRISHNAN and SNELL (1958) and transaminase by SEECOF and WAGNER (1959). All data reported on enzyme activities were obtained by testing crude extracts prepared by homogenizing mycelium grown for the indicated periods of time with a Ten Brock grinder or a Virtis homogenizer at the appropriate pH and then centrifuging ½ hr in a Spinco Model L centrifuge at 82,000 × *g*.

Overall activity for the production of valine from pyruvate and other substrates was determined by the method described previously by WAGNER and BERGQUIST (1963) with the following modifications. The homogenates were prepared in an 0.5 M sucrose solution containing 0.05 M Tris buffer at pH 8.5. The valine synthesizing abilities of the 39,000 × *g* pellets and the 39,000 × *g* supernatants were tested as previously described with the same mixture of coenzymes, etc. In general, 2.5 to 5 mg of protein was present per ml of assay mixture. When pyruvate was tested as substrate it was present in the amount of 200 μmoles per 3 ml of incubation mixture. The other substrates, α-acetolactate, α, β-dihydroxyisovalerate and α-ketoisovalerate were tested at levels of 100 μmoles per 3 ml.

All of the 20 original mutants were tested for growth response on the following known precursors of isoleucine and valine: α-hydroxy-α-acetobutyrate + α-acetolactate, α, β-dihydroxyisovalerate + α, β-dihydroxy-β-methylvalerate, α-ketoisovalerate + α-keto-β-methylvalerate. In addition all 50 mutants were tested for growth response to α-keto-hydroxyisovalerate + α-keto-β-hydroxy-β-methylvalerate. These compounds had previously been shown to be active in stimulating the growth of certain isoleucine-valine mutants of *Salmonella typhimurium*, (WAGNER and BERGQUIST 1960). Since these compounds, except for the α-keto acids, were available only in small quantities, tests were carried out by auxanography. Conidia from each mutant strain were suspended in liquid agar and poured into petri dishes. Whatman No. 1 filter paper discs, 1.2 cm in diameter were placed on the gelled agar surfaces separated by a distance of approximately 1 to 1.5 cm. Approximately 50 μg of supplement was added to each disc. By this means the supplements were tested in pairs. Positive evidence that the supplements were adequate for sustaining growth was demonstrated by a band of growth between the discs where the diffusing supplements had produced a region of adequate relative concentrations. α-Keto acids were also tested for growth promoting activity in standing culture in 125 ml flasks containing 25 ml of supplemented medium.

EXPERIMENTAL AND RESULTS

Genetic characterization of new mutants: After isolation of the new mutants each of them was crossed to the previously characterized isoleucine-valine mutants T322, T304 and T327 which represent the previously defined groups 1, 2 and 3 respectively, in linkage group V. Those mutants not showing linkage to the tester stocks or *lys-1* were crossed to tester strains carrying markers for the other chromosomes.

At least 10^5 ascospores from each of the crosses to T322, T304 and T327 were plated on minimal medium supplemented with lysine and the number of prototrophs was observed following the method described by KIRITANI (1962). The recombination frequencies for those new mutants found to be linked to the linkage group V markers were calculated from these data. Crosses to the strains carrying markers on the other chromosomes were analyzed for recombination frequencies by picking at random sufficient ascospores to produce about 100 viable cultures. These were then each tested for growth on minimal and supplemented media containing 1 percent sorbose and 0.05 percent each of fructose and glucose (BROCKMAN and DE SERRES 1963).

Fourteen of the 30 new mutants were found to be linked to T327 and T304. Linkage to T327 was found to range from 2.0 to 8.9 percent recombination with an average of 4.9 percent, and linkage to T304 averaged 2.0 percent with a range of 0.6 to 3.7 percent. Eleven of these 14 mutants were assigned to group 1 because of their linkage to T327 and T304, their lack of reductoisomerase activity as described in the following sections, and their high carbinol accumulation. None of these mutants crossed to the T322 tester strain. Carbinol accumulation (see Figure 1) ranged from 103 to 1092 μg/ml of medium after three days growth. This is in contrast to carbinol accumulation by the wild-type and the other isoleucine-valine mutants not in group 1 (except certain group 2 mutants) which showed an accumulation in the range of 1.0 to 3.4 μg/ml after an identical period of growth. Extracts of three of the *lysine-1* linked mutants showed a definite reductoisomerase band after electrophoresis and have been tentatively placed in group 2 since they did not accumulate dihydroxy acids. One of them, T362, accumulated 535 μg/ml of carbinols in three days, however.

Twelve of the 30 new mutants were found to be not linked to *lys-1*, but definitely linked to *pan-1* (5531) in linkage group IV. Linkage values of 3.0 to 8.7 percent were obtained to *pan-1*. None of these mutants accumulated carbinols or dihydroxy acids, and all had activities in the wild-type range. They have been assigned to a new group, group 4, and are discussed in more detail in the following section.

The remaining four mutants gave inconclusive results from crosses so that linkage to none of the markers could be established, or we were unable to get them to cross.

Data concerning mutants in each of the groups: The group 1 mutants, as previously described, accumulate large quantities of carbinols in the growth medium after three to four days of growth (WAGNER, KIRITANI and BERGQUIST 1962). Since the WESTERFELD (1945) method of determination of the total carbinols does not discriminate between the various types of carbinols, qualitative determinations were made as described by WAGNER, BERGQUIST and FORREST (1959). This method is based on the principle of converting the carbinols to pteridines by reacting them with 2,4,5-triamino-6-hydroxypyrimidine dihydrochloride, and then identifying the pteridines by paper chromatography. Medium from four- to five-day old cultures from each of the group 1 mutants grown at 25°C was treated as previously described and the pteridines identified as those expected from acetylmethylcarbinol and acetylethylcarbinol. These are the decarboxylation products of α-acetolactate and α-aceto-α-hydroxybutyrate respectively. It is evident, then, that the group 1 mutants are blocked at the step at which the α-aceto acids are converted to the dihydroxy acids (see Figure 1).

The reductoisomerase enzyme which catalyzes this step has been found to be low in activity in the group 1 mutants (WAGNER, KIRITANI and BERGQUIST 1962). These determinations were made spectrophotometrically by following the oxidation of $NADPH_2$ (reduced nicotinamide-adenine dinucleotide phosphate) in the presence of the α-aceto acid substrates (RADHAKRISHNAN, WAGNER and SNELL 1960). The possibility existed, therefore, that the low reductoisomerase activity

observed in the mutants was in fact the result of other reactions involving the α-aceto acids and $NADPH_2$ which resulted in products other than the dihydroxy acids.

To test this possibility electrophoresis on starch gel was carried out on supernatant extracts from three day old mycelium prepared from homogenates centrifuged at 82,000 $\times$ g for ½ hour. The electrophoretic procedure and the method of determination of $NADPH_2$ oxidizing activity on the gels was identical to that described by ARMSTRONG and WAGNER (1961b) for the reductoisomerase of Salmonella. Figure 2 shows the type of results obtained with wild type and a sample of isoleucine-valine mutants. It will be noted that three bands appear on the gel for wild type. These represent areas on the gel where $NADPH_2$ is oxidized to NADP in the presence of either α-acetolactate or α-aceto-α-hydroxybutyrate. They do not appear in the absence of these substrates or $NADPH_2$. When certain other related compounds such as the ethyl esters of the α-aceto acids, and the hydroxy-keto acids, α-keto-β-hydroxyisovaleric acid and α-hydroxypyruvic acid are used as substrates, spots appear in the same general region on the gels as shown in Figure 3.

The results obtained from electrophoresis of certain of the mutants are given in Figure 2 where they are compared to wild type. It will be noted that the group 1 mutants T320, T325, T305 and T324 all show an absence of the lowest of the three bands found in wild type and the group 3 mutants T330, T329 and T328. All suspected group 1 mutants have been analyzed by us in this fashion and found to have this band missing on the gels after electrophoresis. All other mutants produce this band with both α-aceto acids as substrates.

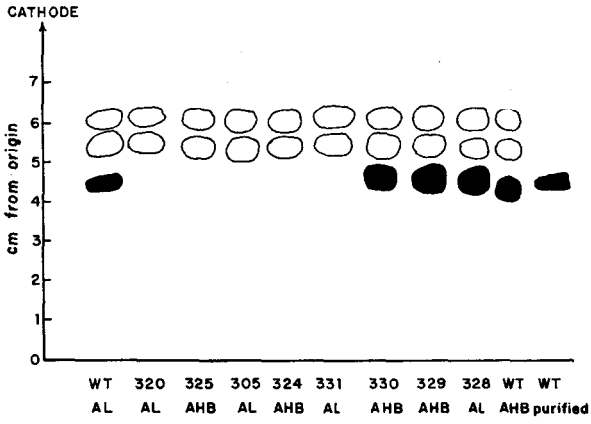

FIGURE 2.—Tracings of patterns obtained on starch gel after electrophoresis of 82,000 $\times$ g supernatant of Neurospora homogenate. The bands represent areas of activity on the gel for the oxidation of $NADPH_2$ in the presence of the substrates AL (α-acetolactate) and AHB (α-aceto-α-hydroxybutyrate). The dark bands represent the areas which have activity for the conversion of the α-aceto acids to the dihydroxy acids. The nature of the reaction(s) in the other areas of activity is unknown. WT = wild type. Mutants 320, 325, 305, and 324 are group 1 mutants. Mutants 331, 330, 329, and 328 are group 3 mutants.

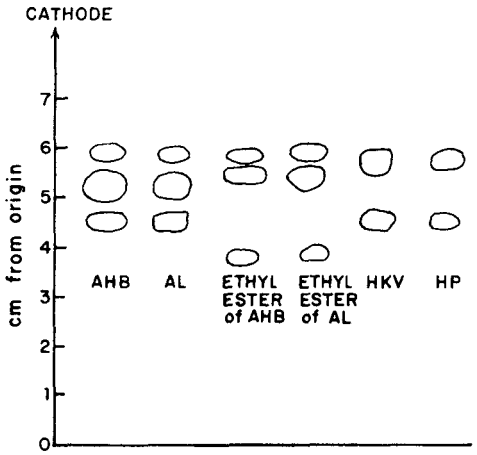

FIGURE 3.—Tracings of patterns obtained on starch gel after electrophoresis of 82,000 × *g* supernatant of wild-type Neurospora homogenate. The bands represent areas of activity on the gel for the oxidation of $NADPH_2$ in the presence of the substrates AHB (α-aceto-α-hydroxybutyrate), AL (α-acetolactate), their respective ethyl esters, HKV (α-keto-β-hydroxyisovaleric acid) and HP (hydroxypyruvic acid).

To determine whether the actual reductoisomerase is located in the lower band, gels were sliced transversely to separate the three areas of activity, and the enzyme extracted from the gel by breaking it up partially by forcing it through a hypodermic needle (ARMSTRONG and WAGNER 1961b). The extruded material was then centrifuged at 82,000 × *g* for ½ hour and the supernatant material tested for activity.

This was done with the following incubation mixture: 9 mg protein extract from the gel, 100 μmoles glucose-6-phosphate, 20 K units of glucose-6-phosphate dehydrogenase, 10 μmoles $MgSO_4$, 1 μmole NADP, 100 μmoles of α-aceto-α-hydroxybutyrate or 200 μmoles of α-acetolactate, all in a total volume of 2 ml of 0.05 M phosphate buffer at pH 7.5. Gel extracts from two strains, the wild type and a group 3 mutant, T323, were incubated in this mixture, separately, for 3 hr at 37°C. The incubates were then placed in a boiling water bath for 5 min and the precipitate was removed by centrifugation. The supernatants were analyzed for the presence of the dihydroxy acids, α, β-dihydroxyisovalerate or α, β-dihydroxy-β-methylvalerate by paper chromatography as described by WAGNER, RADHAKRISHNAN and SNELL (1958).

No dihydroxy acids were identified in the incubation mixtures containing protein from the upper two bands in either strain or from the lowest band from wild type. However, the lowest band from T323 produced dihydroxy acids from both α-aceto acids as identified by their Rf values and their reaction with periodate.

The surprising absence of activity for the production of dihydroxy acids from these three areas of the gels by the wild-type strain led us to test the remaining areas of the gels containing protein with similar negative results. Therefore, an attempt was made to purify the wild-type reductoisomerase with the objective of removing a possible inhibitor present in crude extract.

A partial purification was achieved by making an initial precipitation in crude, centrifuged extract with protamine sulfate using a 1.5 percent solution of the protamine in a proportion of

3.4 ml to 1 g of extract protein. The supernatant from this was treated with ammonium sulfate at 4°C. The precipitate from 65 to 70 percent saturated ammonium sufate at 4°C was collected and resuspended in 0.1 M phosphate buffer at pH 7.5. It was found to have a specific activity five to ten times greater than the crude extract. Attempts at further purification by means of DEAE and methyl cellulose columns only resulted in complete loss of activity.

When the partially purified preparation was tested with the above described incubation mixture both dihydroxy acids were synthesized from their respective α-aceto acids. Electrophoresis of the purified fraction resulted in the production of a single $NADPH_2$-oxidizing active band on the gels shown in Figure 2.

Additional evidence that the purified preparation was indeed the reductoisomerase was provided by noting the effects of omitting Mg^{++} from the assay mixture. Table 1 presents results which show that the omission of Mg^{++} results in the complete loss of activity by the enzyme. This is in agreement with the findings of RADHAKRISHNAN, WAGNER and SNELL (1960) and ARMSTRONG and WAGNER (1961a) with the reductoisomerases of *Escherichia coli*, *Neurospora crassa* and *Salmonella typhimurium*.

These results indicate that some inhibitory material is present in the wild-type extracts, but not in T323, which moves with the reductoisomerase on the gel during electrophoresis. Taken together they also indicate that the bottom spot of the three which does not appear in the group 1 mutants is indeed the reductoisomerase enzyme. Furthermore, it is also evident from these considerations that a spectrophotometric determination of reductoisomerase activity in crude extracts is quite meaningless because of the presence of at least two other enzymes actively oxidizing $NADPH_2$ in the presence of the α-aceto acid substrates.

The demonstrated apparent complete absence of reductoisomerase activity in the group 1 mutants together with the evidence that they accumulate the α-aceto acids makes it certain that they are blocked at the reductoisomerase step. In agreement with this conclusion are the results obtained from a determination of the 39,000 $\times$ *g* pellet and supernatant activity of two of the group 1 mutants, T305 and T322, given in Figure 4.

It has been shown that the pellet obtained from a crude homogenate of wild type centrifuged down at 39,000 $\times$ *g* contains activity for the conversion of pyruvate to valine and α-ketobutyrate + pyruvate to isoleucine (WAGNER and BERGQUIST 1963). In addition to pyruvate, the other known valine precursors, α-acetolactate, α,β-dihydroxyisovalerate and α-ketoisovalerate, were also active, the latter

TABLE 1

Effect of Mg^{++} *on activity of partially purified reductoisomerase enzyme**

Substrate	0.001 M Mg^{++}	Specific activity μmoles/mg/hr
α-aceto-α-hydroxybutyrate	+	28.3
α-aceto-α-hydroxybutyrate	—	0.0
α-acetolactate	+	4.5
α-acetolactate	—	0.0

* 7.7-fold purification of wild-type crude enzyme preparation.

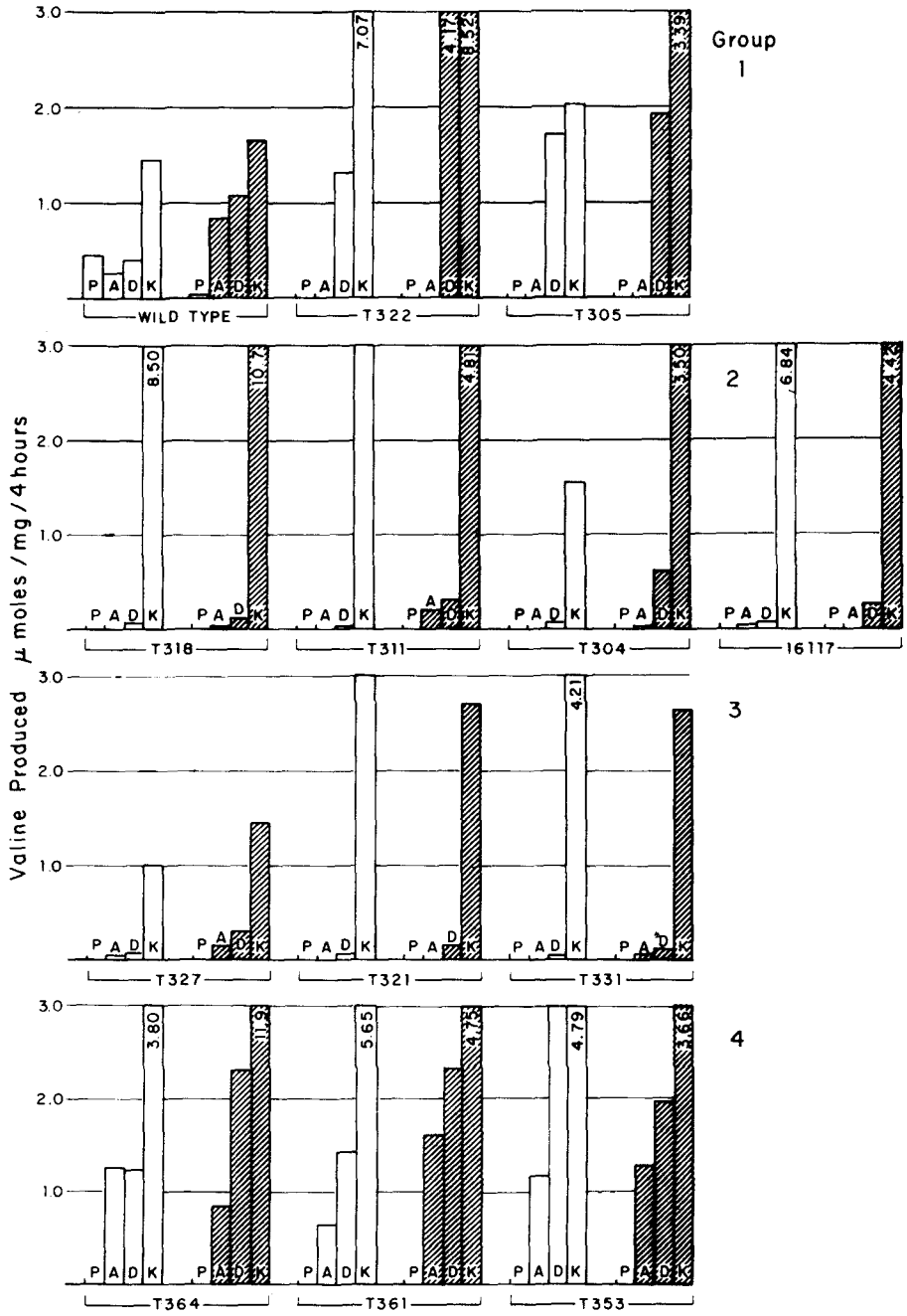

FIGURE 4.—Activities of the 39,000 × *g* pellets (open bars) and 39,000 × *g* supernatants (hatched bars) of wild-type and certain isoleucine-valine mutants for the production of valine from the indicated substrates. P = pyruvate, A = α-acetolactate, D = α, β-dihydroxyisovalerate, K = α-ketoisovalerate. The numbers in some of the bars indicate specific activities for those cases in which the scale was exceeded. The mutants are arranged according to the groups defined in the text.

three also being active with the supernatant as shown in Figure 4. In agreement with the data presented for the absence of this enzyme in the supernatant of the group 1 mutants, the pellets of T305 and T322 had no measurable activity for the production of valine from pyruvate or α-acetolactate, but both α,β-dihydroxyisovalerate and α-ketoisovalerate are active precursors.

A previous report presented data to show that the 82,000 $\times$ g crude extract supernatants from the group 1 mutants have dihydroxy acid dehydrase activities in the wild-type range (WAGNER, KIRITANI and BERGQUIST 1962). Table 2 presents data showing that they also have activity for the condensing enzyme and the transaminase in the range demonstrated for wild type grown on isoleucine and valine. The presence of isoleucine and valine in the medium of wild type has a decided effect on the activity of the condensing enzyme, as shown in the table during the early phase of growth. A similar effect is not noted for the transaminase or the dehydrase (Table 3). It may be assumed, therefore, that the relatively low, but measurable condensing enzyme activity found for the mutants is due to repression by the end products. This is in part borne out by the data in

TABLE 2

*Condensing enzyme and transaminase activities of several group 1 mutants**

Strain	Condensing enzyme			Transaminase		
	48 hr‡	68 hr	115 hr	48 hr	68 hr	115 hr
T305	0.05	0.05	0.09	24.7	8.7	4.5
T322	0.07	0.07	0.06	11.8	4.8	7.2
T324	0.10	0.16	0.08	8.6	6.6	8.3
T325	0.09	0.05	0.12	16.6	6.2	3.4
T320	0.02	0.02	0.11	8.6	7.8	6.1
T319	0.09	0.10	0.12	17.7	3.9	8.2
KJT1960	0.01	0.39	0.16	5.9	1.0	4.2
KJT1960†	0.41	0.44	0.21	4.3	1.5	2.2

* Mycelium was grown on minimal medium supplemented with 10 μmoles of L-isoleucine and 10 μmoles of L-valine per 25 ml in standing culture at 25°C for periods indicated. Specific activities are given as μmoles/mg protein/hr.
† No isoleucine + valine added.
‡ Age of mycelium.

TABLE 3

*Condensing enzyme, dehydrase and transaminase activities of several group 2 mutants**

Strain	Condensing enzyme			Dehydrase			Transaminase		
	48 hr‡	68 hr	115 hr	48 hr	68 hr	115 hr	48 hr	68 hr	115 hr
T311	0.00	0.02	0.02	0.12	0.07	0.11	7.9	2.7	2.6
T318	0.00	0.11	0.11	0.10	0.32	0.15	7.7	3.1	3.6
T304	0.03	0.05	0.03	0.12	0.26	0.22	9.3	3.2	2.4
16117	0.01	0.09	0.05	0.10	0.29	0.19	5.1	3.8	2.5
KJT1960	see Table 2			0.43	0.47	0.55	see Table 2		
KJT1960†	see Table 2			0.58	0.51	0.56	see Table 2		

* Conditions as in Table 2.
† No isoleucine + valine added.
‡ Age of mycelium.

Table 2 since as the mycelium gets older, and the isoleucine and valine presumably are incorporated into protein or degraded, the condensing enzyme activity increases.

In addition to lacking reductoisomerase, the group 1 mutants uniformly fail to grow on isoleucine and valine supplemented media at a pH higher than 7.5. Furthermore, they appear to have a complete block, since they show no growth on a minimal medium after ten days.

Since the group 1 mutants are obviously blocked at the reductoisomerase step, and *in vitro* production of valine, at least, has been demonstrated from α,β-dihydroxyisovaleric acid and α-ketoisovaleric acid, they should all be expected to grow on the dihydroxy acids or one of them plus the other amino acid. Auxanographic tests have, however, shown complete inactivity of the dihydroxy acids for all of the mutants tested. Of the 18 group 1 mutants tested for growth on the two α-keto acid precursors, only nine gave measurable growth after four days in minimal medium supplemented with 20 μmoles/25 ml of both acids. Auxanography also produced negative results for the nongrowers showing that it is probably not the proportion of α-keto acids added that is the limiting factor.

The group 2 mutants, T304, T318, T311 and 16117, have been difficult to classify biochemically because they do not have a uniform phenotype such as is found for the group 1 mutants. They are closely linked (recombination ca. 0.05 percent) to the group 3 mutants, and some share in common with them the accumulation of the dihydroxy acids and carbinols (WAGNER, KIRITANI and BERGQUIST 1962) in the growth medium. Also similar to the group 3 mutants is the fact that their supernatant extracts have a somewhat lower activity for the dihydroxy acid dehydrase enzyme than wild type, as shown in Table 3. But the activity of this enzyme depends in part on the age of the mycelium, and the method of preparation, and only a quantitative difference is evident between them and wild type. T311 exhibits the most significant difference from wild type in this respect.

Table 3 also gives the condensing enzyme and transaminase activities of the supernatant extracts. Compared to the wild type the condensing enzyme is somewhat lower in all four mutants. The transaminase is higher, however, particularly in extracts from young mycelium. Of the four mutants listed in Table 3 only T304 does not grow at pH 7.5 or above. In this respect it resembles the group 1 mutants, though in other respects it resembles the group 3 mutants because of its accumulation of both dihydroxy acids (WAGNER, KIRITANI and BERGQUIST 1962).

It is only when the activity of the 39,000 $\times$ *g* pellets and supernatants of these mutants is examined that a clear picture is obtained of their biochemical deficiency. Figure 4 gives data for four of them showing that they are clearly unable to use either pyruvate, α-acetolactate or α,β-dihydroxyisovalerate for the efficient production of valine *in vitro*.

None of the group 2 mutants grow in the presence of the α-aceto acids or the dihydroxy acids as supplements in place of isoleucine plus valine, but all of them grow on a combination of the two α-keto acids giving from 14 to 100 mg dry

weight of mycelium after four days growth at 25°C in 25 ml of medium.

The group 3 mutants display a uniform phenotype with two exceptions to be discussed. All nine strains in the group accumulate dihydroxy acids, and seven of them have lower dihydroxy acid dehydrase activity than the wild type, but this activity is never completely absent. This same group of seven is also capable of growing on unsupplemented minimal medium after three to four days at 25°C, and is unaffected by a change in pH to pH 9.0 for growth on medium supplemented with isoleucine and valine (WAGNER, KIRITANI and BERGQUIST 1962).

Two mutants, T321 and T331, are classified in group 3 because of their undoubted close linkage to the others (KIRITANI 1962). However, in addition to accumulating the dihydroxy acids (Table 4) they also accumulate carbinols (WAGNER, KIRITANI and BERGQUIST 1962) and one of them, T331, is lacking in reductoisomerase as determined by the electrophoretic method as shown in Figure 2. A previous report that it possessed reductoisomerase activity was based on the spectrophotometric determination of activity of crude extracts.

All group 3 mutants have been tested for the production of the α-keto acids by incubating extracts from each of them with the dihydroxy acids.

Two ml of 82,000 $\times$ *g* supernatant containing approximately 10 mg of protein per ml was added to a mixture containing 2 ml of one of the dihydroxy acids (containing 70 μM of α, β-dihydroxyisovaleric acid or 86 μmoles of α,β-dihydroxy-β-methylvaleric acid), 0.5 ml of 0.1 M $MgSO_4$ and 2 ml of 0.1 M phosphate buffer at pH 8.5. Appropriate controls were prepared in which all ingredients except the dihydroxy acids were present. The mixture was incubated for 4 hr at 37°C, the protein precipitated by adding 1 ml 6N HCl, and 2 ml of 2, 4 dinitrophenylhydrazine in 2N HCl added. The dinitrophenylhydrazones formed after 20 min were dissolved in ethyl acetate and chromatogramed using the method described by WAGNER and BERGQUIST (1955). The dinitrophenylhydrazone spots were identified by comparing with known standards.

Incubation mixtures from all nine strains tested showed the presence of the expected α-keto acid analogues of isoleucine and valine. However, the control

TABLE 4

*Quantitative estimate of dihydroxy acids accumulated by certain isoleucine-valine mutants**

Strain	μmoles/mg dry weight of mycelium	
	α,β-dihydroxy-isovaleric acid	α,β-dihydroxy-β-methylvaleric acid
T311	0.60	0.32
T331	0.74	0.48
T321	1.1	0.36
T326	1.2	0.17
T327	1.2	0.34
T330	1.3	0.36
T328	1.0	0.45
T329	0.94	0.41
T323	0.88	0.39
T332	1.9	0.71

* All isoleucine-valine mutants were tested to determine if the dihydroxy acids were accumulated. The ones listed in this table are all that demonstrated both acids in the growth medium.

mixtures of three of them also contained keto acids so it could not be determined that keto acids were made *in vitro* in the presence of the dihydroxy acids, but they obviously are made *in vivo*.

To demonstrate the accumulation of the dihydroxy acids, α,β-dihydroxyisovalerate and α,β-dihydroxy-β-methylvalerate in the growth media of the group 3 mutants, they were grown on minimal medium supplemented with 20 μmoles each of L-valine and L-isoleucine per 25 ml for five days at 25°C in 125 ml flasks.

The mycelium was removed, brought to dry weight and the weight recorded. The medium was reduced to dryness *in vacuo*, and 0.5 ml of 50 percent H_2SO_4 added for each 25 ml of original volume. After all soluble material was in solution in the acid, 1 g of anhydrous Na_2SO_4 was added and allowed to stand 15 min. This was extracted successively with two 5 ml portions of ethyl ether. The ether soluble fraction was reduced to 1 ml volume and spotted on Whatman No. 1 filter paper. Chromatography was carried out with a solvent system, and the spots developed by the method, described by WAGNER, RADHAKRISHNAN, and SNELL (1958). After the spots were identified by comparing the Rf's with known standards, they were cut out of the papers, dried and weighed. The weights determined were converted to μmoles of dihydroxy acids by reading from a standard curve prepared by subjecting known quantities of dihydroxy acids to the same chromatography procedure.

The results of the determinations are given in Table 4 as μmoles dihydroxy acid/mg dry weight mycelium. The data show that all group 3 mutants, and, in addition, the group 2 mutant, T311, accumulate considerable quantities of both dihydroxy acids, and that generally, the dihydroxy acid analogue of valine is accumulated in significantly higher amounts than the isoleucine analogue.

Table 5 gives the specific activities of the condensing enzyme, dehydrase and transaminase of the group 3 mutants. These activities, as in the case of the previous two groups, were determined with extracts prepared from three different ages of mycelia. It is evident from the data that all strains have measurable activity for all three enzymes. All the group 3 mutants have reductoisomerase activity as determined electrophoretically.

The activities of the 39,000 × *g* pellets and supernatants from several of the

TABLE 5

*Condensing enzyme, dehydrase and transaminase activities for the group 3 mutants**

Strain	Condensing enzyme			Dehydrase			Transaminase		
	48 hr†	68 hr	115 hr	48 hr	68 hr	115 hr	48 hr	68 hr	115 hr
T331	0.02	0.01	0.06	0.38	0.66	0.33	7.9	7.2	2.1
T321	0.00	0.18	0.28	0.16	0.19	0.35	7.4	2.8	2.8
T326	0.07	0.37	0.21	0.17	0.46	0.34	10.8	6.1	3.2
T327	0.01	0.02	0.12	0.17	0.00	0.35	12.8	8.0	4.0
T330	0.06	0.17	0.17	0.17	0.20	0.34	10.0	5.8	2.9
T328	0.04	0.27	0.16	0.26	0.42	0.28	10.0	6.2	2.6
T329	0.05	0.47	0.15	0.19	0.27	0.32	8.2	5.8	3.8
T323	0.04	0.27	0.13	0.11	0.42	0.42	9.7	2.2	3.7
T332	0.01	0.05	0.19	0.07	0.27	0.12	4.8	6.6	2.2

* Conditions as in Table 2. See Tables 2 and 3 for wild-type activities.
† Age of mycelium.

group 3 mutants were determined. Figure 4 shows that they possess slight activity for valine production from α,β-dihydroxyisovalerate and α-acetolactate.

Like the group 1 and 2 mutants the group 3 mutants did not respond to the α-aceto acids or the α,β-dihydroxy acids as growth supplements. However, all except two, T331 and T328, responded in varying degrees to the presence of a combination of the α-keto acid analogues of the amino acids. This response ranged from 5.6 to 71 mg dry weight of mycelium after four days growth at 25°C in 25 ml of medium, and is in addition to the trace of growth given by these mutants after four days on minimal medium.

The group 4 mutants, as described previously, are in linkage group IV, and hence on a different chromosome from the three previously described mutants. All the group 4 mutants have been tested electrophoretically for the presence of reductoisomerase activity on starch gel and found to possess it. In addition, the supernatants from centrifuging crude homogenate of the mutants at 82,000 × *g* have activity for the condensing enzyme, dehydrase and transaminase steps as shown in Table 6. None of them grow at pH 7.5 or above in standing culture on minimal medium supplemented with isoleucine and valine.

Despite the evident presence of condensing enzyme in the 82,000 × *g* supernatant extracts, the 39,000 × *g* pellet and supernatant preparations are not able to synthesize measurable valine from pyruvate. The data supporting this conclusion are given in Figure 4 in which determinations are given for three representative mutants from group 4.

None of the group 4 mutants have been tested for growth on precursors other than the α-keto acids. None gave measurable growth on the two α-keto acids after four days in minimal medium supplemented with 20 μmoles/25 ml of both acids.

DISCUSSION

It is evident from the data presented here that the definitive analysis of the group 2, 3 and 4 mutants with respect to their inability to produce isoleucine and valine sufficient for normal growth is made possible by determining the overall

TABLE 6

*Condensing enzyme, dehydrase and transaminase activities of several group 4 mutants**

Strain	Condensing enzyme	Dehydrase	Transaminase
T354	0.09	0.23	10.6
T361	0.10	0.29	16.3
T345	0.50	0.28	9.3
T353	0.02	0.79	10.2
T364	0.03	1.15	5.8
T346	0.04	0.89	7.0
T355	0.13	1.17	8.1
T363	0.10	1.00	7.9

* Mycelium 24 hours old grown in shake culture except for T346 and T355 which were grown in standing culture for 66 hours. Other conditions as in Table 2.

ability of the mutant strains to synthesize valine from pyruvate, and precursors following valine. Examining the supernatants for the soluble individual enzymes in the pathway gives results which are sufficiently ambiguous to make it impossible to determine with certainty where the blocks occur.

We have hypothesized that the system for the synthesis of valine and isoleucine is an organized one associated with the mitochondria, either inside or outside of these organelles, and that it operates *in vivo* as a unit enzyme complex (WAGNER and BERGQUIST 1963). Further, it is possible that the enzymes which are found in the supernatant of the wild type are either artifacts of preparation of the homogenate, or the result of the natural breakdown of mitochondria in the intact mycelium, or both. This may be true for the soluble enzymes found in the mutants, but an additional factor may enter here, that being that the hypothetical functional unit may not be put together properly in the group 2, 3 and 4 mutants and therefore some of the enzymes may never have been attached. The fact that the activity of some of the soluble enzymes in certain of the mutants is higher than in the wild type would support this latter possibility, although this may also be the result of release of enzyme repression in the mutants which are not making endogenous isoleucine and valine.

The group 2 and 3 mutants, although separable genetically, are otherwise quite similar in their ability to carry out readily the transamination step in the particulate and supernatant fractions, but are relatively inefficient in converting α,β-dihydroxyisovaleric acid or α-acetolactate to valine. We can assume from this that they are probably effectively blocked at the dihydroxy acid dehydrase step, but not because they cannot produce the enzyme which is obviously active *in vitro*.

These considerations raise the interesting possibility that a mutation may change the organization of an organized complex with the result that it may no longer function satisfactorily. This may be visualized as occurring in at least two ways. First, the mutation may alter the configuration of an enzyme in such a way as not to drastically affect its catalytic ability, but to affect its capacity to bind and orient in a particular way into the unit in which it ordinarily functions. Second, the mutation may affect a protein which has no catalytic function but acts only as a binder to hold enzymes together in a functional complex (WAGNER, KIRITANI and BERGQUIST 1962).

Thus the group 2 and 3 mutants may have gene changes affecting the dihydroxy acid dehydrase moiety of the postulated complex in such a way that it is improperly oriented in the complex. The lack of uniformity of phenotype among the group 2 mutants, and the differences between T331, T321 and the other group 3 mutants may be a manifestation of the number of different ways in which the complex may be altered by mutation of a particular genetic segment controlling the formation of just one polypeptide. Mutant T318 in group 2 is particularly puzzling in that it accumulates carbinols, but not the dihydroxy acids, yet has the reductoisomerase according to the electrophoretic studies. Nonetheless, its pellet activity and supernatant activity for synthesis of valine indicates a definite inability to utilize any precursor prior to α-ketoisovaleric acid at a rate compar-

able to wild type. Mutant T304 in group 2 also accumulates carbinols, and has a slight dehydrase activity in the pellet fraction, but in addition seems to accumulate α,β-dihydroxyisovalerate. The accumulation of both types of carbinols and both types of dihydroxy acids by T331 and T321 presents similar difficulties for explanation. Neither of these mutants show activity for the conversion of α-acetolactate to valine in the pellet fraction. T331 shows slight activity in the supernatant for this conversion as do the other group 3 mutants with the exception of T321. A possible explanation is that both mutants have some capacity to carry out the synthesis of the α-aceto acids (which become carbinols on decarboxylation as shown by WAGNER, BERGQUIST and FORREST 1959) and dihydroxy acids from pyruvate, but the synthesis of dihydroxy acids is so slow carbinols accumulate, and the conversion of dihydroxy acids to α-keto acids is likewise slow causing an accumulation of dihydroxy acids. This would require that both the reductoisomerase and dehydrase steps be affected by the mutation. This is apparently true for T331 which shows no reductoisomerase activity by the relatively crude method used to demonstrate the enzyme on starch gel after electrophoresis, but T321 appears to have the reductoisomerase in sufficient amounts to be demonstrable. It should be noted that both mutants have the soluble dehydrase in a range comparable to wild type, but neither shows a wild-type rate for the synthesis of valine from the dihydroxy acid analogue. These considerations indicate that the disorganization of the valine synthesizing system may be more drastic in these two mutants than in the other group 3 mutants which accumulate only the dihydroxy acids.

The group 4 mutants present a relatively uniform phenotype in so far as they accumulate nothing that we can detect in the medium after four days growth, and show activity for all four enzymes, although the condensing enzyme in some of them is low in the young mycelium tested as shown in Table 6. The data from testing the activity of the 39,000 × *g* pellets and supernatants from these mutants clearly indicate that synthesis from pyruvate is blocked at the condensing enzyme step. Whether this is the result of an inadequate amount of enzyme activity *per se* or the result of a disorganization of postulated synthesizing system cannot be determined from the present data. The latter possibility would seem more likely, however, because the soluble condensing enzyme activities are in the wild-type range. An additional alternative also might be that we are not determining the formation of acetylmethylcarbinol from α-acetolactate formed from pyruvate and active acetaldehyde, but rather carbinol formed directly from acetaldehyde derived from pyruvate (JUNI and HEYM 1956). There is no indication that this pathway exists in wild-type Neurospora, however (RADHAKRISHNAN and SNELL 1960).

The group 1 mutants are the only ones which appear to be the result of an orthodox type of mutation of a structural gene, since no reductoisomerase activity can be found in the soluble fraction of the homogenate. It was not surprising therefore to find that neither pyruvate nor acetolactate can act as precursors for valine synthesis in the homogenate fractions tested. The activities of the other three enzymes do not appear to be affected except that the soluble dehydrase

and transaminase show in general a higher activity than wild type (Table 2, and WAGNER, KIRITANI and BERGQUIST 1962).

The reductoisomerase is in itself an interesting enzyme. It catalyzes the transformation of the α-aceto acid precursors of valine and isoleucine to the respective dihydroxy acids. This involves two quite distinct changes, an isomerization and a reduction. All attempts to separate these two steps by the detection of intermediates such as α-keto-β-hydroxyisovalerate and α-keto-β-hydroxymethylvalerate, which would be expected to be formed if the α-aceto acid precursors were first isomerized before reduction, have been unsuccessful in studies on reductoisomerase activity *in vitro* (RADHAKRISHNAN, WAGNER and SNELL 1960). There is present, however, in Neurospora, and in a number of other organisms tested, an enzyme termed the reductase which converts the α-keto-β-hydroxy acids to the dihydroxy acids (RADHAKRISHNAN, WAGNER and SNELL 1960; ARMSTRONG and WAGNER 1961a, b). This enzyme has been separated from the reductoisomerase activity in both Neurospora and Salmonella and shown to be inactive with the α-aceto acid precursors at least with respect to converting them to the dihydroxy acids. Alternatively, purified reductoisomerase is not active with the α-keto-β-hydroxy acids. If α-keto-β-hydroxy acids are tested as substrates to determine whether they are reduced in the presence of $NADPH_2$ on starch gel after electrophoresis, two bands of activity are found as shown in Figure 3. These correspond to the upper and lower bands obtained with the α-aceto acid substrates with respect to their location on the gels. A similar situation is found on comparing the reductoisomerase and reductase of Salmonella (ARMSTRONG and WAGNER 1961b). These findings indicate that a relationship may exist between the reductase and reductoisomerase. This is supported by the demonstration that Salmonella strains with a deficiency of reductoisomerase (the *ilvaA* mutants) produce a reductase with a different electrophoretic mobility than wild type (ARMSTRONG and WAGNER 1962). We have not, however, noted a difference in the electrophoretic mobility of the reductases from the group 1 mutants of Neurospora (unpublished data).

That the presumed intermediates, the α-keto-β-hydroxy acids, occur naturally has been demonstrated (RADHAKRISHNAN, WAGNER and SNELL 1960). The growth medium of the isoleucine-valine mutant, 16117, accumulates them as well as the dihydroxy acids. We have examined the growth medium of the group 1 mutants to determine if they too accumulate the α-keto-β-hydroxy acids, but have been unable to detect them. However, all of these mutants have the reductase present at the wild-type level or higher (unpublished data). Thus the mutations in the group 1 mutants do not eliminate the reductase activity while eliminating the reductoisomerase activity. Since carbinols are accumulated by these mutants, it may be tentatively concluded that if the reductase is indeed part of the reductoisomerase complex *in vivo*, the group 1 mutations affect the isomerase step in the complex, and not the reductase step.

SUMMARY

A number of isoleucine-valine mutants of *Neurospora crassa* which had been previously characterized genetically and placed in three groups in linkage

group V have been more specifically characterized biochemically. The group 1 mutants have been definitely shown to lack the reductoisomerase enzyme. The group 2 and 3 mutants, some of which show a somewhat lower soluble dihydroxy acid dehydrase activity than the wild type, have been shown by analyzing the ability of fractions of their homogenates to carry out the overall synthesis of valine from pyruvate to have a partial block at the step at which the dihydroxy acid precursor of valine is converted to the α-keto acid analogue. This effectively prevents them from synthesizing sufficient valine (and presumably isoleucine) from pyruvate for normal growth. All of the group 2 and 3 mutants are shown to have sufficient dehydrase activity to demonstrate synthesis of the α-keto acids *in vitro*.

A new group of isoleucine-valine mutants, the group 4 mutants, is described. These have been located in linkage group IV. They have been found to be blocked at the condensing enzyme step, although they appear to have soluble condensing enzyme in their homogenates.

It is suggested that the group 2, 3 and 4 mutants are organizational mutants in the sense that they are able to produce the necessary complement of enzymes, but are unable to organize them *in vivo* for an effective synthesis of the required amino acids.

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