THE *iv-3* MUTANTS OF *NEUROSPORA CRASSA*[1]
I. GENETIC AND BIOCHEMICAL CHARACTERISTICS

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A group of isoleucine-valine requiring mutants of *Neurospora crassa* referred to as the group 4 mutants have been described in an earlier publication from this laboratory (WAGNER, BERGQUIST, BARBEE and KIRITANI 1964). Genetic analyses indicated that they were located in linkage group IV. Mitochondrial fractions and supernatants from them were unable to carry out the overall synthesis of valine from pyruvate, but were able to do so from α-acetolactate. The data indicated that they were blocked at the step at which pyruvate and α-ketobutyrate are condensed with "active" acetaldehyde to form α-acetolactate and α-aceto-α-hydroxybutyrate, the two acetohydroxy acid precursors of valine and isoleucine, respectively.

This present communication further characterizes these mutants genetically and biochemically, and presents data which make it clear that they should now be referred to as the *iv-3* mutants according to the standard Neurospora genetic nomenclature rather than group 4 mutants. An accompanying communication by CAROLINE, HARDING, KUWANA, SATYANARAYANA and WAGNER (1969) describes the enzyme deficiency found in the *iv-3* mutants

MATERIALS AND METHODS

Strains: The wild-type strain used in the crosses and as a source of enzyme was KJT1960A. This strain was derived from a cross between EM 5256A and EM 5297a. The mutant strains were either isolated in this laboratory or obtained from the Fungal Genetics Stock Center. The *iv-3* (group 4) mutants 344, 345, 346, 349, 353, 354, 355, 357, 360, 361, 363, 364 and 366 were all isolated after ultraviolet irradiation of the *lys-1* strain, 33933A, and therefore were originally obtained as *lys-1, iv-3* double mutants (WAGNER *et al.* 1964). Single *iv-3* mutants were derived from these by outcrossing. The *iv-3* mutant Y7110, and the marker strains *me-5* (9666), *pdx-1* (37803), *ad-6* (28610), *pan-1* (5531), and *leu-2* (37501) were supplied by the Stock Center. Several *iv* strains not linked to the *iv-3* strain were also used. These, 305 and 322, alleles at the *iv-2* locus, and 304, 311, 321 and 326, alleles at the *iv-1* locus, have been described in detail by WAGNER *et al.* (1964).

Genetic analysis: The genetic analysis of the *iv-3* locus was made by random spore isolation and the plating method of NEWMEYER (1954). The ascospores were treated with 0.1% sodium hypochlorite before heat treatment at 60°C for 30 min to induce germination. The density of the ascospore suspensions used in plating was estimated by means of a hemocytometer.

Complementation studies: Conidia were washed by suspending in deionized water and cen-

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trifuging. Resuspended conidia were then added to minimal agar plates in spots of about 4 mm diameter. The conidial density was maintained at about 10^8 per ml. Conidia from two *iv-3* strains to be tested for complementation were inoculated in single spots and in combination. The plates were then observed for hyphal growth over a period of 7 days. Significantly greater growth in spots where there were mixtures of conidia of two strains than in the control spots was taken as positive evidence of complementation in heterokaryons.

Determination of pyruvate in the culture medium: The mutant strains were cultivated in 125 ml flasks in 25 ml of minimal medium supplemented with 1.6 mM DL-isoleucine and DL-valine. After growth for the appropriate period of time, 3 ml of the culture medium or its diluent was added to 1 ml of 2, 4-dinitrophenylhydrazine reagent made by saturating 2N HCl with the phenylhydrazine. After 10 min, 8 ml of ethylacetate was added. This mixture was aerated for 2 min with a glass capillary tube. Additional ethylacetate was added to restore the original volume and the ethylacetate layer containing the hydrazone of pyruvate removed. This was concentrated to 1/10 its volume over a steam cone, and 0.03 ml of the concentrate applied to Whatman No. 1 filter paper. Solutions of sodium pyruvate in concentrations of 20, 50, 100, 200, 300 and 400 μg/ml were treated by the same procedure, and also applied to the filter paper as reference standards. Chromatography was carried out with a solvent mixture containing n-butanol, ethanol and 0.5 N NH_4OH (7:1:2, v/v). The spots of hydrazones were cut out of the paper after drying and eluted with 1.5N NaOH. The effluent was diluted to a 4 ml volume with 1.5N NaOH and its absorbance determined at a wave length of 420 mμ. The amount of pyruvate hydrazone was determined by comparing the absorbance of the unknowns with the known standards.

RESULTS

Location of the iv-3 *locus:* It was evident from the earlier work of WAGNER *et al.* (1964) that the group 4 (*iv-3*) mutants were linked to *pan-1* in linkage group IV. A more precise determination of their location was undertaken by crossing a number of the group 4 mutants to a variety of group IV markers. A sample of the results is given in Table 1 which gives detailed data from 3-point

TABLE 1

Linkage data on random segregants from 3-point test crosses involving iv-3 *mutants**

Zygote genotype and recombination percent	Parental combination	Recombination: Singles region 1	Recombination: Singles region 2	Recombination: Doubles region 1 and 2	Total and percent germination	Marker isolation numbers
1. *pdx-1* + *pan-1*	52	8	4	0	150	37803
+ *iv-3* +	68	14	3	1	(94%)	T344
15.3 4.7						5531
2. *pdx-1* + *pan-1*	42	4	0	0	80	37803
+ *iv-3* +	26	6	2	0	(50%)	Y7110
12.5 2.5						5531
3. *pdx-1* *leu-2* +	69	0	4	0	102	37803
+ + *iv-3*	19	3	7	0	(68%)	37501
10.8 2.9						T344
4. *leu-2* + *ad-6*	65	3	7	0	191	37501
+ *iv-3* +	104	0	12	0	(23%)	T364
4.0 9.3						28610

* Cross 4 was analyzed by the plating method of NEWMEYER (1954). Crosses 1 and 2 differ in the *iv-3* strain involved, as indicated in the last column.

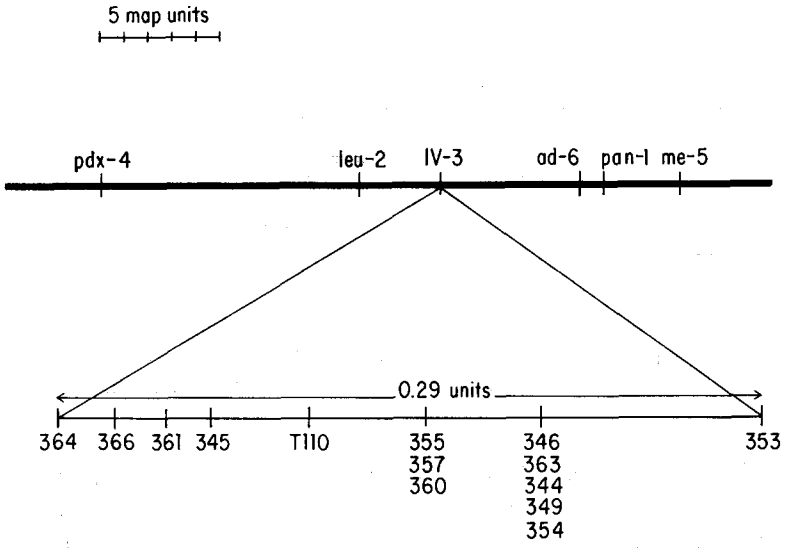

FIGURE 1.—Top, location of *iv-3* gene in linkage group IV and its relation to several other group IV markers. Bottom, proposed fine structure map of *iv-3* mutants.

test crosses. In addition, random spore isolations were made between all the available group 4 mutants and *me-5a* giving an average map distance of 12.4. The original *iv-3* mutants Y7110 and 8 of the group 4 mutants were also crossed to a *pdx-1 pan-1* double mutant. The results of all these crosses showed all to map between *pan-1* and *pdx-1*. Figure 1 summarizes these and other linkage data we have obtained. Our data are in essential agreement with MALING's (1959) for linkage group IV and place the group 4 *iv* mutants in the *iv-3* region at the indicated position in the right arm of IV between *leu-2* and *ad-6*.

Fine structure of the iv-3 locus: Fourteen of the *iv-3* mutants were crossed in all pairwise combinations. Though many of the crosses were infertile, a sufficient number produced adequate numbers of ascospores for a rough genetic analysis of the locus to be accomplished. The map given in Figure 1 was made utilizing most of the data from 80 successful crosses giving ascospores with a germination percentage of 2 to 56%. In general, 10^4 to 10^5 germinating ascospores were tested for each cross. Nineteen of the crosses gave unexpectedly high numbers of prototrophs. The results from these were not used in constructing the map, since they did not agree with the remaining data. It is assumed that these exceptional results are either due to the occurrence of pseudo-wild types or the "selfing" phenomenon (KIRITANI 1962; BAUSUM and WAGNER 1965) or both.

Complementation in heterocaryons: The fourteen strains of the *iv-3* were tested for intragenic complementation in all pairwise combinations in both the single mutant form and combined with other mutant genes such as *lys-1*, *me-5*, *me-7* and *pan-1*. The complementation map given in Figure 2 is a summary of the results obtained.

Accumulation of pyruvate: Because the block in the *iv-3* mutants seemed to

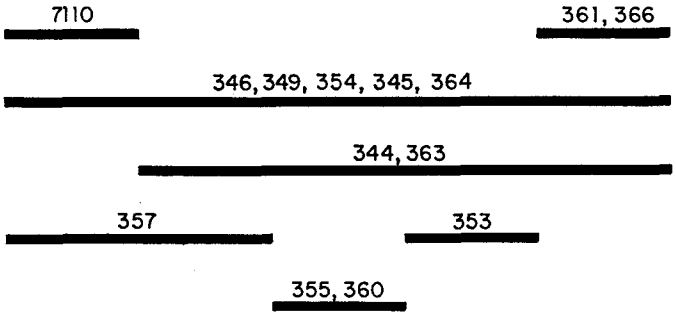

FIGURE 2.—Heterokaryon complementation map of *iv-3* mutants. The map was constructed following the usual convention of showing overlapping bars when complementation does not occur.

be located between pyruvate and α-acetolactate (and between pyruvate plus α-ketobutyrate and α-aceto-α-hydroxybutyrate) on the basis of the results obtained by WAGNER *et al.* (1964), the culture medium of the *iv-3* mutants was examined for the presence of excess pyruvate. Figure 3 shows that the *iv-3* mutants 344, 7110 and 364 accumulate large amounts of pyruvate. These mutants are not leaky (i.e. do not grow in the absence of isoleucine and valine). Those, like strain

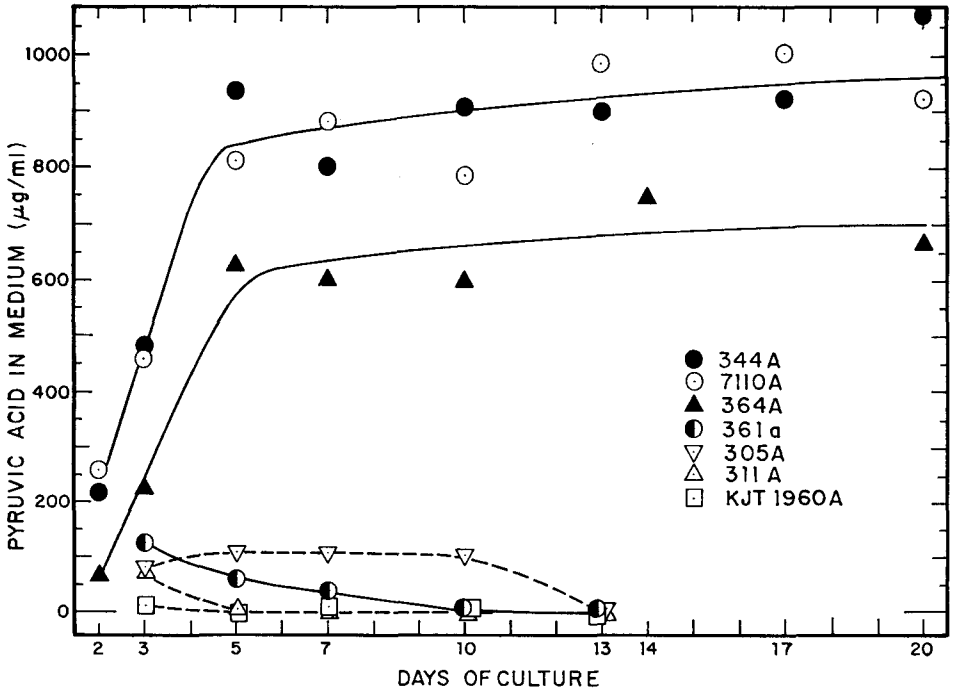

FIGURE 3.—Accumulation of pyruvic acid in the culture media of various strains as a function of time. The accumulation of the strains 344A and 7110A is shown by the same curve. For explanation of the strains see Figure 4.

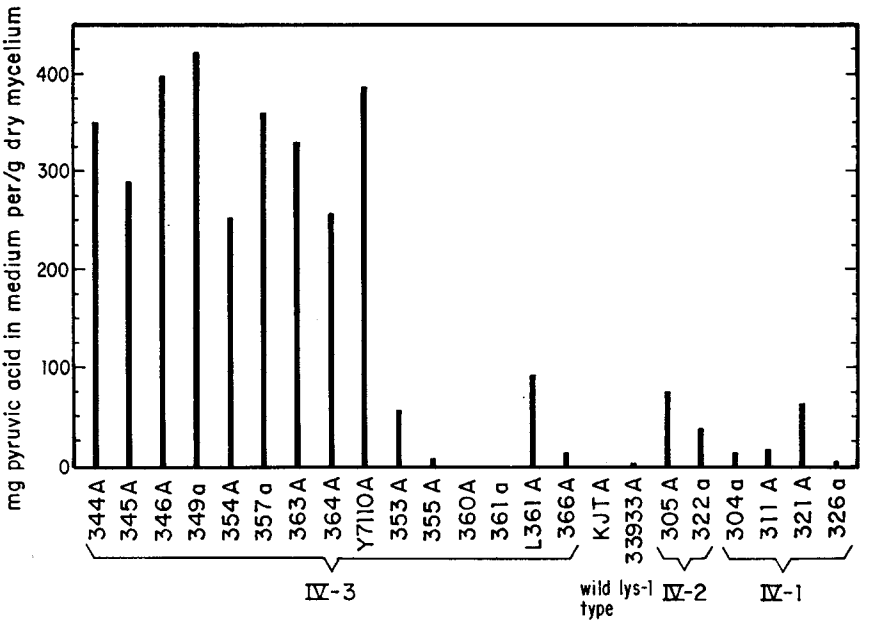

FIGURE 4.—Accumulation of pyruvic acid in the culture media of various strains after 7 days cultivation at room temperature without agitation. *iv-3* mutants between 344A and 7110A inclusive are not leaky. *iv-3* mutants between 353A and 366A inclusive are leaky. KJTA is the wild type, KJT1960A. 33933A is a *lys-1* mutant and the parent strain of all *iv-3* mutants except Y7110A. 305A and 322a are *iv-2* (formerly group 1 mutants), 304a and 311A are *iv-1* (formerly group 2), and 321A and 326a are *iv-1* (formerly group 3).

361 which is leaky, accumulate much less pyruvate, and this tends to disappear from the medium over a period of days (Figure 3). Figure 4 presents the results of pyruvate determinations in the culture medium of all available *iv-3* strains after 7 days of incubation. Some of the *iv* strains with mutations at loci located in the V linkage group also accumulate pyruvate as previously reported (WAGNER *et al.* 1964) and as indicated in the bottom part of Figure 4, but these accumulations are not as great as in the case of most of the *iv-3* mutants, except for 353, 355, 360, 361 and 366 which are all leaky mutants.

Accumulation of carbinols and pyruvate by double mutants: The *iv-2* mutant 305 (formerly referred to as a group 1 mutant) accumulates carbinols in its growth medium (WAGNER, SOMERS and BERGQUIST 1960; WAGNER, KIRITANI and BERGQUIST 1962). This is expected since the mutant is blocked at the reductoisomerase step at which α-acetolactate and α-aceto-α-hydroxybutyrate are converted to the dihydroxy acid precursors of valine and isoleucine. The α-aceto acids are not accumulated as such, but as the carbinol derivatives which result from their decarboxylation (WAGNER, BERGQUIST and FORREST 1959).

Mutant 305 was crossed to 7110, an *iv-3* mutant, and two double mutant segregants isolated. These were then tested for the accumulation of pyruvate and carbinols. The results are given in Table 2. It is evident that the double mutants

TABLE 2

Accumulation of carbinols and pyruvate in the culture media of 305A and Y7110 and their double mutants
(Carbinols and pyruvate given as μg per ml of culture medium)

Strain	305A (*iv-2*)		Y7110A (*iv-3*)		305-Y7110-32A (*iv-2, iv-3*)		305-Y7110-42a (*iv-2, iv-3*)	
Days of culture	Carbinol	Pyruvate	Carbinol	Pyruvate	Carbinol	Pyruvate	Carbinol	Pyruvate
2	6.8	0	0.3	0	0.2	32	0.0	0
4	634	48	3.5	584	1.0	416	0.5	192
7	2000	40	1.3	824	0.3	696	4.0	552
10	3330	0	0.0	1040	0.7	624	5.8	600
15	1850	0	0.8	1010	0.1	1010	5.5	696

accumulate only about as much carbinol as 7110 alone, but continue to accumulate large quantities of pyruvate.

These results provide further evidence that the block in the *iv-3* mutants is indeed prior to the reductoisomerase step at the point at which the α-aceto acids are synthesized.

DISCUSSION

The *iv-3* mutants of Neurospora had earlier been shown to complement to a limited extent by BERNSTEIN and MILLER (1961) who were able to construct an essentially linear complementation map with their results. We have found that our mutants, which were independently derived, give essentially the same results in complementation tests. The linear map constructed from the recombination data from crosses between the *iv-3* mutants shows some correlation with the complementation map which was, however, made using the results from a relatively small number of mutants. Further, the arrangement of the mutation sites on the fine structure genetic map that we have constructed can at best only be considered tentative, since only two point test crosses were carried out.

The accumulation of pyruvate by the *iv-3* mutants is of considerable interest considering the large number of reactions that pyruvate engages in in metabolism. However, the accumulation of pyruvate has been reported for other Neurospora mutants. For example, STRAUSS and PIEROG (1954) have shown that certain acetate requiring mutants (*ac*) accumulate pyruvate particularly in the presence of a suppressor gene, *sp*, which relieves the glucose inhibition of the *ac* sp^+ mutants. Additionally, STRAUSS (1956) has reported that mutants such as *suc* which require dicarboxylic acids also accumulate pyruvate. Various explanations have been offered for these accumulations such as a deficiency in pyruvic carbozylase (STRAUSS and PIEROG 1954) or a deficiency in a dicarboxylic acid source required for two carbon fragment breakdown in the tricarboxylic acid cycle (STRAUSS 1956). It has also been reported that actidione, an inhibitor of protein synthesis in fungi, causes the accumulation of pyruvate by *Neurospora sitophila*. This accumulation is partially relieved by the addition of thiamine (SEYDOUX and TURIAN 1962).

Obviously a number of different kinds of metabolic changes can cause the accumulation of pyruvate in Neurospora none of which are at present clearly understood. On the other hand, the accumulation of pyruvate by the *iv-3* mutants is in agreement with their being blocked at the step at which the α-acetohydroxy acid precursors of isoleucine and valine are synthesized.

SUMMARY

The *iv-3* locus of *Neurospora crassa* is located between *leu-2* and *ad-6* in linkage group IV, and includes those isoleucine-valine mutants formerly referred to as group 4 mutants. Complementation and recombination occur within the locus.—The *iv-3* mutants are apparently blocked at the step at which pyruvate and pyruvate plus α-ketobutyrate are converted to α-acetolactate and α-aceto-α-hydroxybutyrate, respectively. The accumulation of pyruvate in the culture medium of the mutants is one indication of the existence of the block at this point.

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