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*SYNTHESIS OF VALINE AND ISOLEUCINE IN THE PRESENCE OF A PARTICULATE CELL FRACTION OF NEUROSPORA**

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It has been established that valine is synthesized from pyruvate in a series of at least four steps, and that isoleucine is synthesized in a series of four similar steps from pyruvate and α-ketobutyrate. The four enzymes involved are apparently common to both pathways, but this has not been definitely proved in any one case. The enzymes will be described here as the *condensing enzyme*, which produces α-acetolactate and α-acetohydroxybutyrate from pyruvate, and pyruvate + α-ketobutyrate, respectively; the *reductoisomerase*, which converts the α-aceto-acids to the dihydroxy acids; the *dehydrase* which causes a dehydration of the dihydroxy acids with the formation of the α-keto acids; and the *transaminase* which transaminates between phenylalanine and the respective α-keto acids to form valine and isoleucine.

In at least two organisms, Salmonella and Neurospora, it has been shown that two or more of the genes affecting these enzymes are closely linked.[1-3] In *Neurospora crassa* at least two of the enzymes, reductoisomerase and dehydrase, are apparently controlled by two genes which are estimated to be about four map units apart.[4] Between them lies a region in which mutations have been detected only within a small segment. These mutations cause an absolute requirement for both isoleucine and valine, but the mutant strains do not show a deficiency for any of the four enzymes. That the blocks in these are *not* prior to pyruvate and α-acetobutyrate has been established by showing that they accumulate either the α-aceto-acids or dihydroxy acids or both. It was postulated that the defect in these mutants, the group II mutants, lies not in their inability to produce the enzymes, but in their inability to organize them properly to be active *in vivo*.[3] The experiments described below were designed to explore this possibility by first determining whether there exists an isoleucine-valine forming system in the particulate fraction of the wild-type mycelial homogenate.

Methods and Materials.—The assay procedures for the individual enzymes, the condensing enzyme, reductoisomerase, dehydrase, and transaminase have been described in detail previously.[5-8] Succinic cytochrome c reductase activity was determined by the method of Crane *et al.*[9] Protein was determined by the method of Lowry *et al.*[10]

The wild-type strain employed here, *KJT1960a*, is a segregant from a cross between the Emer-

son wild strains *Em5256A* and *Em5297a*. The mutant strain, *T304*, is a group II isoleucine-valine mutant which has been described in detail in previous publications.[3, 4, 11]

All mycelium used in these experiments was grown in Vogel's minimal medium in shake culture for a period of 24 hr. The mycelium was washed after harvesting with several portions of cold 0.5 *M* sucrose solution containing 0.01 *M* Tris buffer at pH 7.5. Homogenates from washed mycelium were prepared by grinding the mycelium in the above sucrose-Tris buffer (0.5 ml buffer to 1 gm wet weight mycelium) with a teflon-glass, and then a glass Ten Brok homogenizer. The homogenates were then strained through glass wool which effectively removed unground mycelium and most of the large fragments, as determined by microscopic observation with phase optics. The strained homogenate is referred to as the filtrate in what follows.

Supernatant and pellet activity: Figure 1 describes some representative results obtained by

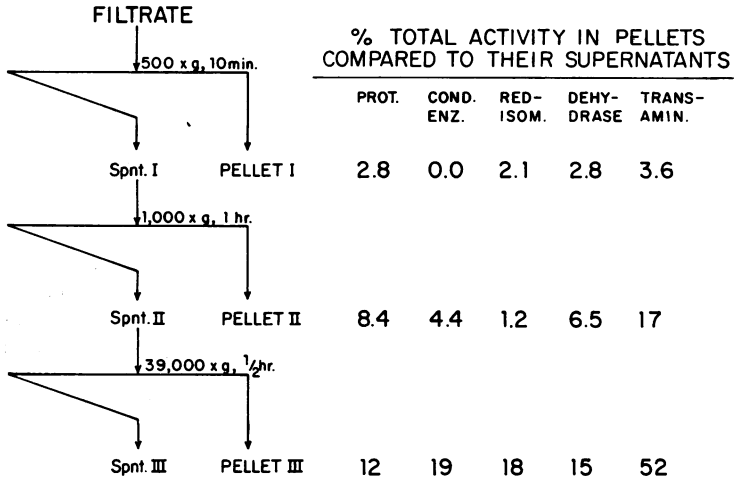

FIG. 1.—Total activity of indicated enzymes in a series of supernatants and pellets presented as percentage activity in pellets. The enzymes were determined individually.

centrifuging filtrate as indicated. In all of these the pellet material was resuspended in sufficient 0.5 *M* sucrose + 0.01 *M* Tris buffer at pH 7.5 to give about 10 mg/ml of protein before assaying for activity. The results show quite clearly that the activity for the four enzymes, as determined individually, remains principally in the supernatant, except for the transaminase.

If, however, instead of testing for activity for each enzyme individually, the ability of supernatant and pellet to carry out the over-all conversion from pyruvic acid to valine and isoleucine is measured, different results are obtained. The supernatant and pellet material used in this type of experiment were obtained by centrifuging the filtrate at 500 × *g* for 10 min to remove large particles not removed by the glass wool, and then centrifuging for 1 hr at 39,000 × *g*. The 39,000 × *g* supernatants and pellets were used in all of the experimental work described below. The 500 × *g* pellet was discarded after it was determined that it had only low fractional activity.

Supernatant and pellet (resuspended in 0.5 *M* sucrose + 0.01 *M* Tris pH 7.0) were incubated separately with the following mixture for the valine assay: 250μ moles L-phenylalanine, 250 μmoles glucose-6-phosphate, 50 μmoles $MgSO_4$, 500 μg TPN^+, 600 μg thiamine pyrophosphate, 250 μg glucose-6-phosphate dehydrogenase (Sigma Type V), 300 μg pyridoxal phosphate, and 500 μmoles sodium pyruvate. Isoleucine production was tested for in the same way, except that 250 μmoles each of pyruvate and α-ketobutyrate were present.

Figure 2 describes the results from an experiment in which supernatant fractions containing 117 mg of protein and pellet fractions containing 119 mg of protein were mixed with the above valine mixture and brought to identical volumes of 18.65 ml with 0.1 *M* phosphate buffer at pH 7.0. Control mixtures were also prepared which lacked the pyruvate precursor. These mixtures were incubated with gentle shaking for 6 hr at 37°C. Similar mixtures containing isoleucine precursors, instead of pyruvate alone, were also made, but incubated for only 4 hr. Aliquots of 3 ml were removed from each every hour and the amount of valine and isoleucine determined by

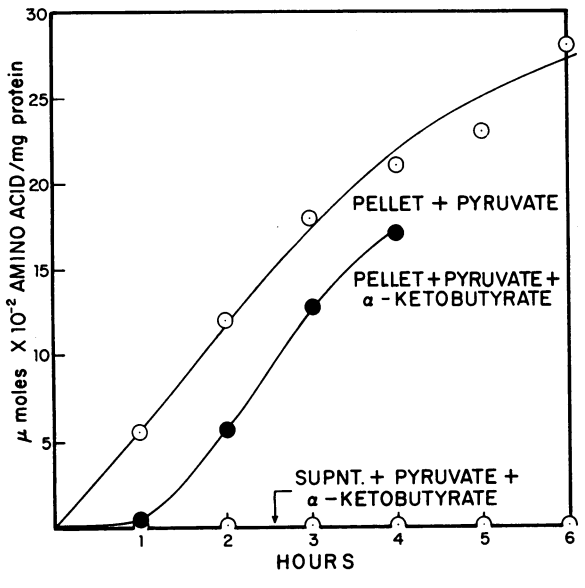

FIG. 2.—Synthesis of valine and isoleucine in the presence of 39,000 × *g* pellet material and the necessary cofactors and substrates. Open circles = valine, closed circles = isoleucine.

assay with *Leuconostoc mesenteroides* P-60.[12] Figure 2 shows that there is a net synthesis of valine and isoleucine in the presence of the pellet material, but not in the presence of the supernatant. The supernatant did, however, have activity for the four enzymes presumably involved. The specific activities (μmoles/mg/hr) were determined to be as follows: condensing enzyme, 0.18; reductoisomerase, 2.64; dehydrase, 0.32; and transaminase, 4.30. The possibility that there is an inhibitor in the supernatant which prevents the synthesis of valine was excluded by showing that the uncentrifuged filtrate has activity for the synthesis of valine. In one experiment filtrate containing 19.3 mg of protein was shown to produce 0.073 μmoles of valine/mg protein in 4.5 hr.

Additional evidence that it is indeed valine which is being formed from pyruvate was obtained by adding C^{14}-labeled pyruvate and demonstrating the appearance of radioactive valine and isoleucine after paper chromatography. In addition, a sample of pellet mixture incubated for 5 hr with pyruvate was freed of protein and analyzed for amino acid content by means of a Spinco amino acid analyzer. Three amino acids were found in significant amounts, alanine 19.5%, valine 15.4%, and phenylalanine 61.3%. The appearance of alanine may be ascribed to the transamination of pyruvate.

Fractionation of pellet: The 39,000 × *g* pellet material was determined to contain a high concentration of mitochondria with the electron microscope. Therefore, a sucrose gradient which has been shown by Luck[13] to band Neurospora mitochondria was employed. Three 0.5 ml samples of pellet material in 0.5 *M* sucrose, each containing 18.5 mg of protein, were layered on top of 4 ml sucrose gradients which ranged from 0.58 *M* to 1.9 *M* sucrose. The buffer was 0.01 *M* Tris at pH 7.5. The tubes were spun in a Spinco SW 39 L bucket head in a Spinco Model L for 4.5 hr at 40,000 rpm. The temperature of the head was maintained at 5.5°C. Ten fractions were removed from each tube dropwise from a puncture at the bottom. Since the ten fractions from each tube were equal in volume, the corresponding fractions were pooled. Each pooled fraction was then analyzed for protein activity for the mitochondrial enzyme succinic cytochrome c reductase, and valine-synthesizing activity. The results are given in Figure 3. They show quite clearly that the valine-synthesizing activity is carried in particles which have the same density as the particles which carry succinic cytochrome c reductase.

Activity of pellet fraction in an isoleucine-valine requiring mutant: T304, a representative of the group of mutants which possess all four enzymes in the soluble part of the supernatant, but yet requires isoleucine and valine for growth, was tested for valine-synthesizing ability in the pellet. First, an homogenate of a 24 hr culture was treated as described in Figure 1. It was found that there was no significant difference in pellet and supernatant enzyme activity between *T304* and the wild type when the enzymes were determined individually. The only exception was a relatively low condensing enzyme activity. This enzyme cannot be missing, however, because *T304* accumulates α-acetolactate and α-acetohydroxybutyrate, as well as α,β-dihydroxyisovaleric acid.[11] A test of activity for the over-all production of valine from pyruvate gave, however, the results summarized in Table 1. These data show that there is no net increase of valine over the controls in the presence of pyruvate either in the pellet or the supernatant.

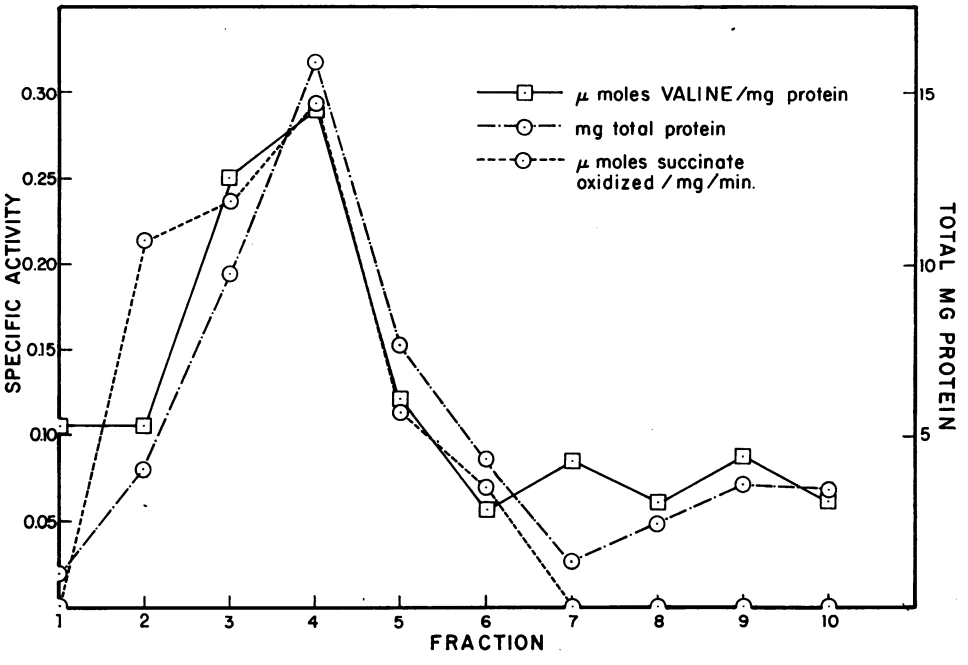

FIG. 3.—Specific activities for the valine-synthesizing system and succinic cytochrome c reductase in the different fractions of 39,000 × *g* pellet material after centrifugation in a sucrose gradient. Increasing density is to the left. Valine activity was determined by using the mixture of cofactors and substrates described in the text, and incubating for 4 hr at 37°C. Total protein is protein in each combined fraction as determined by the Lowry *et al.* method.[10]

TABLE 1

FORMATION OF VALINE FROM PYRUVATE IN THE PRESENCE OF SUPERNATANT AND PELLET FROM MUTANT T304

Hours incubation	μmoles valine/mg protein × 10^{-2}			
	Pellet control	Pellet + pyruvate	Supernatant control	Supernatant + pyruvate
2	0.14	0.11	0.27	0.46
3	0.20	0.18	0.40	0.56
4	0.25	0.20	0.46	0.66
5	0.31	0.22	0.56	0.67

Discussion and Summary.—The results described above demonstrate the existence of valine- and isoleucine-synthesizing systems in the 39,000 × *g* particulate fraction of wild-type Neurospora homogenate. The particles with valine-synthesizing activity have approximately the same sedimentation rate as the mitochondria known to be in the 39,000 × *g* pellet. The 39,000 × *g* supernatant has no detectable valine- or isoleucine-synthesizing activity from pyruvate even though it has all four enzymes.

It is known that the four enzymes of this system are not present in the supernatant bound in a complex. Unpublished results from this laboratory show that the dehydrase, the condensing enzyme, and the reductoisomerase have different sedimentation coefficients. The transaminase and dehydrase may be separated from the other two by electrophoresis on starch gel. These observations lead to the tentative conclusion that the soluble enzymes in the supernatant have become dissociated from particles of relatively high density. Once in solution they no

longer have the capacity to carry out a rapid synthesis of isoleucine and valine from pyruvate. However, wild-type supernatant does have the capacity to convert the dihydroxy acid to isoleucine at a low rate.[14] The active *in vivo* system(s) are presumably these enzymes properly oriented and organized on the particles, which could be mitochondria. Whether soluble enzymes are present free of these particles in the intact cell is not known. It is possible that all the soluble activity detected by the assays is a result of solubilization of the enzymes during preparation of the homogenate. Hence, the soluble enzymes may be no more than artifacts so far as the biosynthesis of isoleucine and valine is concerned.

The finding that the isoleucine-valine mutant, *T304*, does not have an active particulate fraction for the synthesis of valine from pyruvate, even though it possesses all the enzymes individually active in the soluble fraction, supports the hypothesis that it may differ from wild type because the valine-isoleucine enzymes are disorganized and hence inactive for the over-all series of reactions. This disorganization may either be the result of disorientation on particles, or because "cement" substances which attach the enzymes to the particles are not made. Failure of the enzymes to attach may make them relatively ineffective in biosynthesis, since product molecules must contact randomly distributed enzyme molecules in solution.

If the amino acid synthesizing system(s) described here actually proves to be attached to the mitochondria, it may provide a powerful tool for studying the genetic control of mitochondria, as well as of organization at the macromolecular level in general. The recent finding by Luck[13] that the mitochondria of Neurospora probably arise from pre-existing mitochondria by some sort of division raises some interesting questions regarding the nuclear control of particles in the cytoplasm, such as mitochondria.

Finally, the results described here may prove to be of some significance in helping to understand if cytoplasmic organization and genetic organization are related. That is, it may be possible to make an unqualified answer as to why certain groups of genes having related functions in some organisms are clustered on the linkage map.

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*A RELATIONSHIP BETWEEN DNA CONTENT, NUCLEAR VOLUME, AND MINIMUM MITOTIC CYCLE TIME**

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Mitosis is the culmination of many processes integrated in such a manner that two almost exact replicas of a nucleus or cell can be produced time and time again, each replica containing the necessary genetic information to repeat the process. The term mitotic cycle has been applied to those events which occur between the same stage in two successive divisions.[1] The biochemical changes that take place between two successive divisions have been the subject of recent reviews.[2-4] Although much information can be obtained by studying the processes in cells of a single species or a single cell type within an individual, equally useful information can be obtained from investigating similar cells from different species. The purpose of these studies was to determine whether or not a relationship existed between three cellular characteristics, namely, DNA content per cell, nuclear volume, and the minimum mitotic cycle time.

Material and Methods.—Minimum mitotic cycle time measurements: The full details of the technique used to measure minimum mitotic cycle time are published elsewhere.[5, 6] The technique involves the production of tetraploid cells by treating the meristems with colchicine for short periods of time which varied with different species. The number of tetraploid cells produced during the treatment depends upon (1) the number of cells that entered metaphase while colchicine was effective, and (2) the concentration of colchicine used. The affected cells undergo karyokinesis but no cytokinesis, and enter interphase as usual. The next time these affected cells divide they will be tetraploid and hence distinguishable from normal diploid cells. The period of time between the colchicine treatment and the initial appearance of tetraploid cells in division is the minimum mitotic cycle time. All cycle time measurements were performed at 23 ± 1.0°C.

Nuclear volume measurements: Collections for nuclear volume studies were made from growing plants before floral transition. Root meristems were killed, fixed in Craf III, dehydrated, and infiltrated with paraffin by the use of a tertiary butyl alcohol series. Sections were cut at 10 μ and stained with safranin-fast green. The diameters of interphase nuclei of meristematic cells just above the root cap were measured with a Zeiss ocular micrometer. Ten nuclei on each of two slides were measured for each species, and average nuclear volumes were calculated.

DNA measurements: The deoxyribonucleic acid (DNA) was extracted from root-tip material with sodium chloride, a modification of the Schmidt-Thannhauser procedure,[7] and the amount of DNA was estimated by the diphenylamine reaction.[8]

Cell counts: The number of cells per meristem was determined by excising the terminal 2 mm of 40 meristems and randomly selecting 10 of these excised segments. Cell separation was accomplished by hydrolyzing the segments with 1 *N* HCl until the tissue was soft. Following hydrolysis the segments were mascerated and suspended in 1 cc of 1 *N* HCl. Four 0.05 cc aliquots were removed from this suspension for cell counts with a hemocytometer.

Results.—Figures 1*A* and 1*B* show the time at which the tetraploid cells appeared