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*AMINO ACID PRODUCTION BY A MITOCHONDRIAL FRACTION OF NEUROSPORA CRASSA**

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Communicated by Wilson S. Stone, December 1, 1965

Recent work in this laboratory has demonstrated that the enzymes responsible for the synthesis of isoleucine and valine in *Neurospora* from at least pyruvate and α-ketobutyrate are located in the particulate fraction of the cell. This fraction is made up largely of mitochondria.[1-3] We were curious to know whether this particulate nature was unique to the isoleucine-valine enzymes, or whether it might be shared with other amino acid-synthesizing enzymes. We started by testing the leucine-synthesizing system because of its known relationship to the valine pathway. The experiments described below demonstrate that crude mitochondrial preparations (CMP) from *Neurospora* are capable of producing leucine and a number of other amino acids in addition to isoleucine and valine.

Materials and Methods.—The wild-type strain KJT 1960a of *Neurospora crassa* was used throughout these experiments. The origin of this strain and the methods used to propagate mycelium have been described previously.[1] Mycelium from 20- to 24-hr cultures was harvested by filtration through cheesecloth and washed with a 0.1 *M* sucrose solution containing 0.05 *M* Tris at pH 8.0. The mycelial mass was ground with a mortar and pestle at 5°C with $2^1/_2$ times its wet weight of acid-washed sand and $2^1/_2$ times, volume to wet weight, of a 0.25 *M* sucrose solution containing 0.15% bovine serum albumin and 0.005 *M* EDTA. This procedure has been used by Hall and Greenawalt[4] to isolate *Neurospora* mitochondria capable of coupling oxidation to phosphorylation. Subsequent operations, except for incubation, were carried out at 5–10°C. The homogenate resulting from sand grinding was filtered through glass wool. The filtrate was then centrifuged at 1500 × *g* for 10 min. The resulting supernatant was recentrifuged at the same speed. The supernatant from the second 1500 × *g* centrifugation was then centrifuged at 10,000 × *g* for 30 min. The resulting pellet was resuspended in the bovine serum albumin solution described above. This suspension is referred to below as the crude mitochondrial preparation (CMP). The corresponding supernatant is referred to as the 10,000 × *g* supernatant (10 GS).

Protein was determined by the method of Lowry *et al.*[5] Sucrose density gradients were prepared as described by Luck[6] and Wagner and Bergquist.[1] Succinate cytochrome *c* reductase activity was measured by the methods of Green, Mii, and Kahont.[7]

CMP, 10 GS, or fractions collected from the sucrose density gradients were tested for their ability to produce amino acids by incubation, with the reaction mixtures described below, at 37°C on a reciprocal shaker for 4 hr unless otherwise indicated. The reactions were routinely halted by freezing the incubate to −20°C. The amount of amino acids produced during incubation was determined either by use of a bioassay with *Leuconostac mesenteroides* P-60 or by use of a Spinco amino acid analyzer. Leucine and valine were routinely assayed by the former method, and all other amino acids by the latter. In some experiments pyruvate labeled with C^{14} at carbon-2 was added to incubation mixtures, and the amino acids produced separated by paper chromatography. The spots corresponding to known amino acids were checked for incorporated C^{14} using an Ansitron liquid scintillation counter. No attempt was made to quantitate this procedure.

Production of Amino Acids.—The work of Burns *et al.*, Gross *et al.*, and Jungwirth *et al.*[8–10] has elucidated the necessary cofactor requirements for the four terminal enzymes of leucine biosynthesis. Table 1 shows the relative capabilities of the CMP and 10 GS to produce leucine when incubated in a reaction mixture containing the cofactors required by the leucine enzymes as well as ATP (the complete reaction mixture is given in the table). It can be seen that the CMP is capable of producing considerable amounts of leucine under these conditions, while the 10 GS is not. It can also be seen in this table that the production of leucine by the CMP is independent of externally supplied substrate, but is dependent on ATP and Mn^{++}. In similar experiments in which the leucine intermediate β-carboxy-β-hydroxyiso-

TABLE 1

ABILITY OF PARTICULATE FRACTION TO PRODUCE LEUCINE

Incubation mixture	Specific production of leucine (μmoles/mg protein/4 hr)
CMP, Tris	0.034
CMP, TRM	0.204
CMP, TRM less ketoisovaleric acid	0.356
CMP, TRM less coenzyme A and acetyl-phosphate transacetylase	0.382
CMP, TRM less manganous chloride	0.019
CMP, TRM less ATP	0.051
10 GS, Tris	0.000
10 GS, TRM	0.007

The total reaction mixture (TRM) contained: 0.1 *M* Tris-HCl buffer, pH 8.0; 3×10^{-4} *M* NAD; 1×10^{-2} *M* $MnCl_2$; 5×10^{-2} *M* KCl; 4.3×10^{-4} *M* coenzyme A; 5×10^{-2} acetyl phosphate; 5×10^{-2} *M* α-ketoisovaleric acid; 10 enzyme units of phosphotransacetylase; 1×10^{-2} *M* ATP; 1×10^{-4} *M* pyridoxal phosphate; 2.5×10 *M* phenylalanine, and 3 mg of either crude mitochondrial protein (CMP) or 10,000 × *g* supernatant (10 GS) in a total volume of 1.0 ml. Leucine production determined by microbiological assay.

TABLE 2

ABILITY OF THE PARTICULATE FRACTION TO PRODUCE AMINO ACIDS IN ADDITION TO LEUCINE

Amino acid produced	Specific Production (μmoles of amino acid/mg protein/4 hr) Total reaction mixture	Background
Alanine	0.28	Trace
Arginine	0.08	0
Aspartic acid	0.08	Trace
Glutamic acid	0.08	Trace
Glycine	0.14	Trace
Isoleucine	0.15	Trace
Leucine	0.33	Trace
Lysine	0.22	Trace
Methionine	0.06	Trace
Serine	0.208	Trace
Threonine	0.142	Trace
Tyrosine	0.360	0
Valine	0.12	0

The total reaction mixture contained: 0.1 *M* Tris-HCl buffer; pH 7.8; 3×10^{-4} *M* NAD; 1×10^{-2} *M* $MnCl_2$; 5×10^{-2} *M* KCl; 1×10^{-2} *M* ATP; 1×10^{-4} *M* pyridoxal phosphate; 2.5×10^{-2} *M* phenylalanine, and 3.0 mg of crude mitochondrial protein in a total volume of 1.0 ml. Background refers to the amount of any given amino acid produced when the CMP is incubated with Tris buffer alone. Trace is used to indicate that the amino acid was present in detectable, but not measurable, quantities. All amino acids were determined on a Spinco amino acid analyzer.

caproate (kindly supplied by Dr. Samson Gross of Duke University) was used as a substrate, a considerable enhancement of leucine production occurred. This shows that at least part of the enzymes necessary for leucine synthesis are present in the CMP.

When tests for valine production by the CMP were performed under conditions similar to those described in Table 1, valine was also found to be produced by the CMP. This production was also found to be independent of externally supplied substrate and dependent upon the presence of ATP and Mn^{++} in the incubation mixture. Consequently, the CMP was tested for its ability to produce all amino acids under similar conditions. Table 2 shows that when the CMP is incubated in the presence of ATP and Mn^{++} (the complete reaction mixture is given in the table), a number of amino acids are produced in relatively large quantities, even though no substrate source is supplied.

In order to ascertain whether this synthetic activity is actually associated with the mitochondria, sucrose density gradient studies were done on the CMP. Figure 1 shows the result of such a study. It can be seen that the ability to produce leucine is found in the same peak with succinic cytochrome *c* reductase activity (the reaction mixture used was the same as that described in Table 2).

Origin of Amino Acids.—The origin of these free amino acids by the CMP and the purified mitochondrial fraction may conceivably be the result of at least three different mechanisms. (1) *De novo* synthesis: this would require that some source of substrate be present in the CMP. It is conceivable that ATP and Mn^{++} would act as mitochondrial-preserving agents in such a scheme. (2) The leaching of an amino acid pool: this would require that the CMP contain a pool of amino acids that could be leached only in the presence of ATP and Mn^{++}. (3) The hydrolysis of protein: this would require a proteolytic process dependent upon ATP and Mn^{++}.

Time Course.—It would seem unlikely that production of the amino acids could be sustained for a very long period of time by any of these mechanisms. Figure

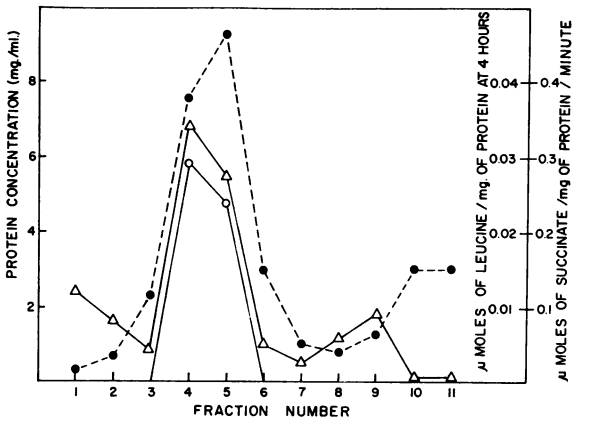

FIG. 1.—Total protein, succinic cytochrome *c* reductase activity, and ability to produce leucine of the various fractions from a sucrose density gradient. The incubation mixture for leucine production is given in Table 2. —○—, Leucine production; —●—, total protein; —Δ—, succinic cytochrome *c* reductase activity.

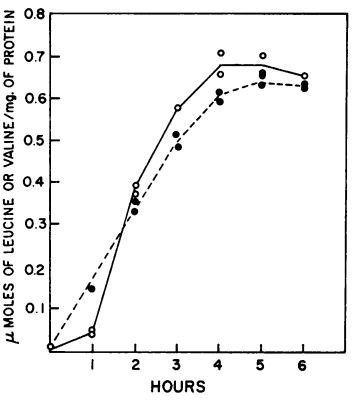

FIG. 2.—Time curve for leucine and valine production. Incubation mixture is the same as given in Table 2. *Open circles* represent leucine production. *Closed circles* represent valine production.

2 shows the time course of leucine and valine production. The complete incubation mixture was similar to that described for Table 2. The production of both leucine and valine was seen to reach a maximum under these conditions after 3–4 hr. It can also be seen that for the first 3 hr, valine production was relatively linear, whereas a definite lag followed by a relatively rapid "burst" was seen in the leucine production. This is inconsistent with what one would expect from either a hydrolytic or amino acid pool hypothesis.

Effect of Protein Concentration.—Of interest, particularly from the standpoint of the hydrolytic hypothesis, is the effect of protein concentration on the production of these amino acids. Figure 3 shows the effect of protein concentration on the specific production of leucine and valine. The complete reaction mixture was similar to that described for Table 2. It is evident that at low protein concentrations the specific production of leucine was very different from that of valine. This is inconsistent with what one would expect of either a hydrolytic or an amino acid pool hypothesis.

Incorporation of C^{14}.—If a synthetic process is in operation, it would seem logical that even though externally supplied substrates did not increase the production of leucine or valine, it might be possible to equilibrate the internal pool of substrate with externally supplied labeled substrate. For this purpose, pyruvate labeled with C^{14} at carbon-2 was added, along with unlabeled succinate or malate, to a reaction mixture similar to that described for Table 2. The chromatographic procedures used were not sufficiently refined to allow certainty of the complete separation of leucine from isoleucine; consequently, leucine and isoleucine have not been distinguished in the results shown in Table 3. It can be clearly seen that some C^{14} from pyruvate is incorporated into both valine and leucine-isoleucine, indicating that at least part of the amino acid production occurs by a synthetic process.

Externally Added Substrates.—In view of the apparent synthetic nature of part of the amino acid production, it was of some interest to know what effect added

TABLE 3

INCORPORATION OF C^{14} FROM PYRUVATE INTO LEUCINE-ISOLEUCINE AND VALINE

Contents	Cpm Leucine-isoleucine	Cpm Valine
Total reaction mixture + Pyruvate and succinate	145.25	2943.25
Total reaction mixture + Pyruvate and malate	118.90	3389.80

Contents of the total reaction mixture were the same as given in Table 2. Values given are in terms of counts per minute above background.

TABLE 4

EFFECT OF EXTERNALLY ADDED SUBSTRATES WITH 6-HR INCUBATIONS

Contents	Per Cent Specific Production (μmoles of amino acid/mg protein/6 hr) Leucine	Valine
Background	16.5	0.0
Total reaction mixture	100.0	100.0
+ Malate and pyruvate	108.0	121.0
+ Succinate and pyruvate	114.0	157.0

Contents of the incubation mixture are described in the text. The values given are in terms of per cent specific production of the total reaction mixture without added substrates.

substrates would have on leucine and valine production, after production had ceased from internal substrates. Consequently, crude mitochondrial preparations were incubated for 6 hr with incubation mixtures similar to those indicated for Table 2, and with similar incubation mixtures containing pyruvate (0.05 *M*) and either malate (0.005 *M*) or succinate (0.005 *M*). It is clear from results shown in Table 4 that these substrates can enhance leucine and valine production if the mixture is allowed to incubate for periods of time longer than 3–4 hr.

Thus, it would seem that the hypothesis that best explains the data so far considered is one which postulates a synthetic process. This is consistent with the data, if one assumes that there is a limited substrate pool of some kind within the mitochondrion which can be utilized for the synthesis of amino acids, and that the rate at which the mitochondrion uses the substrates to fashion amino acids cannot be increased by increasing the substrate level with externally supplied substrates.

Optimal Conditions.—Optimal conditions for the production of leucine and valine by the CMP in the absence of added substrate were determined and in all cases they were found to be identical. The optimal Mn^{++} concentration was 5×10^{-2} *M*. The optimal Tris-HCl concentration was 9×10^{-2} *M*. The optimal pH was 7.8.

ATP concentration was found to be optimal at 20 μmoles/ml. Higher ATP concentrations were strongly inhibitory (see Fig. 4). This very pronounced inhibition by high levels of ATP was completely unexpected; however, it is in accord with work reported by Klingenberg and Schollmeyer,[13] which demonstrated an inhibition of respiration in mitochondria under conditions of high ATP concentration. ADP was capable of replacing the ATP requirement to a large extent.

Discussion and Summary.—The experiments described above show that the mitochondrial fraction (CMP) from *Neurospora* is capable of producing a number of amino acids by what appears to be a synthetic process. The amino acids produced by the CMP are largely those that are normally derived either from pyruvate or Krebs cycle intermediates. Tyrosine, the exception produced in the largest quantities, is possibly produced from the phenylalanine which is added as the amino group donor.

The fact that added substrates have little, if any, effect on leucine and valine synthesis necessitates a mechanism for substrate exclusion. Perhaps the simplest such mechanism would be a permeability barrier such as the mitochondrial membranes. This would be in accord with other known mitochondrial systems, particularly the Krebs tricarboxylic acid cycle. It is well known[11, 12] that many of the

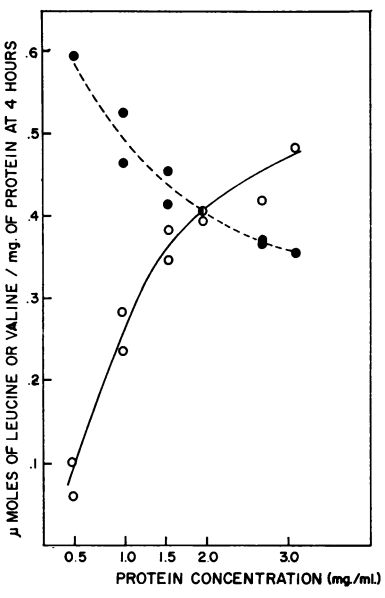

FIG. 3.—Effect of varying protein concentration on production of leucine and valine. Contents of the incubation mixture are given in Table 2. *Open circles* represent leucine production. *Shaded circles* represent valine production.

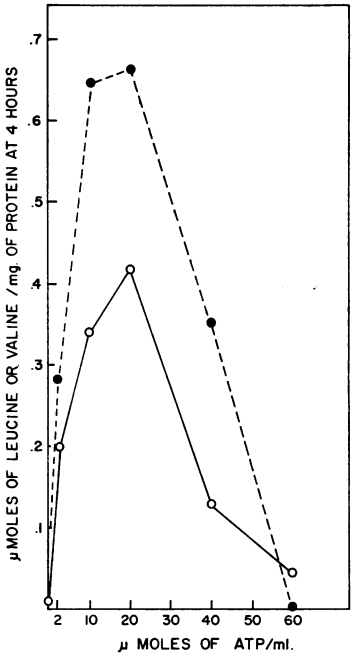

FIG. 4.—Effect of increasing ATP concentration on leucine and valine production. The contents of the incubation mixture are given in the text. *Open circles* represent leucine production. *Shaded circles* represent valine production.

Krebs cycle intermediates are excluded from intact mitochondria even though these mitochondria contain the necessary enzymes for their oxidation.

Alternatively, it is conceivable that the mitochondrial preparations from *Neurospora* are operating at a regulated rate using some internal substrate, and that additional substrate does not greatly affect this rate. It is also possible that both of these mechanisms are in operation.

In order to resolve the fact that these preparations make amino acids without externally supplied substrate, it is necessary to postulate either that they contain a large substrate pool, or that they contain invertase and the glycolytic enzymes. This latter possibility would render the mitochondrial preparations capable of utilizing the sucrose added to stabilize the mitochondria (0.25 *M* in the isolation solution, 0.025–0.012 *M* in the reaction mixture).

A distinction between these alternative hypotheses is not possible at present.

The mitochondrial preparations previously described by members of this laboratory have been able to synthesize isoleucine and valine only in the presence of added substrates, and have shown no activity for the production of other amino acids.[1–3] These earlier preparations differed from those reported here largely in the method used for isolation. Tris buffer containing sucrose was used in the preparation of mitochondria in the earlier studies. The mitochondrial preparations used in the work described here were isolated in Tris buffer containing sucrose to

which had been added bovine serum albumin. The latter has been shown to aid in the preservation of *Neurospora* mitochondria capable of coupling oxidation to phosphorylation.[4] It would seem then that the inclusion of bovine serum albumin in the medium has allowed us to isolate mitochondrial preparations capable of demonstrating substrate-independent amino acid production.

Summary.—The mitochondria of *Neurospora*, or a particulate fraction with the same density, have been shown to produce several amino acids in the presence of ATP and manganous ion. The process seems to be synthetic.

* This work was supported in part by grants GM-12323 and 5T1-GM-337 from the National Institutes of Health, USPHS, and a grant from the Robert A. Welch Foundation, Houston, Texas. The senior author was supported by a predoctoral training grant award from the Robert A. Welch Foundation during a portion of this work.

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‡ Career award 5-K6-GM-18, 383, National Institutes of Health, USPHS.

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*TESTOSTERONE BIOSYNTHESIS BY RABBIT TESTIS SLICES: GLUCOSE-UL-C^{14} AS A CARBON SOURCE FOR TESTICULAR STEROIDS AND TESTICULAR PROTEINS**

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Communicated by Oscar Riddle, December 13, 1965

Numerous studies have indicated that both the testis and the ovary respond to gonadotrophin addition *in vitro* by an increased production of steroid hormones. Thus, testosterone production and incorporation of isotopically labeled precursor into labeled testosterone by rabbit testis slices are stimulated by interstitial cell-stimulating hormone (ICSH), follicle-stimulating hormone (FSH containing ICSH), human chorionic gonadotrophin (HCG), and pregnant mare serum gonadotrophin (PMS).[1] Hall and Eik-Nes,[2] moreover, studying the incorporation of labeled amino acids (1-C^{14}-valine and 1-C^{14}-tryptophan) into testicular proteins, have shown that ICSH will enhance this incorporation. This effect of gonadotrophin *in vitro* could