REFERENCES

Adelberg, E. A. & Meyers, J. W. (1953). *J. Bact.* **65**, 348.
Barker, H. A., Smyth, R. D., Weissbach, H., Toohey, J. I., Ladd, J. N. & Volcani, B. E. (1960). *J. biol. Chem.* **235**, 480.
Cross, M. J. & Woods, D. D. (1954). *Biochem. J.* **58**, xvi.
Davis, B. D. & Mingioli, E. S. (1950). *J. Bact.* **60**, 17.
Gibson, F. & Woods, D. D. (1960). *Biochem. J.* **74**, 160.
Guest, J. R. (1959). *Biochem. J.* **72**, 5P.
Guest, J. R. (1960). *Biochem. J.* **76**, 405.
Guest, J. R., Helleiner, C. W., Cross, M. J. & Woods, D. D. (1960). *Biochem. J.* **76**, 396.
Hatch, F. T., Takeyama, S. & Buchanan, J. M. (1959). *Fed. Proc.* **18**, 243.
Helleiner, C. W., Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.
Kisliuk, R. L. & Woods, D. D. (1957). *J. gen. Microbiol.* **18**, xv.
Kisliuk, R. L. & Woods, D. D. (1960). *Biochem. J.* **75**, 467.
Layne, E. (1957). In *Methods in Enzymology*, vol. 3, p. 451. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Smith, E. L., Parker, L. F. J. & Gant, D. E. (1956). *Biochem. J.* **62**, 14P.
Szulmajster, J. & Woods, D. D. (1960). *Biochem. J.* **75**, 3.
Weissbach, H., Toohey, J. & Barker, H. A. (1959). *Proc. nat. Acad. Sci., Wash.*, **45**, 521.
Woods, D. D. (1958). *Proc. 4th int. Congr. Biochem., Vienna*, **11**, 302.

Biochem. J. (1960) **77**, 430

The Metabolism of C_2 Compounds in Micro-organisms

4. SYNTHESIS OF CELL MATERIALS FROM ACETATE BY *ASPERGILLUS NIGER**

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Many fungi grow readily on C_2 compounds as the sole source of carbon and excrete, into their growth media, intermediates of the tricarboxylic acid cycle or compounds derived therefrom. The tricarboxylic acid cycle has been shown to be the major route both for the catabolism of C_2 compounds such as acetate or ethanol and for the provision of the carbon skeletons of most cellular constituents and of compounds excreted (for review, see Chain, 1955; Foster, 1958; Kornberg, 1959). Its continued operation under these conditions necessitates the occurrence of reactions, ancillary to the tricarboxylic acid cycle, which lead to the net formation of intermediates of that cycle from the C_2 growth substrate.

The main purpose of this paper is to show that *Aspergillus niger* growing on acetate as sole carbon source incorporates isotope from [2-^{14}C]acetate in a manner which excludes the primary formation of succinate from acetate (Thunberg, 1920) and which indicates that the required syntheses of intermediates of the tricarboxylic acid cycle are effected via the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957). Cell-free extracts contain a high activity of the requisite enzymes of the glyoxylate cycle and can catalyse the net formation of malate and succinate from acetyl-coenzyme A and *iso*citrate. Extracts of *A. niger*, grown on substrates the metabolism of which does not necessitate such syntheses of C_4 compounds from acetate, are virtually devoid of a key enzyme of the glyoxylate cycle, *iso*citratase: this supports the view that that cycle plays an essential role in growth on acetate.

This work has been presented in part to the Biochemical Society (Kornberg & Collins, 1958).

* Part 3: Kornberg & Madsen (1958).

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METHODS

Maintenance and growth of the mould. The strain of *A. niger* studied had a high ability for producing citric acid. The organism was maintained on slopes consisting of 50 mM-ammonium acetate, 20 mM-sodium–potassium phosphate buffer, pH 7·4 (prepared by mixing 80·4 vol. of 20 mM-Na_2HPO_4 with 19·6 vol. of 20 mM-K_2HPO_4), salts (25 mg. of $MgSO_4,7H_2O$, 0·1 mg. of $FeSO_4,7H_2O$, 0·1 mg. of $ZnSO_4,7H_2O$, 10 μg. of $MnSO_4,4H_2O$, 1 μg. of $CuSO_4,5H_2O$ and 0·2 μg. of $K_2Cr_2O_7$/100 ml. of medium), 0·05% (w/v) of 'Difco' yeast extract and 2% (w/v) of agar (Hopkin and Williams Ltd., Chadwell Heath, Essex). Stock cultures were grown at 30° until sporulation had taken place (4–7 days), and were then stored at 2°. For growth in liquid medium, a suspension of spores in sterile water was used to inoculate Carrel culture flasks (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of medium which, with the omission of the agar, was identical with that used for the slopes. The flasks were shaken in air at 30° for 20–

24 hr. Larger quantities of cells were grown after inoculation of 9 l. of medium, contained in an aspirator bottle (10 l.) fitted with a sterile air supply to maintain aeration and agitation, at 30°. For growth on glucose, the liquid medium contained the same buffer and salts as before but the ammonium acetate was replaced by 50 mM-glucose and 50 mM-ammonium chloride.

Incorporation of [2-^{14}C]*acetate by whole cells. A. niger* (700 mg. wet wt.) was harvested by filtration after 20 hr. growth on acetate, washed with water and suspended in 60 ml. of 20 mM-potassium phosphate buffer, pH 7·1. One-fifth of this suspension was shaken at 30° in a conical flask, together with 0·4 ml. of 0·5 M-ammonium acetate. The flask was shaken for 10 min. at 30°. Sodium [2-^{14}C]acetate (44·4 μmoles, 200 μc) in 1 ml. of water was mixed with 2 ml. of 20 mM-phosphate buffer pH 7·1; 0·25 ml. of this mixture was added to 3 ml. of hot ethanol together with 1 ml. of the *A. niger* suspension. This constituted the first (zero-time) sample. The remainder of the [2-^{14}C]acetate solution was added to the mould suspension, and samples (1 ml.) were taken at measured intervals and pipetted into tubes containing 3 ml. of hot ethanol, standing in a bath at 80°. The subsequent treatment of these samples, extraction with aqueous ethanol, chromatographic analysis, radioautography, assay and identification of the labelled compounds, was as described by Kornberg (1958).

Preparation of extracts from crushed Aspergillus niger. *A. niger* was harvested by filtration after 24 hr. growth, and was washed with 0·9% (w/v) potassium chloride solution. The damp mould was frozen and crushed at −25° in a Hughes press (Hughes, 1951). Extracts were prepared by stirring some crushed mycelia with 4 vol. of 0·1 M-potassium phosphate buffer, pH 7·2, at 0°. Some of the suspension was dried overnight at 100° for determination of the dry weight of mould present. The rest was centrifuged for 10 min. at approximately 20 000 *g* in an International refrigerated centrifuge at 0°. The supernatant solution was used in experiments involving enzymic assays.

Incorporation of [^{14}C]*acetate by cell extracts.* The incubation mixtures used for these experiments contained 100 μmoles of potassium phosphate buffer, pH 7·4, 10 μmoles of reduced glutathione, 5 μmoles of magnesium chloride, 0·08 μmole of coenzyme A, 1·9 μmoles of potassium [^{14}C]acetate (containing 10 μc of isotope), 10 μmoles of other unlabelled substrates as indicated, 0·2 ml. of *A. niger* extract and water to 0·95 ml. After incubation for 3 min. at 30°, 5 μmoles of adenosine triphosphate (ATP) tetrasodium salt were added. The reaction was stopped by adding 3 ml. of ethanol; the resulting aqueous ethanolic solutions were analysed as previously described (Kornberg, 1958; Kornberg & Madsen, 1958).

Incorporation of [1-^{14}C]*glyoxylate by cell extracts.* The incubation procedure was similar to that described for incorporation experiments involving [^{14}C]acetate, but the acetate was replaced by 7·5 μmoles of sodium [1-^{14}C]glyoxylate (containing 4·5 μc of isotope), which had been prepared from the calcium salt by treatment with Dowex-50 (Na^+ form).

Enzyme assays. Acetic thiokinase was determined as described by Jones & Lipmann (1955). *iso*Citratase was measured by the procedure of Olson (1959). *iso*Citrate was assayed with the *iso*citric dehydrogenase of *Pseudomonas* KB1 (Kornberg & Madsen, 1958) by spectrophotometric measurement of the reduction of triphosphopyridine nucleotide at 340 mμ (Kornberg, 1955). Succinate was estimated manometrically with the succinoxidase of washed pigeon-breast muscle (Krebs, 1937; Umbreit, Burris & Stauffer, 1957).

Identification of glyoxylate. Glyoxylate semicarbazone, produced by enzymic cleavage of *iso*citrate and interaction of the formed glyoxylate with semicarbazide (Olson, 1959), was decomposed with 6 N-hydrochloric acid and a slight excess of saturated 2:4-dinitrophenylhydrazine hydrochloride at room temperature overnight. The 2:4-dinitrophenylhydrazone was extracted into ethyl acetate and this non-aqueous layer was extracted with M-sodium carbonate. This solution was acidified with 6 N-hydrochloric acid and re-extracted with ethyl acetate. The quantity of keto acid present in a portion of this solution was estimated by the method of Friedemann & Haugen (1943); the ratio of the extinction at 445 mμ to that at 520 mμ was 2:1, indicating that glyoxylate 2:4-dinitrophenylhydrazone was the sole derivative present. This was confirmed by comparison of the spectra of the enzymically formed material and that of authentic glyoxylate 2:4-dinitrophenylhydrazone. The remainder of the material extracted into ethyl acetate was analysed by ascending chromatography in the solvent of El Hawary & Thompson (1953): again, the product behaved identically with that of authentic glyoxylate 2:4-dinitrophenylhydrazone.

Materials used. Potassium dihydrogen L$_s$-*iso*citrate was a gift from Dr H. B. Vickery and highly purified sodium glyoxylate from Dr I. Zelitch (both at the Connecticut Agricultural Experiment Station, New Haven, Conn., U.S.A.). Pyridine nucleotides and coenzyme A were obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A.; ATP (tetrasodium salt) from the Zellstoff-Fabrik Waldhof, Wiesbaden, Germany; glutathione from The Distillers Co., Speke, Lancs.; and isotopic materials from The Radiochemical Centre, Amersham, Bucks. All other reagents used were of A.R. grade or of the highest purity commercially available.

RESULTS

Incorporation of [2-^{14}C]*acetate by* Aspergillus niger. When [2-^{14}C]acetate was added to suspensions of *A. niger*, shaken at 30° in acetate growth medium, isotope was rapidly incorporated into cellular constituents. The incorporation into the fraction of the cells soluble in aqueous ethanol was approximately linear with time over the first 4 min. of the experiment (Fig. 1).

Distribution of ^{14}C *amongst the labelled constituents of the soluble fraction.* Two-dimensional chromatography and radioautography of the aqueous ethanol-soluble fractions obtained from each sample of the incubated suspension showed that ^{14}C from acetate rapidly appeared in many of the intermediates of the tricarboxylic acid cycle and in amino acids derived therefrom: even after the shortest time of incubation (13 sec.) isotope was found in malate, citrate, aspartate, glutamate and succinate. Isotope was detected in alanine after 50 sec., but in phosphorylated materials only after more than 5 min. This latter finding showed that

carbon dioxide-fixing reactions, such as operate in photosynthetic (Calvin, 1955) or autotrophic (Santer & Vishniac, 1955; Trudinger, 1955, 1956; Aubert, Milhaud & Millet, 1956, 1957; Kornberg, Collins & Bigley, 1960*a*) organisms were not of primary importance in the incorporation of acetate.

The difficulties of accurately and speedily obtaining samples of uniform size from the heterogeneous suspension of *Aspergillus* were such that only approximate estimates can be made of the rate at which the added [2-^{14}C]acetate was utilized; moreover, materials which are volatile (such as carbon dioxide) or are lost in the chromatographic procedure (such as pyruvate or oxaloacetate) were not assayed. However, the relative distribution of isotope within each sample fairly reflects the time course of incorporation of isotope from acetate.

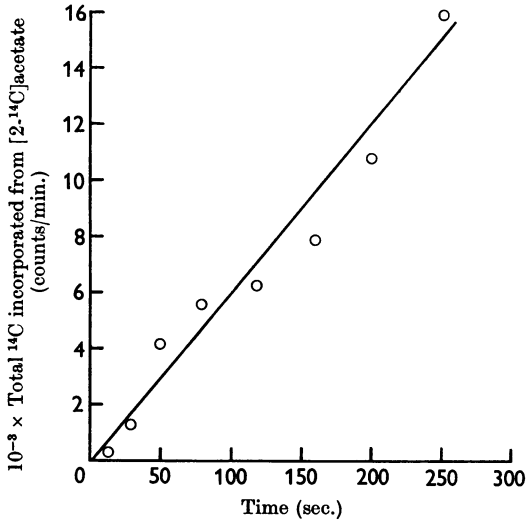

Fig. 1. Incorporation of ^{14}C from [2-^{14}C]acetate into the fraction soluble in aqueous ethanol of *Aspergillus niger* growing on acetate. For conditions, see Methods section.

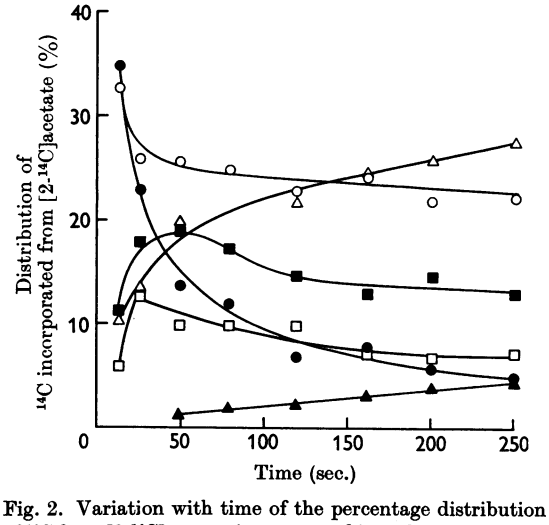

Fig. 2. Variation with time of the percentage distribution of ^{14}C from [2-^{14}C]acetate incorporated into the constituents of the fraction, soluble in aqueous ethanol, of *Aspergillus niger* growing on acetate. For conditions, see Methods section. ●, Citrate; ○, malate; □, succinate; ■, aspartate; △, glutamate; ▲, alanine.

When the proportion of the total radioactivity of each sample contributed by each labelled compound, expressed as the percentage of the total isotope in the sample, was plotted against time, smooth curves could be drawn through the points obtained (Fig. 2). Initially, approx. 35% of the incorporated ^{14}C was present in citrate, and approx. 33% in malate, whereas aspartate and glutamate contained respectively 11 and 10%. Some 5% of the isotope was present in succinate. These proportions rapidly altered with time: both citrate and malate decreased sharply with time, whereas succinate and aspartate, after an initial rise, reached a steady level at which they contributed respectively 9 and 15% of the total radioactivity of each sample.The proportion of isotope present in glutamate rose steadily throughout the incubation, as did that in alanine. After 100 sec., a 'steady state' had been reached: the labelling of the intermediates of the tricarboxylic acid cycle at and after this time may be taken as a reflexion of the 'pool sizes' of these compounds, since, by this time, these intermediates appear to be saturated with the isotope entering from the administered acetate.

The rapid initial decrease with time in the proportion of isotope contained in citrate and the concomitant rise of that in succinate indicates, as expected from the tricarboxylic acid cycle, that isotope from acetate entered citrate before it appeared in succinate. But the behaviour of malate, which is similar to that of citrate, is at variance with that expected from the operation of the tricarboxylic acid cycle; at the earliest times, malate contained nearly 6 times as much isotope as did succinate, whereas, after establishment of the 'steady state', this ratio had decreased to slightly over 2. This showed that at the earliest times succinate could not have been an isotopic precursor of malate, but that isotope from the added [2-^{14}C]acetate must have entered malate without passing through the stage of succinate. These results, which are similar to those reported for *Pseudomonas* KB1 (Kornberg, 1958) therefore offer presumptive evidence for the operation of the glyoxylate cycle in *A. niger* growing in acetate.

Incorporation of [1-^{14}C]*acetate by extracts of* Aspergillus niger. Cell-free extracts of acetate-grown *Aspergillus* catalysed the formation of

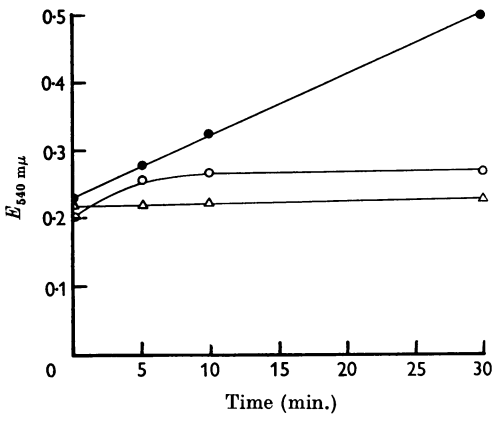

Fig. 3. Time course of the formation of acetohydroxamic acid by extracts of acetate-grown *Aspergillus niger*. For conditions, see Methods section. ●, Complete system; ○, acetate omitted; △, coenzyme A omitted.

acetyl-coenzyme A from acetate, ATP and coenzyme A (Fig. 3). The formation of acetyl-coenzyme A, measured as acetohydroxamic acid in the colorimetric assay of Jones & Lipmann (1955), depended on the presence of acetate and coenzyme A and proceeded linearly with time over the period of incubation (30 min.). The activity of acetic thiokinase in the extracts fell rapidly on storage: the most active preparation tested, assayed less than 1 hr. after harvesting of the cells, catalysed the formation of 1·74 μmoles of acetohydroxamic acid/mg. dry wt. of cells/hr., which (if the activity of this enzyme were the rate-limiting step in growth on acetate) would allow a doubling of cell mass in about 12 hr. and is of the order of the observed mean generation time (9·1 hr.).

When [1-^{14}C]acetate was added to cell extracts supplemented with ATP, magnesium chloride, glutathione and coenzyme A, small amounts (4–9 μm-moles) of radioactive citrate, malate, succinate and fumarate were formed (Table 1). Further supplementation of this mixture with α-oxoglutarate, succinate, fumarate or pyruvate did not promote any major increase in the amounts of isotope incorporated. However, addition of oxaloacetate resulted in the formation of 816 μm-moles of labelled citrate and 90 μm-moles of labelled glutamate, which indicated that the extract contained the citrate-forming condensing enzyme (Ochoa, Stern & Schneider, 1951) and probably also some aconitase, *iso*citric dehydrogenase and either glutamic dehydrogenase or α-oxoglutaric–amino acid transaminases. A similarly large incorporation of isotope from acetate was effected by addition of glyoxylate: the content of labelled malate rose from 9 to 822 μm-moles and that of fumarate from 5 to 187 μm-moles. This indicated the presence in the extract of fumarase and of one of the key enzymes of the glyoxylate cycle, malate synthetase (Wong & Ajl, 1956; Dixon, Kornberg & Lund, 1960) which catalyses the reaction (i) shown at foot of page.

A major incorporation of isotope into malate was also observed when *iso*citrate was added to the reaction mixture: 234 μm-moles of labelled malate and 50 μm-moles of labelled fumarate were formed, these compounds being the only labelled products of the reaction. This indicates that the extract contained, in addition to malate synthetase, the other enzyme unique to the glyoxylate cycle, *iso*citratase (Campbell, Smith & Eagles, 1953; Smith & Gunsalus, 1955, 1957; Olson, 1954, 1959; Saz, 1954; Saz & Hillary, 1956), which catalyses the reaction (ii) shown at foot of page.

The combined effect of these reactions, reaction (iii) shown at foot of page, would explain the observed patterns of isotope incorporation in the presence of *iso*citrate or glyoxylate.

Malate synthetase and iso*citratase in extracts of acetate-grown* Aspergillus. Further evidence for the presence of malate synthetase in extracts of acetate-grown *Aspergillus* was obtained with experiments in which either [2-^{14}C]acetate or [1-^{14}C]glyoxylate was used as precursor of labelled malate (Table 2): little or no labelled malate was formed from labelled acetate in the absence of added unlabelled glyoxylate, or from labelled glyoxylate in the absence of unlabelled acetate, and only a mixture of the two substrates resulted in the formation of [^{14}C]malate.

Evidence for the presence of *iso*citratase in such extracts was obtained in several ways. When extracts were incubated as previously described with *iso*citrate, magnesium chloride and either glutathione (Kornberg & Madsen, 1957, 1958) or sodium sulphide (Kornberg & Beevers, 1957*a*, *b*) keto acids (as determined by the assay procedure of Friedemann & Haugen, 1943) were rapidly produced. Chromatographic analysis (El Hawary & Thompson, 1953) of the 2:4-dinitrophenylhydrazones of such keto acids showed that the material predominantly formed was glyoxylate, although small quantities of α-oxoglutarate were also produced. No glyoxylate was formed in the absence of either *iso*citrate or cell extract, or in the complete

[^{14}C]Acetyl-coenzyme A + glyoxylate + water → [^{14}C]malate + coenzyme A (i)

*iso*Citrate ⇌ succinate + glyoxylate (ii)

[^{14}C]Acetyl-coenzyme A + *iso*citrate + water → succinate + [^{14}C]malate + coenzyme A (iii)

Table 1. *Incorporation of isotope from* [1-^{14}C]*acetate catalysed by extracts of acetate-grown* Aspergillus niger

The incubation mixture contained 100 μmoles of potassium phosphate, pH 7·4, 10 μmoles of glutathione, 5 μmoles of magnesium chloride, 0·08 μmole of coenzyme A, 1·9 μmoles of sodium [1-^{14}C]acetate (containing 10 μc of isotope), 10 μmoles of potassium acetate, 5 μmoles of ATP, 0·2 ml. of freshly prepared extract (containing approx. 1·1 mg. of soluble protein), 10 μmoles of additional substrates as indicated and water to 1 ml. All tubes were incubated at 30° for 30 min., after which time 3 ml. of absolute ethanol was added. The labelled products formed were analysed as described by Kornberg & Madsen (1958).

Additional substrate (10 μmoles)	Quantities of labelled products formed (μm-moles)				
	Citrate	Succinate	Malate	Fumarate	Glutamate
None	4	8	9	5	0
None: boiled extract	2	0	2	0	0
Oxaloacetate	816	12	19	6	90
Citrate	6	9	30	10	5
L$_s$(+)-*iso*Citrate	8	9	234	50	0
α-Oxoglutarate	9	12	12	21	7
Glyoxylate	6	6	822	187	0
Succinate	5	3	7	6	0
Fumarate	8	0	0	0	3
Pyruvate	10	2	3	25	3

Table 2. *Formation of labelled malate from* [2-^{14}C]*acetate or* [1-^{14}C]*glyoxylate in extracts of acetate-grown* Aspergillus niger

The incubation mixture contained 100 μmoles of potassium phosphate, pH 7·4, 5 μmoles of magnesium chloride, 0·08 μmole of coenzyme A, 10 μmoles of glutathione, 5 μmoles of ATP, 0·2 ml. of cell extract (containing approx. 1·1 mg. of soluble protein), other substrates as indicated and water to 1 ml. All tubes were incubated at 30° for 30 min., after which time the reactions were terminated by the addition of 3 ml. of absolute ethanol. The labelled products were analysed as described by Kornberg & Madsen (1958).

Tube no.	Isotopic material added	Additional substrate	Quantities of labelled products (μm-moles)				
			Citrate	Glutamate	Fumarate	Malate	Succinate
1	Sodium [2-^{14}C]acetate (10 μc, 2·2 μmoles)	None	65	31	1	0	6
2	As 1	Glyoxylate (10 μmoles)	72	36	32	154	81
3	Sodium [1-^{14}C]glyoxylate (4·5 μc, 7·5 μmoles)	None	6	2	1	6	2
4	As 3	Acetate (10 μmoles)	60	80	100	234	69

Table 3. *Quantities of succinate and glyoxylate formed from* isocitrate *by extracts of acetate-grown* Aspergillus niger

The amounts of succinate formed from *iso*citrate (Expt. A) were estimated manometrically with succinoxidase from washed pigeon-breast muscle. The conditions of the incubation were similar to those described by Kornberg & Beevers (1957*b*) but glutathione was used as the thiol compound. *iso*Citrate was estimated spectrophotometrically as the extinction change at 340 mμ consequent upon the reduction of triphosphopyridine nucleotide by *iso*citrate and the *iso*citric dehydrogenase of *Pseudomonas* KB1 (Kornberg & Madsen, 1957, 1958). The amounts of glyoxylate formed from *iso*citrate (Expt. B) were measured spectrophotometrically as the total change of extinction at 252 mμ, pH 6, consequent upon the formation of glyoxylic acid semicarbazone (Olson, 1959). The semicarbazone formed was decomposed with 6 N-HCl and 2:4-dinitrophenylhydrazine hydrochloride; chromatography (El Hawary & Thompson, 1953) of the 2:4-dinitrophenylhydrazones showed that of glyoxylic acid to be the only one formed.

Expt.	*A. niger* extract	*iso*Citrate (μmoles) present at		*iso*Citrate utilized (μmoles)	Succinate present at		Succinate formed (μmoles)	Glyoxylate formed (μmole)
		0 min.	30 min.		0 min.	30 min.		
A	Fresh	46·4	32·5	13·9	6·8	19·9	13·1	—
A	Boiled	45·9	45·7	0·2	6·8	6·8	0	—
B	Fresh	3·28	2·43	0·85	—	—	—	0·81
B	Boiled	3·28	3·28	0	—	—	—	0

system if the enzyme was boiled before incubation. Addition of purified aconitase (kindly donated by Dr W. Bartley) and [2-^{14}C]citrate (eluted from chromatograms after reaction of [2-^{14}C]acetate and oxaloacetate, as described in Table 1) further showed that the appearance of glyoxylate was also accompanied by the formation of [^{14}C]succinate as the only labelled product, as expected from the stoicheiometry of reaction (ii).

These results, although providing evidence for the presence of *iso*citratase, were not sufficient for a quantitative estimate of the activity of this enzyme. Unlike the behaviour of extracts of *Pseudomonas* (Smith & Gunsalus, 1957; Kornberg & Madsen, 1957, 1958) or castor beans (Kornberg & Beevers, 1957*a*, *b*), extracts of *Aspergillus* appeared to contain enzymes which destroyed glyoxylate rapidly and without concomitant gas uptake or evolution. The action of the enzyme system found in *A. niger* by Franke & de Boer (1958), which catalyses the dismutation of glyoxylate to oxalate and glycollate, would account for this destruction of glyoxylate. It was thus impossible to obtain from such extracts any quantitative relation between the rates and amounts of *iso*citrate disappearance and glyoxylate formation. Attempts to purify the *iso*citratase by the method described for *Pseudomonas* by Smith & Gunsalus (1957) were unsuccessful: although considerable purification was achieved, the enzyme was rapidly destroyed on storage.

Use was therefore made of the assay procedure of Olson (1959), of which Dr Olson kindly provided details before publication. This technique employs the trapping of glyoxylate formed from *iso*citrate at pH 6 with semicarbazide, the rate of formation of the –C=N– linkage of the semicarbazone being measured spectrophotometrically at 252 mμ. By these means, it was shown that at least 1·04 μmoles of glyoxylate/hr./mg. dry wt. of crushed mould was formed in fresh extracts of acetate-grown *Aspergillus*: since the activity of *iso*citratase is considerably lower at pH 6 than under more alkaline conditions (under which, however, the speed of condensation of glyoxylate and semicarbazide would impose a rate-limiting step), it is likely that the observed value is a minimum one but is sufficient to allow of a doubling of cell mass in 9 hr. When the semicarbazone formed in this reaction was decomposed by incubation with 2:4-dinitrophenylhydrazine in 6N-hydrochloric acid, and the resultant 2:4-dinitrophenylhydrazones were extracted and analysed by paper chromatography (El Hawary & Thompson, 1953), glyoxylate was found to be the only keto compound formed from *iso*citrate. Estimation of the amounts of succinate formed from *iso*citrate, and the amounts of glyoxylate produced under conditions under which the glyoxylate was trapped (Table 3) further confirmed that for each mol.prop. of *iso*citrate utilized, one mol.prop. of glyoxylate and one of succinate were produced.

Enzymes of the glyoxylate cycle in extracts of Aspergillus niger *grown on compounds other than acetate.* Cell-free extracts of *A. niger* which had been grown on the usual growth medium but in which the carbon source was glucose contained less than 5% of the acetic-thiokinase activity found in extracts of acetate-grown *A. niger*: although the latter extracts catalysed the formation of 1·7 μmoles of acetohydroxamic acid/hr./mg. dry wt. of cells, the former formed only 0·04 μmole of acetohydroxamic acid/hr./mg. dry wt. of cells. This low activity was also reflected in the decreased ability of extracts of glucose-grown cells to catalyse the incorporation of isotope from [1-^{14}C]acetate (Table 4). However, the small amounts of radioactive materials formed from acetate are adequate to show that both the citrate-forming condensing enzyme and malate synthetase were present: addition of oxaloacetate promoted the formation of 35 μm-moles of labelled citrate, and addition of glyoxylate that of 10 μm-moles of labelled malate. Addition of *iso*citrate did not stimulate the incorporation of isotope into malate, which indicated that the extract contained little or no *iso*citratase. This was confirmed by spectrophotometric assay (Olson, 1959): whereas extracts of the acetate-grown mould catalysed the formation of 1·04 μ-moles of glyoxylate/hr./mg. dry wt. of crushed mould, extracts of glucose-grown cells formed less than 0·007 μmole/hr./mg. dry wt. and those of succinate-grown cells less than 0·02. This indicates that in *A. niger*, as in other micro-organisms (Smith & Gunsalus, 1955, 1957; Olson, 1959; Callely, Dagley & Hodgson, 1958; Kornberg, Gotto & Lund, 1958; Kornberg & Lund, 1959; Kornberg *et al.* 1960*a*; Kornberg, Phizackerley & Sadler, 1960*b*) *iso*citratase is formed only under conditions

Table 4. *Incorporation of isotope from* [1-^{14}C]*acetate catalysed by extracts of glucose-grown* Aspergillus niger

The cell extract used was obtained from *A. niger* grown on the usual growth medium but in which ammonium acetate had been replaced by 50 mM-glucose, 50 mM-ammonium chloride and 0·05% of Difco yeast extract. Each tube received 0·2 ml. of extract (containing 1·0 mg. of soluble protein). Other conditions are as in Table 1.

Additional substrate (10 μmoles)	Labelled products formed (μm-moles)			
	Succinate	Malate	Citrate	Fumarate
None	11	12	0	9
Oxaloacetate	10	11	35	7
Glyoxylate	9	22	0	9
*iso*Citrate	13	10	0	7

necessitating the net formation of cellular materials from acetate, and thus supports the postulated role of the glyoxylate cycle in the growth of the organism on acetate. Malate synthetase was found to be present in extracts of *Aspergillus* under all conditions of growth.

DISCUSSION

The oxidation of acetate by *A. niger* is known to proceed via the tricarboxylic acid cycle (Martin, 1954; Ramakrishnan & Martin, 1954*a*, *b*; Ramakrishnan, 1954). This pathway provides both energy to the cell and intermediates, the carbon skeletons of which are the precursors of cell constituents (Krebs, Gurin & Eggleston, 1952; Roberts, Abelson, Cowie, Bolton & Britten, 1955; Ehrensvärd, 1955). Growth of the organism on acetate as sole carbon source therefore demands the occurrence of reactions effecting the net synthesis of intermediates of the tricarboxylic acid cycle from acetate.

One such route for the replenishment from acetate of the intermediates drained from the cycle during growth might be the direct oxidative condensation of acetate, to yield succinate, reaction (iv)

$$2 \text{ acetate} \rightarrow \text{succinate} + 2H \qquad \text{(iv)}$$

postulated by Thunberg (1920), Wieland (1922) and Knoop (1923). Although attempts to demonstrate this reaction with purified enzymes have been unsuccessful, it has been widely invoked to account for the growth of micro-organisms on C_2 compounds (for reviews, see Foster, 1949, 1958; Ajl, 1951, 1958; Chain, 1955; Kornberg, 1959). In particular, a reaction of this kind was required to explain the distribution of isotope within compounds synthesized by fungi growing on isotopically labelled acetate (Lewis & Weinhouse, 1951*a*, *b*) or ethanol (Foster & Carson, 1950; Foster *et al.* 1949).

A second route is the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957), the overall reaction of which is identical with reaction (iv) and the operation of which, in conjunction with the tricarboxylic acid cycle, would satisfactorily account for the isotope distribution patterns recorded (for review see Kornberg, 1959).

The evidence presented in this paper favours the second alternative. Incubation of *A. niger*, growing on [2-^{14}C]acetate for brief periods, showed that malate acquired isotope from acetate at the earliest times and without passing through the stage of succinate. The proportion of the total incorporated radioactivity present in malate fell with time, whereas that in succinate rose: this behaviour conforms to that expected from the operation of the glyoxylate cycle, and excludes a direct formation of succinate from acetate. Of the key enzymes of the glyoxylate cycle, *iso*citratase had already been shown to occur in the fungi *Rhizopus nigricans* and *Penicillium chrysogenum* by Olson (1954), who termed the enzyme '*iso*citric lyase'. Assay of the enzymic activity of extracts of *A. niger* confirm this finding: extracts of the acetate-grown mould contain *iso*citratase activity at pH 6 sufficient to allow of a doubling of cell mass in about 10 hr. were the activity of this enzyme to impose the rate-limiting step of growth on acetate. Since the activity of *iso*citratase at pH 6 is less than a tenth of that at pH 7 (Smith & Gunsalus, 1957), it is probable that the measured value is a minimum one, and that the activity of *iso*citratase is therefore more than adequate to account for the doubling of cell mass in 9·1 hr. observed during growth on acetate. The virtual absence of this enzyme in extracts of *A. niger* grown under conditions not necessitating the net synthesis of tricarboxylic acid cycle intermediates from acetate supports the view that *iso*citratase plays an essential role in the growth of the mould on acetate.

A second key enzyme of the glyoxylate cycle, malate synthetase, was discovered in extracts of *E. coli* by Wong & Ajl (1956). Its presence in extracts of *A. niger* is shown by the ability of such extracts, supplemented with cofactors, to catalyse the formation of malate from either [^{14}C]acetate and glyoxylate or [^{14}C]glyoxylate and acetate. The amounts of malate formed were similar to those of citrate produced from [^{14}C]acetate and oxaloacetate. Although no quantitative assay for malate synthetase was available at the time of completion of this work, these results indicate that the activities of the citrate-forming condensing enzyme and of malate synthetase were of the same order of magnitude. The presence of this enzyme (in the virtual absence of *iso*citratase) in extracts of *A. niger* grown on glucose or succinate further indicates that in this mould, as in *Pseudomonas* (Kornberg *et al.* 1958; Kornberg & Lund, 1959), *Micrococcus denitrificans* (Kornberg *et al.* 1960*a*) and *Saccharomyces drosophilarum* (Barnett & Kornberg, 1960), the operation of the glyoxylate cycle is controlled by factors influencing the intracellular activity and/or the formation of *iso*citratase.

SUMMARY

1. Isotope from [2-^{14}C]acetate added to *Aspergillus niger* growing on acetate as the sole carbon source was rapidly incorporated into cellular components. At the earliest times, only intermediates of the tricarboxylic acid cycle and amino acids derived therefrom became labelled.

2. Isotope from acetate entered the tricarboxylic acid cycle at two sites, to form citrate at one and

malate at the other. At the earliest times, labelled malate could not have been solely derived from labelled succinate. These results are consistent with the simultaneous operation of the tricarboxylic acid and glyoxylate cycles.

3. These findings also exclude the formation of succinate from the oxidative condensation of acetate as being of quantitative importance in the growth of *A. niger* on acetate.

4. Cell-free extracts of the acetate-grown mould contained the two key enzymes of the glyoxylate cycle, *iso*citratase and malate synthetase, in quantities adequate to account for the observed rates of growth of the organism. When supplemented with cofactors, such extracts also catalysed the overall formation of malate and succinate from acetate and *iso*citrate.

5. Cell-free extracts of the mould grown on glucose or succinate contained malate synthetase but only traces of *iso*citratase. This indicates that the operation of the glyoxylate cycle is controlled by factors influencing the intracellular activity of *iso*citratase.

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REFERENCES

Ajl, S. J. (1951). *Bact. Rev.* **15**, 211.
Ajl, S. J. (1958). *Physiol. Rev.* **38**, 196.
Aubert, J.-P., Milhaud, G. & Millet, J. (1956). *C.R. Acad. Sci., Paris*, **242**, 2059.
Aubert, J.-P., Milhaud, G. & Millet, J. (1957). *Ann. Inst. Pasteur*, **92**, 515.
Barnett, J. A. & Kornberg, H. L. (1960). *J. gen. Microbiol.* **23**, 65.
Callely, A. G., Dagley, S. & Hodgson, B. (1958). *Biochem. J.* **69**, 173.
Calvin, M. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 211.
Campbell, J. J. R., Smith, R. A. & Eagles, B. A. (1953). *Biochim. biophys. Acta*, **11**, 594.
Chain, E. B. (1955). *Proc. 3rd int. Congr. Biochem., Brussels*, p. 523.
Dixon, G. H., Kornberg, H. L. & Lund, P. (1960). *Biochim. biophys. Acta*, **41**, 217.
Ehrensvärd, G. (1955). *Annu. Rev. Biochem.* **24**, 275.
El Hawary, M. F. S. & Thompson, R. H. S. (1953). *Biochem. J.* **53**, 340.
Foster, J. W. (1949). *Chemical Activities of Fungi*. New York: Academic Press Inc.
Foster, J. W. (1958). *Tex. Rep. Biol. Med.* **16**, 79.
Foster, J. W. & Carson, S. F. (1950). *Proc. nat. Acad. Sci., Wash.*, **36**, 219.
Foster, J. W., Carson, S. F., Anthony, D. S., Davis, J. B., Jefferson, W. E. & Long, M. V. (1949). *Proc. nat. Acad. Sci., Wash.*, **35**, 663.
Franke, W. & de Boer, W. (1958). *Abstr. Comm. 4th int. Congr. Biochem., Vienna*, no. 10–83, p. 134.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Hughes, D. E. (1951). *Brit. J. exp. Path.* **32**, 97.
Jones, M. E. & Lipmann, F. (1955). In *Methods in Enzymology*, vol. 1, p. 585. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Knoop, F. (1923). *Klin. Wschr.* **2**, 60.
Kornberg, A. (1955). In *Methods in Enzymology*, vol. 1, p. 705. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
Kornberg, H. L. (1958). *Biochem. J.* **68**, 535.
Kornberg, H. L. (1959). *Annu. Rev. Microbiol.* **13**, 49.
Kornberg, H. L. & Beevers, H. (1957*a*). *Nature, Lond.*, **180**, 35.
Kornberg, H. L. & Beevers, H. (1957*b*). *Biochim. biophys. Acta*, **26**, 531.
Kornberg, H. L. & Collins, J. F. (1958). *Biochem. J.* **68**, 3P.
Kornberg, H. L., Collins, J. F. & Bigley, D. (1960*a*). *Biochim. biophys. Acta*, **39**, 13.
Kornberg, H. L., Gotto, A. M. & Lund, P. (1958). *Nature, Lond.*, **182**, 1430.
Kornberg, H. L. & Krebs, H. A. (1957). *Nature, Lond.*, **179**, 988.
Kornberg, H. L. & Lund, P. (1959). *Biochem. J.* **72**, 33P.
Kornberg, H. L. & Madsen, N. B. (1957). *Biochim. biophys. Acta*, **24**, 651.
Kornberg, H. L. & Madsen, N. B. (1958). *Biochem. J.* **68**, 549.
Kornberg, H. L., Phizackerley, P. J. R. & Sadler, J. R. (1960*b*). *Biochem. J.* **77**, 438.
Krebs, H. A. (1937). *Biochem. J.* **31**, 2095.
Krebs, H. A., Gurin, S. & Eggleston, L. V. (1952). *Biochem. J.* **51**, 614.
Lewis, K. F. & Weinhouse, S. (1951*a*). *J. Amer. chem. Soc.* **73**, 2500.
Lewis, K. F. & Weinhouse, S. (1951*b*). *J. Amer. chem. Soc.* **73**, 2906.
Martin, S. M. (1954). *Canad. J. Microbiol.* **1**, 6.
Ochoa, S., Stern, J. R. & Schneider, M. C. (1951). *J. biol. Chem.* **193**, 691.
Olson, J. A. (1954). *Nature, Lond.*, **194**, 695.
Olson, J. A. (1959). *J. biol. Chem.* **234**, 5.
Ramakrishnan, C. V. (1954). *Enzymologia*, **17**, 169.
Ramakrishnan, C. V. & Martin, S. M. (1954*a*). *Canad. J. Biochem. Physiol.* **32**, 434.
Ramakrishnan, C. V. & Martin, S. M. (1954*b*). *Arch. Biochem. Biophys.* **55**, 403.
Roberts, R. B., Abelson, P. H., Cowie, D. B., Bolton, E. T. & Britten, R. J. (1955). *Studies of Biosynthesis in* Escherichia coli. Washington: Carnegie Institution of Washington, Publ. no. 607.
Santer, M. & Vishniac, W. (1955). *Biochim. biophys. Acta*, **18**, 157.

Saz, H. J. (1954). *Biochem. J.* **58**, xx.
Saz, H. J. & Hillary, E. P. (1956). *Biochem. J.* **62**, 563.
Smith, R. A. & Gunsalus, I. C. (1955). *Nature, Lond.*, **175**, 774.
Smith, R. A. & Gunsalus, I. C. (1957). *J. biol. Chem.* **229**, 305.
Thunberg, T. (1920). *Skand. Arch. Physiol.* **40**, 1.
Trudinger, P. A. (1955). *Biochim. biophys. Acta*, **18**, 581.
Trudinger, P. A. (1956). *Biochem. J.* **64**, 274.
Umbreit, W. W., Burris, R. H. & Stauffer, J. F. (1957). *Manometric Techniques and Tissue Metabolism*, 3rd ed. p. 202. Minneapolis: Burgess Publishing Co.
Wieland, H. (1922). *Ergebn. Physiol.* **20**, 477.
Wong, D. T. O. & Ajl, S. J. (1956). *J. Amer. chem. Soc.* **78**, 3230.

Biochem. J. (1960) **77**, 438

The Metabolism of C_2 Compounds in Micro-organisms

5. BIOSYNTHESIS OF CELL MATERIALS FROM ACETATE IN *ESCHERICHIA COLI**

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The tricarboxylic acid cycle (Krebs & Johnson, 1937) has been shown to be the major route for the oxidation of acetate by *Escherichia coli* (Swim & Krampitz, 1954; Gilvarg & Davis, 1956) and for the provision of the carbon skeletons of cell constituents (Roberts, Abelson, Cowie, Bolton & Britten, 1955; Gilvarg & Davis, 1956). When the organisms grow on acetate as sole carbon source and intermediates are removed from the cycle to supply cell materials, reactions must occur to replenish from acetate the supply of such intermediates. It has been postulated that this may be effected by condensation of acetate with carbon dioxide, to yield a C_3 compound which, by further carboxylation, yields malate or oxaloacetate (Abelson, Bolton, Britten, Cowie & Roberts, 1954; Roberts *et al.* 1955), or by the direct formation of succinate (Thunberg, 1920) from the oxidative condensation of 2 molecules of acetate (Glasky & Rafelson, 1957, 1959; Glasky, Eichholz & Rafelson, 1958). A third possible route is the glyoxylate cycle (Kornberg & Madsen, 1957, 1958; Kornberg & Krebs, 1957).

The purpose of this paper is to show that isotope from [1-^{14}C]acetate is incorporated into cellular materials by *E. coli* strain *w*, growing on acetate, in a manner consistent with the simultaneous operation of the tricarboxylic acid and glyoxylate cycles, and that extracts of such cells contain the requisite enzymes in high activity. No evidence for the postulated successive carboxylation of acetate or for the direct formation of succinate was obtained. Since this latter finding might have been due to species differences, these experiments were repeated with *E. coli*, Crooks strain, as used by Glasky & Rafelson (1957, 1959) and Glasky *et al.* (1958). The results obtained again exclude the direct formation of succinate from acetate. They support the view that acetate is catabolized via the tricarboxylic acid cycle, and that the glyoxylate cycle operates as a means of supplying intermediates to that cycle. This work has been presented in part to The Biochemical Society (Kornberg, Phizackerley & Sadler, 1959).

* Part 4: Collins & Kornberg (1960).

METHODS

Maintenance and growth of organisms. A culture of *E. coli* strain *w* was the gift of Professor B. D. Davis (Harvard University Medical School, Boston, Mass., U.S.A.). *E. coli*, Crooks strain, was obtained from The National Collection of Industrial Bacteria, Teddington, Middx. (catalogue no. 8545).

The organisms were maintained on agar slopes consisting of 50 mM-potassium phosphate buffer, pH 7·2, 50 mM-ammonium chloride, 50 mM-sodium acetate, essential salts (4 mg. of $CaCl_2,6H_2O$; 8 mg. of $MgSO_4,7H_2O$; 0·4 mg. of $MnSO_4,4H_2O$; 0·4 mg. of $FeSO_4,7H_2O$/100 ml. of medium), solidified with 2% (w/v) of agar agar (Hopkins and Williams Ltd., Chadwell Heath, Essex). Stock cultures of the organism were subcultured every 2 weeks, grown at 30° and stored at 2°.

For growth in liquid medium, a loopful of organisms from a freshly grown slope was suspended in a Carrel culture flask (J. A. Jobling and Co. Ltd., Sunderland) containing 400 ml. of the above-mentioned medium but with the agar agar omitted. The flasks were shaken at 30° for 16–24 hr. on a reciprocal shaker. Growth of the cells was determined by measurements in an EEL nephelometer (Evans Electroselenium Ltd., Harlow, Essex) of the light-scattering of samples of the bacterial suspensions and comparison of the observed readings with a previously constructed calibration curve relating such readings to the dry