The Nature of the Lesion in the Succinate-requiring Mutants of *Neurospora crassa*: Interaction between Carbohydrate and Nitrogen Metabolism

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SUMMARY: Mutants of *Neurospora crassa* requiring dicarboxylic acids for an immediate growth response (*suc* and *at suc*) oxidize acetate, and are inhibited by fluoroacetate with consequent citric acid accumulation to approximately the same extent as the wild-type. The concentration of nitrogen (as ammonium and nitrate salts) present in the conventional growth medium is inhibitory to the growth of these mutants and leads to an accumulation of acetylmethylcarbinol, pyruvic acid and α -ketoisovaleric acid. This inhibition is reduced and growth is stimulated by the addition of dicarboxylic acids or by diminution of the nitrogen present in 'minimal' medium. The addition of nitrogen salts to *suc* mutants probably diverts dicarboxylic acids (already in short supply) from the catalysis of the oxidation of C_2 fragments via the tricarboxylic acid cycle to other reactions. This effect of nitrogen salts upsets the already precarious dicarboxylic acid balance of the *suc* mutants leading to a growth requirement and to the accumulation of intermediates.

This investigation was begun because the existence of mutants of the ascomycete *Neurospora crassa*, which grow on minimal medium only after the addition of small quantities of dicarboxylic acids (succinate-requiring mutants), seemed difficult to reconcile with an apparent use of the tricarboxylic acid cycle for both synthesis and energy by *Neurospora* spp. (Lewis, 1948; Strauss, 1955*a*). Accepting the idea of a 'genetic block' in the cyclic metabolism of dicarboxylic acids by the succinate-requiring mutants seems to require the assumption that acetate is oxidized by some mechanism other than the tricarboxylic acid cycle (Krebs, Gurin & Eggleston, 1952) or that the 'genetic block' is not absolute. Since either of these explanations would be of interest, the properties of the succinate-requiring mutants have been studied in some detail.

It now appears that the succinate-requiring mutants do use the tricarboxylic acid cycle, and that there is no enzymic block to the operation of the cycle. Dicarboxylic acids required to catalyse acetate oxidation are in short supply and, as a result, excess ammonium or nitrate nitrogen inhibits these mutants by withdrawing this limited supply of dicarboxylic acids for synthetic purposes. Inhibition by excess nitrogen is at least partly responsible for the failure of succinate-requiring mutants to grow normally on 'minimal medium' (Beadle & Tatum, 1945).

It is the purpose of this paper to present the evidence for the interaction of carbohydrate and nitrogen metabolism in the succinate-requiring mutants, and to discuss briefly the implications of these findings.

METHODS

The methods used in growing *Neurospora crassa* have been previously described (Strauss & Pierog, 1954; Strauss, 1955*a*). Conidia were prepared by allowing cultures to grow 7 days at room temperature in the light on minimal medium with succinate (0.5 % disodium succinate) and 1.5 % agar added. The conidia were harvested by being suspended in water, filtered through cheese-cloth, washed twice with water by centrifugation and finally suspended in the experimental medium as indicated below. The analytical, enzymic, isotopic and manometric methods used in this investigation were identical with those previously used, except where otherwise indicated.

GENETIC MATERIALS

Two mutations, *suc* and *at*, were used in these studies. The *suc* strains were derived from strain 46005, a succinate-requiring mutant described by Dubes (1953) and obtained from Mrs M. B. Mitchell of the California Institute of Technology, and from strain 86, a double mutant derived as described below from one of the 'glutamate' mutants described by Woodward (1954). Both strain 46005 and Woodward's 'glutamate' mutants are phenotypically similar to the strain described by Lewis (1948) and to strain 46403 (Dubes, 1953; Strauss, 1955*a*); that is they grow readily with either malate, fumarate, succinate or α -ketoglutarate and after a lag (Fig. 1) with acetate and glutamate. The two *suc* strains used (46005 and 86) probably represent independent occurrences of the same *suc* mutation since: (a) heterocaryons growing on minimal medium are not formed by the two strains; (b) wild-type recombinants were not obtained from among 295 single ascospore cultures picked from a cross of 46005 $\times$ 86. The probability of not detecting a wild-type recombinant from this number of random segregants from a cross of non-allelic genes is less than 1 %, even if the genes are as close together as 0.8 map unit. Perithecia are formed only sparsely in this cross, and most of the resulting ascospores do not acquire the black coat characteristic of ripe spores. For our tests, spores were plated on Petri plates containing solid minimal or minimal plus succinate medium, the spores were activated and the plates were incubated at 25°. Only spores which had germinated within 15 hr. incubation were picked for testing.

In this paper the succinate-requiring mutants are designated *suc* with the wild-type allele designated *suc*⁺, the symbolism used by Barratt, Newmeyer, Perkins & Garnjobst (1954). We have used absence of growth in minimal medium and response to both succinate and acetate (tested separately) as growth supplements after 3 days of incubation as a diagnostic test for the *suc* phenotype. The evidence that *suc* represents a single gene mutation is given in Table 1. Of the total of 256 ascospores tested from the type cross *suc* $\times$ *suc*⁺, 130 were *suc*⁺, 126 *suc*, a close fit to the expected 1:1 ratio.

Strain 86 was obtained by the method of Woodward, De Zeeuw & Srb (1954), starting with a 'glutamic' mutant obtained by Dr Woodward from wild-type

74A by X-irradiation. Further X-irradiation of the conidia of this mutant followed by incubation with shaking in minimal medium plus glutamate resulted after repeated filtration in the isolation of a strain which differed phenotypically from the original. This new strain (86), obtained in the course of work at Brookhaven National Laboratory, did not grow within 3 days on either acetate or glutamate supplements, although it still responded to either

Table 1

Parental strains	Presumed parental genotypes	Phenotypes of segregants	Total segregants		No. of classifiable asci for gene indicated	Percentage second division segregation
			Random	From ordered asci		
46005 $\times$ 8a	<i>at⁺ suc</i>	<i>at⁺ suc</i>	—	31	18 (<i>suc</i>)	11
	$\times$					
	<i>at⁺ suc⁺</i>	<i>at⁺ suc⁺</i>	—	43		
15300 $\times$ 46005	<i>alb at⁺ suc⁺</i>	<i>alb at⁺ suc⁺</i>	21	30	12 (<i>alb</i>)	50
	$\times$	<i>alb⁺ at⁺ suc</i>	32	23		
	<i>alb⁺ at⁺ suc</i>	<i>alb at⁺ suc</i>	8	32	12 (<i>suc</i>)	42
		<i>alb⁺ at⁺ suc⁺</i>	16	20		
86 $\times$ 15300	<i>alb⁺ at suc</i>	<i>alb⁺ at⁺ suc⁺*</i>	—	30	16 (<i>alb</i>) 16 (<i>suc</i>) 8 (<i>at</i>)	75 16 0
	$\times$	<i>alb at⁺ suc⁺*</i>	—	70		
	<i>alb at⁺ suc⁺</i>	<i>alb at suc</i>	—	16		
		<i>alb at⁺ suc</i>	—	33		
		<i>alb⁺ at suc</i>	—	22		
		<i>alb⁺ at⁺ suc</i>	—	42		
86 $\times$ 5297	<i>at suc</i>	<i>at⁺ suc⁺*</i>	—	27	4 (<i>suc</i>) 4 (<i>at</i>)	0 0
	$\times$	<i>at⁺ suc</i>	—	14		
	<i>at⁺ suc⁺</i>	<i>at suc</i>	—	7		
27-1† $\times$ 46005	<i>at suc⁺</i>	<i>at⁺ suc⁺*</i>	—	36	10 (<i>suc</i>) 10 (<i>at</i>)	0 0
	$\times$	<i>at⁺ suc</i>	—	23		
	<i>at⁺ suc</i>	<i>at suc</i>	—	18		

Phenotypes: *alb*=albino; colourless mycelium. *alb⁺*=wild type coloration. *at⁺ suc=suc*=no growth in 3 days on minimal medium; growth with succinate or acetate added as supplements. *at suc*=no growth in 3 days on minimal medium or on minimal + acetate; growth on minimal + succinate. *at⁺ suc⁺*=wild type=growth on minimal medium.

* The *at⁺ suc⁺* phenotype includes the class of *at suc⁺* strains since these grow on minimal medium (see Table 2).

† See Table 2 for the origin of this strain.

succinate, fumarate, malate or α -ketoglutarate. The new strain obtained was crossed to wild-type and a high conidiating segregant (86-1) was obtained. The evidence that strain 86-1 is a double mutant is summarized in Table 1. Crosses of 86-1 to wild-type or to the morphological mutant, albino, give asci-yielding nutritional mutants of parent type (no growth on acetate) and recombinants (mutants growing on acetate) with spore pairs always giving the same results. Furthermore, a supposed *at suc⁺* strain selected from ascus # 27 of the cross 86 $\times$ 5297 (Table 2), when crossed to strain 46005 (supposed *at⁺ suc*) gave asci containing spores yielding cultures with *at suc* phenotypes, as would be expected if this cross (27-1 $\times$ 46005) were *at suc⁺ $\times$ at⁺ suc*. On totalling the data in Table 1 it will be noted that a total of 112 *at⁺ suc* strains were obtained

from the type cross *at suc* × *at*⁺ *suc* compared to a total of 63 *at suc*. We believe that this discrepancy from the expected 1:1 ratio probably is due to the relatively low percentage germination of *at suc* ascospores compared to *at*⁺ *suc* spores for which we have other evidence. No classifiable ascus has been obtained that is not in accord with the hypothesis of a single modifier gene. This gene was designated *at* to indicate its effect on the utilization of acetate, and to distinguish it from the acetate requiring mutants described earlier under the designation *ac* (Strauss & Pierog, 1954). The *at* locus apparently acts only as a modifier to the *suc* gene, preventing response of *suc* strains to acetate. In the presence of the wild-type allele of *suc*, the *at* gene apparently has no effect since strain 27-1 (*at suc*⁺) grows normally on minimal medium.

Table 2. *Composition of ascus 27*

	Parents	...	86-1 × 15300	
Growth response of parents				
Minimal			0	+
Minimal + succinate			+	+
Minimal + acetate			0	+
Presumed genotype of parents			<i>alb</i> ⁺ <i>at suc</i>	<i>alb at</i> ⁺ <i>suc</i> ⁺
	Segregants spore pair number			
	1	2	3	4
Growth				
Minimal	+	+	0	0
Succinate	+	+	+	+
Acetate	+	+	+	+
Colour	Normal	Albino	Normal	Albino
Presumed genotype	<i>alb</i> ⁺ <i>at suc</i> ⁺	<i>alb at suc</i> ⁺	<i>alb</i> ⁺ <i>at</i> ⁺ <i>suc</i>	<i>alb at</i> ⁺ <i>suc</i>

RESULTS

Growth studies

Both *suc* and *at suc* strains grow well in the presence of succinate (Figs. 1, 2), *suc* grows on acetate and on glutamate after a short lag, while the *at suc* strain grows on acetate only after a considerable lag period. At about the same time that *at suc* strains show visible growth with an acetate supplement, *suc* strains initiate growth on minimal, and the growth of *suc* on minimal roughly parallels the growth of *at suc* on acetate. The *suc* mutants are not permanently adapted after initiation of growth on minimal medium since subinoculations of cultures that are growing on minimal medium on to fresh minimal medium require a long lag period before again starting to grow. As can be seen from the scatter of points in Fig. 1, the time at which cultures of *suc* on minimal medium or of *at suc* on acetate begin to grow rapidly varies noticeably from flask to flask.

When cultures are grown with varying amounts of nitrogen (added as ammonium sulphate and substituting equimolar amounts of sodium potassium tartrate for the ammonium tartrate usually present) there is an inhibition of mutant growth by quantities of nitrogen that do not inhibit the wild-type (Fig. 3). This inhibition is counteracted by the addition of succinate (Table 3).

Fig. 1

Fig. 2

Fig. 1. Growth of *suc* mutants as a function of time. Additions to 20 ml. minimal medium: $25 \mu\text{M}$ succinic acid or glutamic acid as sodium salts. $50 \mu\text{M}$ acetic acid as the sodium salt: $\times$, succinate added; $\circ$, acetate added; $\bullet$, glutamate added; $\cdot$, no addition.

Fig. 2. Growth of *at suc* and *suc* strains as a function of succinate concentration. Growth after 4 days at 28° . Succinic acid added as disodium salt: $\times$, 7A (*at+ suc+*); $\circ$, 46005 (*suc*); $\bullet$, 86 (*at suc*).

Fig. 3. Effect of nitrogen concentration on growth of *suc*. Wild-type curve of growth as a function of nitrogen concentration obtained after 3 days' incubation. *suc* grown with no organic supplement. *at suc* grown with 5 mg. $\text{Na}_2\text{C}_2\text{H}_3\text{O}_2 \cdot 3\text{H}_2\text{O}$ per 20 ml. Nitrogen added as $(\text{NH}_4)_2\text{SO}_4$.

Both *suc* and *at suc* strains are inhibited by the amount of nitrogen ordinarily present in minimal medium (22.2 mg./20 ml. as ammonium tartrate and ammonium nitrate).

Accumulations

The *suc* strains accumulate acetylmethylcarbinol (AMC, 3-hydroxy-2-butanone), pyruvic acid and α -ketoisovaleric acid when grown on limiting succinate concentrations. AMC was first recognized by its characteristic odour and by the Voges-Proskauer reaction (Westerfeld, 1945) given by steam distillates of culture medium. It was identified in the steam distillate from *suc* culture medium by the formation of the characteristic precipitate of nickel dimethylglyoxime after oxidation (of the steam distillate) with FeCl_3 in acid, with subsequent distillation of the oxidation product into neutralized hydroxylamine in the presence of nickel chloride and sodium acetate. This compound (AMC) has been previously identified as an accumulation product of neurospora mutants requiring acetate for growth (Strauss, 1953).

Table 3. *Effect of succinic acid on the inhibition of the growth of a suc strain by ammonium nitrogen*

Succinic acid (mg./flask)	mg. N per flask					Strain
	0.5	1.0	2.0	8.0	25.0	
0	12	13	9	4	2	<i>suc</i> (46005)
0.6	22	31	37	30	21	
1.2	25	39	45	41	42	
0	22	35	50	105	103	<i>suc</i> ⁺ (7A)

Recorded growth as mg. dry wt. after 4 days at 25° in medium using sodium potassium-tartrate. Nitrogen added as ammonium sulphate. Succinic acid added as disodium succinate. Values averages of duplicates.

Accumulation of keto acids in culture media in which *suc* strains had grown was indicated by the formation of 2,4-dinitrophenylhydrazine derivatives which could be extracted from ethyl acetate by sodium carbonate and then returned to an ethyl acetate layer after acidification. Pyruvic acid was identified by chromatography as one of the accumulation products of the dinitrophenylhydrazones after two passages through sodium carbonate; a spot coincided with that given by an authentic sample of pyruvic 2,4-dinitrophenylhydrazone after ascending chromatography in tertiary amyl alcohol : ethanol : water (50 : 10 : 40) or in isobutanol : water (90 : 10). Another major spot (distinct from the second spot given by the pyruvic acid derivatives) was noted with an R_f of 0.83 in the tertiary amyl alcohol mixture and with an R_f of 0.5 in isobutanol : water. These R_f values, higher than those characteristic of pyruvic acid, indicate a compound with more non-polar character than pyruvic acid. We have provisionally identified this compound as the derivative of α -ketoisovaleric acid since the unknown compound gave a spot which was superimposed on the spot given by the 2,4-dinitrophenylhydrazine derivative of an authentic sample of α -ketoisovaleric acid (kindly

provided by Dr H. E. Umbarger) after chromatography with either the tertiary amyl alcohol mixture or with isobutanol : water.

Cultures of the *suc* strain grown in the presence of succinate show a steadily decreasing accumulation of AMC as growth proceeds (Fig. 4). In the absence of succinate relatively more AMC is accumulated up to the point where rapid growth begins. Nitrogen (added as ammonium sulphate) leads to an increase in the amount of AMC accumulated per mg. dry weight (Table 4), whereas succinate reduces the amount of accumulation.

Fig. 4. Amount of acetylmethylcarbinol accumulated by *suc* cultures as a function of time and the presence of succinic acid. Succinic acid = 1.2 mg./flask added as disodium succinate. Lighter lines show dry weight produced. ●—●, *suc* no succinate; ○---○, *suc* plus succinate; ×---×, 7A (*suc*⁺) no succinate.

Table 4. Effect of succinic acid and ammonium nitrogen on the accumulation of acetylmethylcarbinol by a *suc* mutant

Succinic acid (mg./flask)	mg. N per flask			
	1.0	5.0	10.0	20.0
0	329 (4)	—	—	—
0.3	109 (19)	179 (20)	183 (14)	224 (12)
1.2	15 (33)	—	—	—
3.0	1 (33)	—	—	—

Cultures grown 4 days in medium containing sodium potassium tartrate as a substitute for ammonium tartrate.

Nitrogen added as ammonium sulphate. Succinic acid added as disodium succinate.

AMC values as µg. accumulated/mg. dry weight. Values averages of duplicate flasks. Dry weight given in parentheses.

There is a reciprocal relationship between AMC accumulation and germination of the *suc* and *at suc* mutants. Conidia incubated in phosphate buffer with sucrose send out long germ tubes, and if the conidial suspension is dense enough there is visible growth after a 23 hr. shaking period. Nitrogen salts completely inhibit the germination of *at suc* conidia, greatly reduce the germination of *suc* conidia, and cause the accumulation of AMC by both *suc* and *at suc* mutants

Table 5. *Effects of the constituents of minimal medium on germination and acetylmethylcarbinol accumulation*

Addition	Strain 7A (<i>at⁺ suc⁺</i>)		Strain 46005 (<i>at⁺ suc</i>)		Strain 86 (<i>at suc</i>)	
	AMC	Germination	AMC	Germination	AMC	Germination
None	0	Small clumps of mycelium	0	Germination with long germ tubes. Some clumps	0	Good germination with long germ tubes
0.5 % ammonium tartrate	0	Germination but no clumps	38	Some germination but many non-germinated conidia	19	Practically no germination of conidia
0.05 % Mg SO ₄ ·7H ₂ O	0	Clumps of mycelium	0	Heavy mycelial clumps formed	0.5	Germination with much mycelial development
0.01 % NaCl, 0.01 % CaCl ₂ trace elements and biotin*	0	Clumps of mycelium	0	Very heavy clumps of mycelium formed	0	Clumps of mycelium
0.5 % KNO ₃	0	Germination with small germ tubes and no clumps	25	Good germination but some non-germinated conidia	20	No germination

AMC as total μ g. accumulated after 23 hr. shaking at 28° determined by direct analysis of the medium after removal of conidia by centrifugation.

Added 4.7×10^7 *suc*, 1.1×10^8 *at suc* and 9.8×10^7 *at⁺ suc⁺* (7A) conidia to 54 ml. of 0.067M-phosphate buffer pH 6.0 plus 2% sucrose plus additions as shown. The conidia were harvested from 7-day cultures and washed three times with water by centrifugation before use.

* Trace elements and biotin added as in minimal medium.

(Table 5). Conidia of the *suc* and *at suc* strains suspended in minimal medium and shaken 2–4 hr. in manometer vessels at 30° accumulate measurable amounts of AMC. No accumulation is noted when conidia of these strains are shaken under identical conditions with 2% sucrose in phosphate buffer (0.067 M, pH 6.0), although sucrose in phosphate buffer is rapidly oxidized by both *suc* and *at suc* strains.

Oxidation studies

The wild-type, *suc* and *at suc* strains oxidize sucrose and acetate readily with no appreciable time lag (Fig. 5). There are no appreciable differences in the rate of mutant and wild-type oxidation. Sodium fluoroacetate inhibits acetate

oxidation by both wild-type and mutant strains (Table 6), with a concomitant increase in the accumulation of citrate (Table 7) as determined by the method of Ettinger, Goldbaum & Smith (1952). Although the data are given for conidia (because of the greater uniformity of this material) *suc* and *at suc* oxidation of acetate and sucrose, inhibition by fluoroacetate and accumulation of citrate can be readily demonstrated with mycelium from 24 hr. shake cultures.

Fig. 5. Oxidation of acetate and sucrose by *suc* and *at suc*. Oxygen consumption measured at 30°. Reaction mixture contained 2 ml. conidial suspension + 0.3 ml. substrate in 0.067 M-KH₂PO₄-Na₂HPO₄ buffer, pH 6.0. ●---●, endogenous; ●—●, acetate, ×—×, sucrose.

Table 6. *Inhibition of acetate oxidation by sodium fluoroacetate*

Strain	Addition			
	None	Sodium acetate, 10 μM	Sodium fluoroacetate, 20 μM	Sodium acetate plus sodium fluoroacetate
<i>at suc</i>	24	202	8	25
<i>suc</i>	28	233	9	29
7 A (<i>at</i> ⁺ <i>suc</i> ⁺)	17	194	5	20

Recorded μl. O₂ taken up in the first 90 min. after tipping. Final volume in the main compartment = 2.5 ml.

Added suspensions of conidia grown 7 days and washed twice with water, once with buffer after harvesting. Suspensions made up to approximately the same turbidity in 0.067 M-phosphate buffer pH 6.0. Two ml. of conidial suspensions added to each vessel.

It is possible to demonstrate an active isocitric-dehydrogenase and glutamic-dehydrogenase in extracts of *suc* and *at suc* prepared and tested by a method similar to that used by Fincham (1954). Extracts of *at suc* prepared from material grown 2 days in shake culture on minimal medium plus succinate contain an active glutamic-dehydrogenase, even though these strains do not show significant amounts of growth with glutamate as a growth supplement before the end of a 7-day lag period (Fig. 6). α-Ketoglutaric acid, sterilized by filtration, produces a growth response by *at suc* as rapidly as does succinate. Although glutamate does not initiate growth within the first 3 days, it does

stimulate growth in this time period in the presence of a limiting amount of succinate (Table 8).

Yemm & Folkes (1954) showed that nitrogen-deficient cultures of *Torulopsis utilis*, suspended under aerobic conditions in a carbohydrate-free medium, doubled or tripled their rate of oxygen uptake on the addition of ammonium nitrogen. Since this appeared to be a method of studying the systems of

Fig. 6. Presence of glutamic dehydrogenase in an *at suc* strain. Reaction mixtures: isocitrate 400 μ g., 0.6 ml. 0.05 M-tris(hydroxymethyl)aminomethane buffer pH 7.2, 0.1 ml. 0.02 M-MnCl₂, 50 μ g. TPN (Sigma Chemical Company), 0.1 ml. enzyme extract. Total volume = 3 ml. Dotted line without isocitrate. Added TPN at arrow A. At arrow B added 5 μ M- α -ketoglutarate, 10 μ M-NH₄Cl. Enzyme extracted from 2-day shake cultures grown in minimal medium with succinate, collected, washed, ground with alumina in 0.05 M-KH₂PO₄-Na₂HPO₄ buffer pH 7.2 and centrifuged in the cold at 11,000 g for 20 min. Supernatant used as enzyme preparation.

Table 7. Accumulation of citric acid in the presence of sodium fluoroacetate

Strain	None	Addition		
		Sodium acetate, 100 μ M	Sodium fluoro-acetate, 50 μ M	Acetate plus fluoroacetate
<i>suc</i> (46005)	64	98	93	160
	—	89	120	190
<i>suc</i> ⁺ (7 A)	32	57	110	170
	40	69	92	130

Recorded total μ g. of citric acid accumulated in a total volume of 2.5 ml. Added 3.4×10^8 *suc*⁺ conidia, 3.7×10^8 *suc* conidia. Citric acid extracted and reaction stopped after two hours' incubation at 30° with shaking by heating in a boiling water bath with 0.5 ml. 9 N-H₂SO₄ and diluting to 10 ml.

carbohydrate metabolism under conditions of maximum utilization, an experiment similar to, but not identical with, that of Yemm & Folkes was performed. Conidia of the various strains were prepared as usual and washed

Table 8. *Stimulation of growth of at suc by glutamate in the presence of limiting concentrations of succinate*

Disodium succinate (mg./flask)	Monosodium glutamate (mg./flask)				
	0	1	2	4	8
0	0	0	0	0.2	0.8
1	5	7	8	10	25
2	13	10	18	26	39
4	21	28	32	37	46
8	32	43	43	46	54

Recorded mg. dry weight produced by *at suc* (86-1A) after 3 days in minimal medium plus additions as shown. All values averages of duplicate determinations.

Table 9. *Effect of nitrogen on oxygen uptake by suc conidia incubated with sucrose*

Expt. no.	Preincubation period (hr.)	Nitrogen	Strain			Time after tipping
			<i>at⁺ suc⁺</i> (7 A)	<i>suc</i> (46005)	<i>at suc</i> (86)	
1	1	0	351	239	374	105 min.
			359	232	375	
		+	450	172	284	
			445	182	287	
	24	0	130	246	137	120 min.
			114	183	145	
		+	201	247	144	
			176	210	171	
2	1	0	318	251	257	120 min.
			365	244	—	
		+	533	211	184	
			531	215	169	
	24	0	136	65	136	120 min.
			135	54	134	
		+	254	74	208	
			248	73	181	

Conidia grown 7 days. Harvested and washed twice with water by centrifugation. Suspended in buffer as described in text.

2 ml. suspension added to flask. Side arm contained either 0.2 ml. of water or 0.2 ml. of a solution containing 2.75 mg. N/ml. (as $(\text{NH}_4)_2\text{SO}_4$) as indicated. 0.067 M- KH_2PO_4 - Na_2HPO_4 buffer pH 6.0, 2% sucrose plus 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Recorded total $\mu\text{l. O}_2$ taken up in the indicated time interval.

with 0.067 M-phosphate buffer pH 6.0 containing 2% sucrose and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. One set of conidia of each strain was suspended in this buffer 1 hr., and after resuspending in fresh buffer of identical composition, 2 ml. of this conidial suspension were placed in a manometric vessel containing ammonium sulphate in the side arm (Table 7). The second set of conidia was shaken for 24 hr. in the sugar-containing buffer, then the germinated conidia were strained through cheese-cloth, resuspended in fresh sugar-containing buffer and treated in a manner similar to the conidia of set 1 (Table 9). In these experiments the turbidity of the conidial suspensions of set 1 was

adjusted so that approximately the same number of wild-type, *suc* and *at suc*, conidia were added to the manometers. The suspensions after shaking for 24 hr. were not adjusted in this manner.

The addition of ammonium nitrogen to a 'fresh' conidial suspension results in a stimulation of oxygen uptake by wild-type conidia in a manner reminiscent of the Yemm & Folkes phenomenon, but the addition of ammonium nitrogen depresses the uptake of oxygen by mutant conidia. After 24 hr. shaking in the absence of nitrogen, however, even mutant conidia are slightly stimulated by the addition of nitrogen, although not as much as the wild-type.

Carbon dioxide fixation

The different effect of ammonium on the oxygen uptake of *suc* and *suc*⁺ prompted us to look for the mechanisms that might supply dicarboxylic acids for interaction with ammonium. Since carbon dioxide fixation is one mechanism for the *net* synthesis of dicarboxylic acids (Utter & Wood, 1951) it was decided to measure the amount of carbon dioxide fixation by wild-type and *suc* strains. Conidia prepared by the methods used to study the effect of nitrogen on oxygen uptake (cf. above) were incubated with 0.2 μ c. $\text{NaH}^{14}\text{CO}_3$ in closed manometer vessels in the presence and absence of ammonium nitrogen. At the conclusion of a 2 hr. shaking period the conidia were harvested by centrifugation, washed twice with unlabelled 0.1 M- NaHCO_3 and twice with hot water. The conidial suspensions were then transferred to stainless steel planchets, evaporated to dryness, weighed, and their radioactivity determined in a flow counter operated in the Geiger region. Heat inactivated conidia treated in this way gave a count that was never significantly above the background. An attempt was made to use suspensions containing the same number of wild-type and mutant conidia, and in all cases where absolute comparison was desired haemocytometer counts of the suspensions were made.

The addition of nitrogen to a washed conidial suspension causes a large increase in the amount of carbon dioxide fixed by the wild-type, but has only a small effect on carbon dioxide fixation by the *suc* or *at suc* strains (Table 10). As little as 0.1 mg. of N in 2.6 ml. is clearly effective in increasing the carbon dioxide uptake by the wild-type (Fig. 7). The uptake of carbon dioxide by the wild-type strain is linear in the absence of nitrogen but more irregular in its presence (Fig. 8). Under our conditions the fixation of carbon dioxide is not a linear function of the number of conidia; the ratio of carbon dioxide fixation in the presence and absence of nitrogen is higher when the conidial concentration is low than when this concentration is high.

It was suggested to the author by Dr R. C. Fuller that a significant portion of the total fixed carbon dioxide would be removed by the vigorous washing of the conidia involved in our experiments. We therefore studied the *total* amount of carbon dioxide fixed by stopping the reaction at the conclusion of the incubation period by the addition of trichloroacetic acid (final concentration 10%, w/v), and then separating the conidia from the acid soluble fraction by centrifugation after 30 min. at 40–50°. Samples of both fractions were

evaporated to dryness, weighed and their radioactivity determined. The counts recorded in Table 11 are corrected for self-absorption.

The wild-type fixes about as much carbon dioxide into conidia as into the acid soluble fraction, both in the presence and absence of an ammonium salt.

Table 10. *Effect of ammonium nitrogen on the fixation of $\text{NaH}^{14}\text{CO}_3$*

Expt.	Nitrogen	Strain		
		7 A (<i>at</i> ⁺ <i>suc</i> ⁺)	46005 (<i>suc</i>)	86-1 (<i>at</i> <i>suc</i>)
1	0	384	163	369
		396	194	—
	+	2120	217	362
		1650	227	231
		(5.2×10^7)	(2.6×10^7)	(4.3×10^7)
2	0	257	—	168
		425	—	160
		323	—	109
	+	1785	—	245
		1910	—	245
		1890	—	158
		(6.6×10^7)	—	(4.4×10^7)

Recorded total counts per minute after 2 hr. shaking in 2 ml. 0.067 M- KH_2PO_4 - Na_2HPO_4 buffer pH 6.0 with 2% sucrose and 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ plus 0.2 ml. $\text{NaH}^{14}\text{CO}_3$ solution containing 1 μC /25 mg. NaHCO_3 /ml.

Values in parentheses indicate number of conidia added (haemocytometer counts). Experiment 1, final washings with hot water. Expt. 2, final washings with hot ethanol.

Manometer vessels shaken 2 hr. at 30° after tipping $\text{NaH}^{14}\text{CO}_3$.

Expt. 1, 6-day-old cultures; heat inactivated conidia showed no incorporation. Expt. 2, 7-day-old cultures; heat inactivated conidia showed no incorporation.

Fig. 7

Fig. 8

Fig. 7. Effect of ammonium sulphate nitrogen on the incorporation of $\text{NaH}^{14}\text{CO}_3$ by wild-type and *suc* conidia. Measured incorporation of $\text{NaH}^{14}\text{CO}_3$ as total c.p.m. after 2 hr. Ammonium sulphate added to 2.0 ml. conidial suspension in buffer (0.067 M- KH_2PO_4 - Na_2HPO_4 pH 6.0 + 2% sucrose + 0.05% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$) plus 0.2 ml. $\text{NaH}^{14}\text{CO}_3$ containing 1 μC /25 mg. NaHCO_3 /ml. $\times$, *suc*⁺; $\circ$, *suc*.

Fig. 8. Incorporation of $\text{NaH}^{14}\text{CO}_3$ by wild-type conidia as a function of time, conidial concentration and the presence of nitrogen. Dotted curve, 1 ml. conidial suspension; solid curve, 2 ml. conidial suspension. Top curve of each in the presence of 0.5 mg. of nitrogen as $(\text{NH}_4)_2\text{SO}_4$.

The striking feature of this experiment, however, is the effect of ammonium salts in increasing the amount of carbon dioxide fixed into the acid soluble fraction of the mutant tested without causing a concomitant increase in the conidial fraction. More recent experiments indicate that the fixed material in the acid soluble fraction is not ether extractable. Succinate, a compound which will permit growth of the *suc* strains in minimal medium, does not lead to an increase in the amount of carbon dioxide fixed into the conidial fraction, even after 2 hr. preincubation of conidia in succinate-containing buffer.*

Table 11. *Distribution of the $^{14}\text{CO}_2$ fixed by wild-type and at suc conidia*

Fraction	Strain					
	7A (<i>at⁺ suc⁺</i>)		86-1 (<i>at suc</i>)		86-1 (<i>at suc</i>) pre-	
	Nitrogen		Nitrogen		incubated with succinate	Nitrogen
	0	+	0	+		
Conidia	1720	4900	785	655	801	720
	1670	5280	710	711	819	712
Supernatant (acid-soluble)	2230	6880	520	1610	570	2360
	1960	5280	545	1800	545	2480

Recorded total counts per minute in the various fractions after 2 hr. shaking in 2 ml. 0.067 M-KH₂PO₄-Na₂HPO₄ buffer pH 6.0 with 1% sucrose and 0.05% MgSO₄·7H₂O plus 0.2 ml. NaH¹⁴CO₃ solution containing 2 μC /25 mg. NaHCO₃/ml.

Nitrogen added=0.25 ml. of a solution of (NH₄)₂SO₄ containing 2 mg. N/ml. Succinate added to buffer where indicated=0.1% disodium succinate. The conidial concentrations were different for the three strains tested.

DISCUSSION

The unstable balance (Strauss, 1955*b*) between carbohydrate and nitrogen metabolism in the *suc* mutants is seen: (*a*) in the inhibitory effect of nitrogen salts on the germination and growth of *suc* and *at suc* conidia; (*b*) in the relationship between the accumulation of AMC and the presence of ammonium salts; (*c*) in the lack of increased oxygen uptake in the presence of ammonium salts by mutant conidia compared to the wild-type; and (*d*) by the relative accumulation of acid-soluble products of carbon dioxide fixation in the presence of ammonium salts by the mutant tested compared to the wild-type. Assuming that the supply of dicarboxylic acid is limiting in the *suc* mutants (an assumption required by the growth-promoting effects of dicarboxylic acids), addition of nitrogen salts might be expected to compete with C₂ fragments for the limited supply of C₄ compounds available by forming amino acids or other synthetic products, the nature of which has not yet been established (Fig. 8). Ammonium salts are known to be ketogenic when added to liver slices, and a similar competition for dicarboxylic acids has been suggested by Recknagel & Potter (1951) to account for this ketogenic effect. In *neurospora*, when the

* L-asparagine increases the amount of C¹⁴O₂ fixed by mutant conidia to almost the wild-type level.

supply of C_4 acids required for C_2 oxidation is diminished, products such as pyruvic acid α -ketoisovaleric acid and AMC accumulate (Fig. 9).

Notwithstanding the growth-promoting action of intermediate compounds of the tricarboxylic acid cycle, it seems likely that the *suc* and *at suc* strains make the same use of the tricarboxylic acid cycle for acetate oxidation as does the wild-type (Strauss, 1955a). Both *suc* and *at suc* strains oxidize acetate as well as the wild-type, even though the *at suc* strain does not start growth with acetate as a supplement to minimal medium until after a 7-day lag period. Fluoroacetate, which is commonly used as an inhibitor of the tricarboxylic

Fig. 9. Scheme of metabolism in the *suc* and *at suc* mutants of *Neurospora crassa*.

acid cycle (Peters, 1952), inhibits acetate oxidation by both wild-type and *suc* strains, and this inhibition is accompanied by an accumulation of citrate indicating that citrate is an intermediate in acetate oxidation by the *suc* mutants of *Neurospora*. Other mutants (of *Escherichia coli*) requiring dicarboxylic acids for growth and not responding to acetate have been described (Gilvarg & Davis, 1954), but there is presumably no use made of the tricarboxylic acid cycle in these strains since they are unable to oxidize acetate and lack condensing enzyme. Non-utilization of acetate for growth by strains requiring dicarboxylic acids, however, does not preclude use of the tricarboxylic acid cycle as a mechanism of acetate oxidation.

Pyruvic acid, AMC and C_2 condensation products which do not require complete oxidation accumulate in cultures of the *suc* mutants (necessarily grown in the presence of nitrogen salts) because of the deficiency in the dicarboxylic acid catalyst required for C_2 fragment oxidation. The same

accumulation products are produced in the *suc* mutants, where there is no immediate block to pyruvate oxidation as in the *ac* mutants (Strauss & Pierog, 1954), where there is a block in the oxidation of pyruvate: another illustration of the danger of using accumulations as evidence of genetic block (Adelberg, 1953). The relationship between succinate, a growth requirement, and AMC, a by-product of a true intermediate, is another case in which an end product controls the accumulation of a precursor (Strauss, 1955*a, b*; Adelberg & Umbarger, 1953). In this case, however, the 'end product' (succinate) is a catalyst for the metabolism of the intermediate.

At present it is not possible for the author to give an adequate explanation for the dicarboxylic acid deficiency of the *suc* mutants or to locate an absolute 'genetic block' in these strains. The tricarboxylic acid cycle operating in a complete cycle regenerates 1 mole of dicarboxylic acid for every mole supplied. Therefore, some other mechanism must be present to insure a net increase in the amount of dicarboxylic acids in an organism using the cycle for energy liberation and for the production of intermediates (Abelson & Vogel, 1955). At least two possible alternate mechanisms exist for the synthesis of C_4 acids; carbon dioxide fixation in a $C_3 + C_1$ type of condensation (Utter & Wood, 1951), and a $C_2 + C_2$ condensation by the Wieland-Thunberg reaction (Seaman & Naschke, 1955). However, the *suc* mutants are able to fix carbon dioxide, and the amount of carbon dioxide fixed in the acid-soluble fraction is increased on the addition of ammonium salts. The deficiency in the *suc* mutants is apparently in the incorporation of the fixation products into the non-soluble fraction (protein?), but this is what might be expected in any strain unable to grow.

A deficiency in the Wieland-Thunberg condensation might logically be expected in the *at* strain because of the lack of response of the *at suc* strain to acetate. However, Seaman (personal communication) has shown that both wild-type and mutant (*at suc*) preparations show comparable activities of the reversible succinate-cleaving enzyme.

Investigations in biochemical genetics seem to uncover two types of 'biochemical mutants'; one type showing a fairly simple relationship between gene, enzyme and growth requirement (Fincham, 1954; Davis, 1955), the other requiring a complex interpretation in terms of inhibitors and the balance of reactions (Mitchell & Mitchell, 1952). The advantage of studies of this latter type is that they show, somewhat more clearly than is possible by studies with the wild-type, the interactions between multi-enzyme systems (Dixon, 1949) which may be the basis for the intracellular regulation of metabolism (Strauss, 1955*b*). An effect that is present in 'wild-type' preparations, i.e. the ketogenic effect of ammonium (Recknagel & Potter, 1951) may be magnified in a mutant until a growth requirement occurs. Study of such mutants permits a more ready identification of the interactions actually regulating metabolism in normal organisms.

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