THE UTILIZATION OF ACETATE BY *NEOCOSMOSPORA VASINFECTA*

By K. BUDD

Department of Botany, University of Michigan, Ann Arbor, Michigan, U.S.A.

(*Received* 4 *May* 1965)

Summary

Mycelium of *Neocosmospora vasinfecta*, harvested during active growth, was incubated for up to 10 minutes in dilute solutions of [1-^{14}C]acetate or [2-^{14}C]acetate. Radioactivity in the respiratory CO_2 and in identifiable compounds extracted from the mycelium was measured. Closely similar results were obtained with both [1-^{14}C]acetate and [2-^{14}C]acetate except for the loss of radioactivity to CO_2, which took place much more rapidly from the [1-^{14}C]acetate. Water-soluble compounds accounted for over 80% of the radioactivity of the mycelium. With incubation periods of up to 2.5 minutes, most of the radioactivity in the water-soluble material was associated with organic acids, whereas with longer incubation, the label in basic compounds predominated. Glutamate was consistently the most heavily labelled basic compound, with small amounts of radioactivity in glutamine and aspartic acid. Radioactivity in the organic acid fraction was practically confined to citric acid after 2.5 minutes incubation; succinic, malic and other acids were significantly labelled after 10 minutes. The specific activities of these acids after 10 minutes decreased in the order citrate, succinate, malate, suggesting their involvement in the tricarboxylic acid cycle.

The results are discussed in relation to the importance of the tricarboxylic acid cycle in acetate utilization, nitrogen assimilation and respiration.

Introduction

Previous work with *Neocosmospora vasinfecta* (Budd and Harley, 1962) showed the importance of aerobic respiration in the assimilation of ammonia, and identified the chief product of this process as *glutamine*. By analogy with yeast (Yemm and Folkes, 1954) and higher plants, it was suggested that glutamine arises by the formation and subsequent amidation of glutamic acid. This compound is closely related to α-ketoglutaric acid, a key intermediate of the tricarboxylic acid cycle.

Unpublished experiments have shown that ammonia assimilation by either freshly-harvested or 'low-nitrogen' mycelium is stimulated by the tricarboxylic acid cycle acids malate, succinate, citrate and α-ketoglutarate, and by pyruvate and acetate, as well as by glucose (cf. Budd and Harley, 1962). Of these, acetate was most effective and citrate least. Stimulation was not due to the buffering action of these acids on the external pH, suggesting that they contribute carbon to the assimilation process. The cumulative evidence from these observations strongly implies the operation of the tricarboxylic acid cycle in ammonia assimilation, and perhaps also as a major respiratory pathway. Specifically-labelled acetate has successfully been used to investigate the possible operation of the tricarboxylic acid cycle in both higher plants and micro-organisms. Its metabolism in *Neocosmospora* was therefore examined.

Methods

Growth and handling of mycelium

Neocosmospora vasinfecta E. F. Smith (Nectriaceae) was grown in submerged culture as previously described (Budd and Harley, 1962). Vigorously growing mycelium was harvested by filtration and washed in distilled water. It was lightly blended to break up hyphal aggregations and resuspended in demineralized water. Mycelial samples of approximately 100 mg dry wt. were prepared by the techniques previously described, and the moist hyphal pads placed in the experimental solutions as soon as possible.

Acetate labelled in either the C-1 (carboxyl) or C-2 (methyl) carbon was supplied by the Radiochemical Centre, Amersham, Bucks., at a specific activity of 10–12 mc/mM, and was diluted with unlabelled sodium acetate to a specific activity of 0.2 mc/mM. This was presented to the mycelium at a concentration of 0.25 mM/l in 25 mm KH_2PO_4 solution adjusted to pH 5.2 with NaOH. With the counting equipment used, this solution registered approximately 17×10^3 counts/min/ml. Mycelium was incubated with 20-ml portions of labelled medium in stoppered conical flasks of approximately 100 ml capacity. Each flask was fitted with a centre well into which 0.2 ml 10% KOH was pipetted for absorption of respiratory CO_2. Flasks were shaken at 25° C in a water-bath during the incubation period. At the close of this period, the contents of the centre well were transferred quantitatively to excess of 5% $BaCl_2$ in a centrifuge tube. The mycelium was then separated from radioactive medium by filtration, washed with ice-cold buffer, and dropped into 10–15 ml hot (70° C) 80% ethanol. It was then set aside for 24 hours before completing the extraction procedure. Meanwhile the precipitated $BaCO_3$ from the centre well was collected, washed, and plated for radioactive assay as described by Aronoff (1956), and the recorded radioactivity corrected for self-absorption.

Extraction, fractionation and identification of labelled products

Mycelial mats were removed from the fixing alcohol, and extraction completed by boiling under reflux in three changes of 10 ml 80% ethanol (approximately 20 minutes per change). The combined extracts were evaporated to dryness at 38–40° C and lipids separated from the residue with petroleum spirit (b.p. 100–120° C). The remaining material ('water-soluble' fraction) was taken up in demineralized water and, after removing an aliquot for radioactive assay, passed consecutively down 8×1 cm beds of Amberlite CG-120 (H^+ form) and CG-400 (formate form) ion exchange resins. From these, sugars and other non-polar compounds were eluted with water, amino-acids and other bases with 4 N NH_4OH, and acidic compounds with 4 N formic acid. Eluates were concentrated and freed from volatile eluants *in vacuo* at 38–40° C, and the resulting solutions assayed for radioactivity and then subjected to chromatographic analysis as outlined below. Recovery of radioactivity applied to the resin-beds averaged 79.5%.

Insoluble material from the ethanol-extraction procedure was hydrolysed under reflux for 2 hours in 5 N HCl (10 ml per sample). After filtering off acid-insoluble matter, the clear brown filtrate was dried *in vacuo* over solid NaOH and redissolved in water ('acid-hydrolysable' fraction). The solution was assayed for radioactivity and fractionated as described above.

Components of the 'basic' fraction of both water-soluble and acid-hydrolysable extracts were separated by two-dimensional paper chromatography, and radioactive areas on the chromatogram identified by autoradiography. The chromatograms were developed with n-butanol–glacial acetic acid–water (40 : 10 : 40, v/v) in the first direction, and phenol/ammonia (Smith, 1960), containing 0.1% KCN to prevent discoloration, in

the second. Autoradiograms were prepared by exposing Kodak 'Kodirex' X-ray plates to the dried chromatograms for 1–4 weeks, after which amino-acids were located on the paper with Levy and Chung's (1953) reagent.

Neutral components of the extracts were separated by unidirectional paper chromatography using propanol–ethyl acetate–water (7 : 1 : 2, v/v). Radioactive areas on the chromatogram were located by strip-scanning.

Acidic components were separated by gradient elution from 6 × 1 cm columns of Dowex 1 × 10 resin (formate form), using 8 N formic acid in the acid reservoir (Palmer, 1955; Canvin and Beevers, 1961). Succinic, malic, citric, fumaric, α-ketoglutaric and aconitic acids were simultaneously applied to the resin as 'markers'. About 240 × 2-ml serially eluted samples were collected, dried in a warm air-stream (Harley and Beevers, 1963) and made to 2 ml with distilled water. Aliquots were taken from even-numbered tubes for radioassay, and the remainder titrated with N/200 alkali to locate marker acids. Coincidence of radioactive with marker 'peaks' was confirmed in the odd-numbered tubes by paper chromatography. Butanol–formic acid–water (40 : 10 : 50, v/v) was used as solvent system, and the developed chromatogram was strip-scanned.

The quantities of the various organic acids present in fresh mycelium were determined using the above techniques (omitting however the 'marker' acids). For this purpose, 15 g wet wt. (1.724 g dry wt.) of freshly-grown mycelium was extracted as previously described. The results are used below in calculating approximate specific activities of the citrate, malate and succinate pools of the mycelium.

Extraction and identification of α-keto acids

α-Keto acids were extracted into a mixture of methanol and dry ice, using a modification of the method of Isherwood and Niavis (Smith, 1960). The α-keto acids were obtained as the 2,4-dinitrophenylhydrazones, dissolved in ethyl acetate and chromatographed in n-butanol–ethanol–ammonia (Smith, 1960). Radioactive derivatives were located by strip-scanning.

Determination of radioactivity

Samples to be assayed were transferred to flamed nickel planchets. One ml aliquots of lipid extracts were dried down directly on the planchet. To ensure uniform spreading of aqueous solutions over the planchet, 0.5 ml was mixed with 0.2 ml of 1% agar solution on a 60° C hotplate, allowed to solidify at room temperature, and then dried slowly under an infra-red lamp. The planchets were then loaded into an automatic sample-changer and counted with a thin end-window detector.

RESULTS

Overall pattern of uptake and incorporation of radioactivity

Absorption of acetate by the mycelium took place rapidly. Total radioactivity supplied to each mycelial sample was approximately 3.5×10^5 counts/min, representing 5 μ-equiv (μM) acetate. Within 10 minutes over half this amount of radioactivity, equivalent to 2.6 μ-equiv acetate, became incorporated into the mycelium (Table 1). The release of label as respiratory CO_2 was relatively slow, but was clearly more rapid from the [1-^{14}C]acetate than from the [2-^{14}C]. (Small differences occur in radioactivity associated with the mycelium between [1-^{14}C] and [2-^{14}C] series, in Table 1 and elsewhere. These are ascribed to the slightly lower activity of the [2-^{14}C]acetate as compared to the [1-^{14}C].)

Table 1. *Recovery of acetate-carbon (counts/minute per 100 mg dry wt.) after various periods of incubation*

	Time				
	30 sec	60 sec	2.5 min	5 min	10 min
[1-^{14}C]acetate					
Mycelium	12,261	15,818	45,517	93,938	189,797
CO_2	–	–	24	69	489
[2-^{14}C]acetate					
Mycelium	8,424	12,869	39,483	85,238	174,165
CO_2	–	–	6	6	15

Fig. 1 shows the movement of radioactivity from the carboxyl and methyl groups of the acetate molecule into water-soluble, ether-soluble and acid-hydrolysable components of the mycelium. [1-^{14}C] and [2-^{14}C]acetate gave closely similar results, suggesting that the acetate molecule is assimilated as a whole. The radioactivity of the *water-soluble* fraction increased linearly with time, and after 10 minutes accounted for over 80% of the total. Further examination of this fraction is discussed below. Less than 2% of the total radioactivity appeared in *acid-hydrolysable* material; of this, 75–79% was associated with amino-acid residues, derived presumably from cellular protein. The remaining 21–25% of the activity in this fraction was in the form of organic acids. The *ether-soluble* fraction presumably represented lipids, and accounted for approximately 16% of the mycelial radioactivity. Its composition was not further investigated.

Fig. 2 shows the incorporation of radioactivity into the basic, acidic and neutral components of the water-soluble fraction. The activity in each is expressed as a percentage

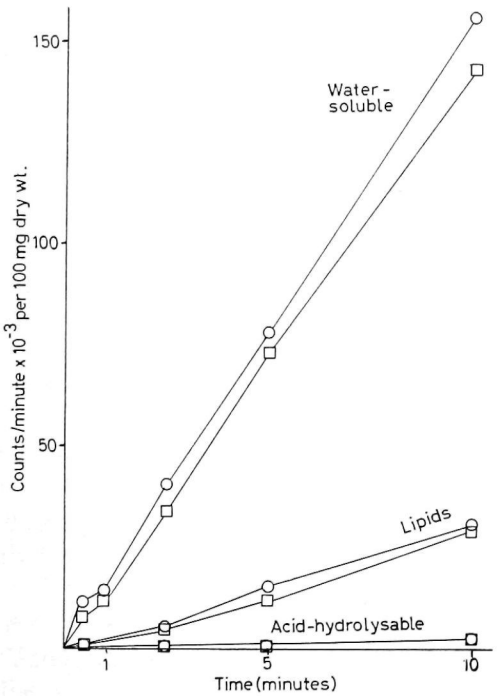

Fig. 1

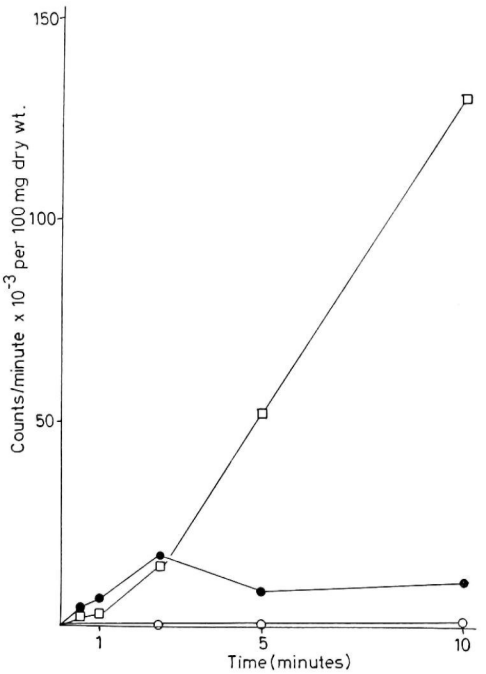

Fig. 2

Fig. 1. Progress of incorporation of radioactivity into major fractions of the mycelium. ○, [1-^{14}C]acetate supplied; □, [2-^{14}C]acetate.

Fig. 2. Progress of incorporation of radioactivity from [1-^{14}C]acetate into the water-soluble fraction. □, Basic compounds; ●, organic acids; ○, neutral compounds.

of the total water-soluble radioactivity in Table 2. Again, the results obtained with [1-^{14}C] and [2-^{14}C]acetate are similar, indicating metabolism of the acetate molecule as a unit. After the shortest periods of exposure to the labelled substrate (up to 1 minute), the bulk of radioactivity is found in the acidic fraction, and the balance in basic compounds (Table 2). With progressively longer exposures, the proportion of label in the acidic

Table 2. *The radioactivity in basic, acidic, and neutral components as a percentage of total water-soluble radioactivity*

Substrate	Component	Time 30 sec	60 sec	2.5 min	5 min	10 min
[1-^{14}C]acetate	Basic	28.1	37.6	51.3	85.8	91.4
	Acidic	71.9	62.3	48.6	13.6	7.8
	Neutral	0	0.1	0.1	0.6	0.8
[2-^{14}C]acetate	Basic	34.2	40.4	39.7	85.4	90.7
	Acidic	65.8	59.6	60.2	13.8	8.4
	Neutral	0	0	0.1	0.8	0.9

fraction decreases and that in the basic fraction increases, until after 10 minutes the latter accounts for over 90% of the water-soluble radioactivity. Neutral compounds accumulate only traces of label throughout. A plausible interpretation of these results is that organic acids act as radioactive precursors for the other compounds. After 2.5–5 minutes, radioactivity in the acid fraction reaches a more or less constant level, while that in the other fractions continues to increase linearly (Fig. 2). These data seem to imply the attainment of a steady-state, in which radioactivity is being continually withdrawn from the acid pool, chiefly into the basic fraction, at a rate equal to that at which it enters. Examination of the specific activities of the individual acids suggests however that a true steady-state may not be attained within 10 minutes (see below).

Identity of labelled compounds

Radioactive components of the *basic* fraction were identified by chromatography and autoradiography. The results were the same after 10 minutes incubation as after 5 minutes, and with either [1-^{14}C] or [2-^{14}C]acetate. Radioactivity in this fraction was confined almost entirely to *glutamate*, with traces also in glutamine and aspartate. In addition to these, at least a dozen other amino-acids have been identified in ethanol extracts of fresh mycelium, including aspartate, glycine, serine, lysine, histidine, arginine, alanine, proline and asparagine (unpublished), but these clearly acquire negligible radioactivity at least during the first 10 minutes of acetate assimilation.

The small amounts of radioactivity in the *neutral* fraction were found to be associated with an unidentified polyhydric alcohol having an Rg value of 1.29 in propanol–ethyl acetate–water. Radioactivity could not be detected in glucose, although small amounts might easily have been overlooked because of the extremely low specific activities involved.

Radioactive components of the *acidic* fraction were examined in greater detail because of their apparent function as radioactive precursors for other soluble constituents. To obtain information on the chief organic acids present in the mycelium, the acidic fraction from a large mycelial sample was fractionated by means of a Dowex 1 formate resin-bed and formic acid gradient as described above ('Methods'). Identity of titratable 'peaks' was determined from elution position relative to known acids, and by paper chromatography. The elution diagram is reproduced in Fig. 3, which also shows the

titrable acidity of certain peaks in μ-equiv/g dry wt. mycelium. Among the acids identified were *succinate* and *malate*, the latter being the most abundant single acid in the growing mycelium. The identity of *fumarate* was also confirmed by its bleaching of acidified potassium permanganate; inorganic phosphate was also present in this peak.

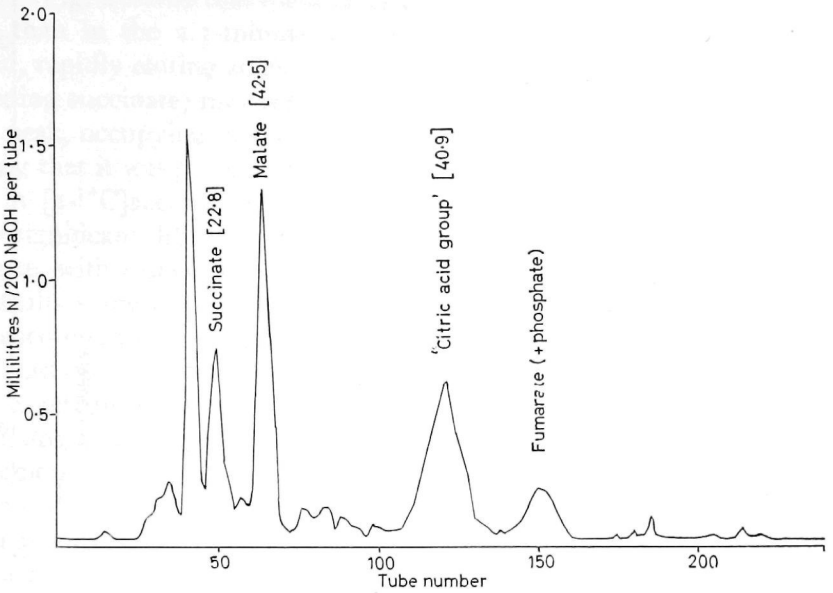

Fig. 3. The organic acids of growing mycelium. Total titrable acidity, 174 μ-equiv/g dry wt. Figures above peaks are in μ-equiv/g dry wt.

The 'citric acid group' contained two unidentified acids besides *citrate*, and hence the specific activities of labelled citric acid, calculated below on the basis of the titrable acidity of this peak, must be taken as minimal estimates. The acid eluting immediately before succinate has been tentatively identified (from paper chromatographic studies) as pyrrolidone carboxylic acid, possibly derived from glutamine during the extraction procedure. The presence of at least four acids of the tricarboxylic acid cycle in detectable amounts in the mycelium suggested the possible operation of the cycle. Authentic samples of these four acids, plus α-ketoglutarate and aconitate, were therefore added to the radioactive organic acid fraction before fractionation by the gradient elution technique, to act as titratable 'markers' with which to compare radioactive peaks. Both the 2.5-minute and the 10-minute samples were fractionated in this way. The elution patterns for marker acids and radioactivity are shown in Figs. 4 and 5. Only the results obtained with samples incubated in [1-^{14}C]acetate are represented, since, with the minor exceptions noted below, identical results were found with samples of the [2-^{14}C] series.

Fig. 4 shows the distribution of radioactivity among the organic acids after 2.5 minutes incubation in [1-^{14}C]acetate; 75.1% of the label applied to the column was recovered in this experiment. Almost all of the activity was found in tubes 110–144, corresponding to the 'citric acid group' of Fig. 3 and to the citric acid marker. The contents of tubes 121–137 were combined, chromatographed on paper and the developed chromatogram was strip-scanned. Radioactivity coincided exactly with citric acid, and none was found in the positions corresponding to the two unidentified 'citric acid group' acids. Small amounts of radioactivity also appear in Fig. 4 to correspond with succinate and malate, and with a component eluting immediately after malate. Attempts to confirm

the identity of these components by paper chromatography were not positively successful. Citric acid therefore appears to be by far the most strongly labelled acid after 2.5 minutes assimilation of labelled acetate.

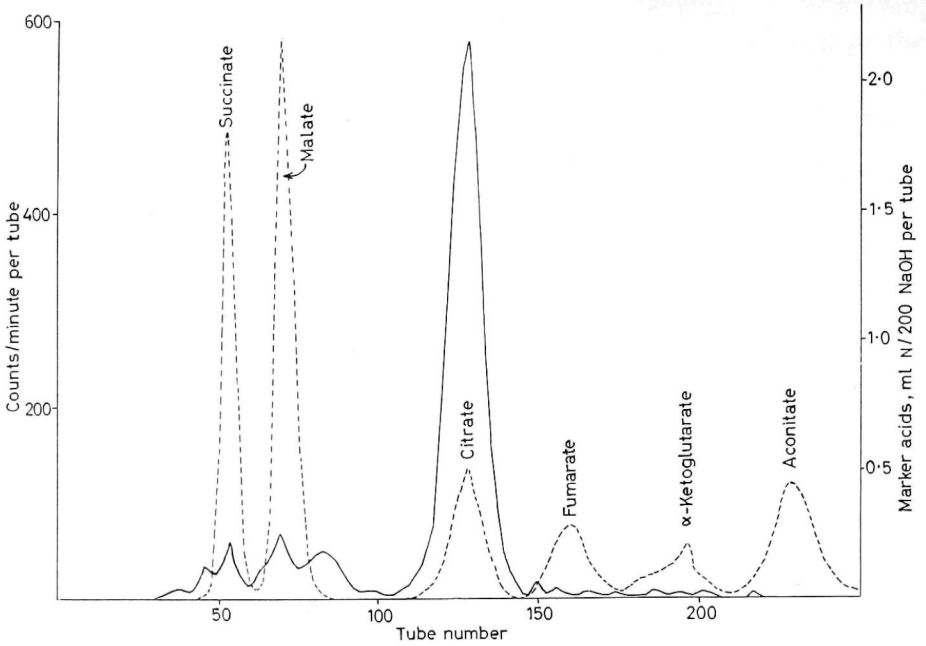

Fig. 4. Distribution of radioactivity among organic acids after 2.5 minutes incubation in [1-^{14}C]acetate. Radioactivity (per 100 mg dry wt.) given by the continuous line; titration of marker acids, by the broken line.

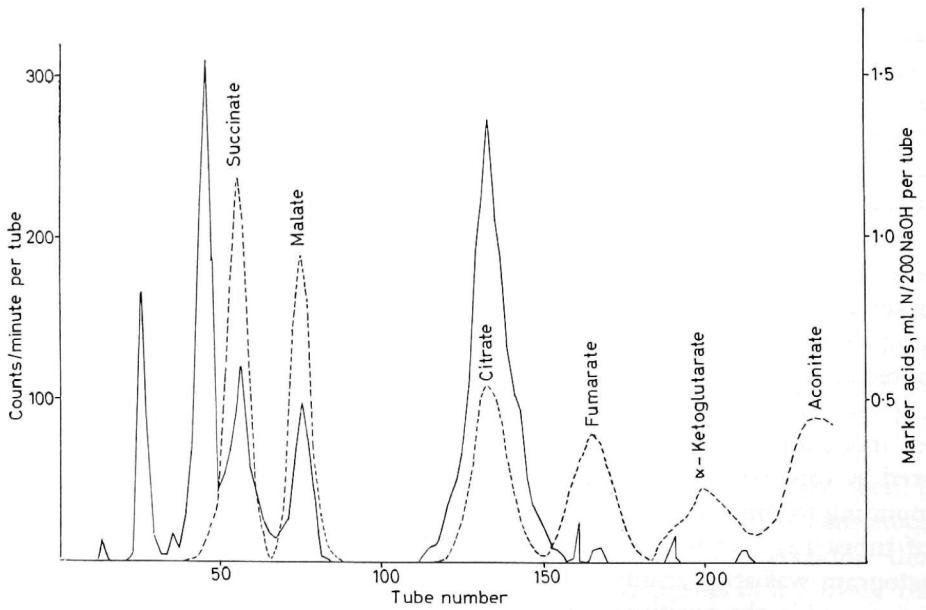

Fig. 5. Distribution of radioactivity among organic acids after 10 minutes incubation in [1-^{14}C]acetate. Radioactivity (per 100 mg dry wt.) given by the continuous line; titration of marker acids, by the broken line.

In Fig. 5, the distribution of radioactivity after 10 minutes incubation in [1-^{14}C]-acetate is shown, and it is clear that many more acids have by this time acquired label. 76.2% of the radioactivity was recovered from the resin beds. Citric acid is still the most strongly labelled acid, but is only half as radioactive as in the 2.5-minute sample. (Reference to Fig. 2 shows that the total radioactivity in organic acids is also lower in the 10-minute than in the 2.5-minute sample). In addition, succinate, malate, and two unidentified, rapidly eluting acids contain radioactivity. One of the latter (that immediately preceding succinate) may represent pyrrolidone carboxylic acid, as already stated. The other peak, occupying tubes 22–30, has not been further examined, but it may be worth noting that it was not found among the organic acids of the corresponding sample incubated in [2-^{14}C]acetate. Apart from this, and some variation in the actual peak heights, no significant differences were found between mycelium incubated in [1-^{14}C] or [2-^{14}C]acetate, with respect to labelling of the organic acids.

These results show that, after 10 minutes, the bulk of the label in the organic acid fraction is accounted for by citrate, succinate and malate, three key members of the tricarboxylic acid cycle. Significant levels of radioactivity were not however found after 10 minutes in α-ketoglutarate, aconitate or fumarate. In the case of the first two acids, this is not surprising since it has already been mentioned that these are not present in easily detectable amounts in the mycelium. (Special methods are required for the isolation and identification of α-keto acids such as α-ketoglutarate, and are described below.) This explanation is apparently excluded for fumarate, which is present in appreciable titre (Fig. 3). It is possible, however, that only a small part of the mycelial fumarate is involved in tricarboxylic acid cycle activity, while the rest is sequestered from this and represents a metabolic end-product.

The labelling of citric acid before succinate and malate is in agreement with the operation of the tricarboxylic acid cycle. The same is true of the specific activities calculated for these acids after 10 minutes incubation. These values, expressed in Table 3, are based on the titration data of Fig. 3, and their general validity is discussed below.

Table 3. *Total counts/minute and specific activity of citrate, succinate and malate after 10 minutes incubation in labelled acetate*

Source of label	Label (counts/minute)			Specific activity (counts/minute/μ-equiv)		
	Citrate	Succinate	Malate	Citrate	Succinate	Malate
[1-^{14}C]acetate	3660	959	688	895	421	162
[2-^{14}C]acetate	3860	1444	1002	944	633	236

Specific activities calculated from the data of Figs. 3 and 5. (Average specific activities in counts/minute per carbon equivalent can be obtained by dividing these values by 2.)

It can be seen that, whether [1-^{14}C] or [2-^{14}C]acetate acts as the source of radio-carbon, the specific activities of citrate, succinate and malate decrease in that order, which is explicable if these acids become labelled as a result of their involvement in the tricarboxylic acid cycle. The specific activity of citrate is in reality much higher than the calculated value of Table 3, since citrate is not the only acid in the 'citric acid group.' Hence the decrease in specific activity between citrate and succinate must be even more marked than appears from Table 3. The most likely explanation for this sharp decrease is the movement of radioactivity into *glutamate*, already noted. If this takes place via α-ketoglutarate, glutamate thus will compete with succinate (and malate) for carbon derived from citric acid.

The fact that citrate, malate and succinate have different specific activities suggests that, in spite of the indications of Fig. 2, a true steady-state has probably not been attained after 10 minutes. Attainment of the steady state might well be delayed by the rapid funnelling of radioactivity into glutamate. (Alternatively it might be postulated that only a part of the total pool of each acid is involved in cycle activity (see below).)

Labelling of α-keto acids

It was desirable to examine the incorporation of radioactivity into α-keto acids for two reasons. Firstly it is difficult to distinguish with certainty between *succinate* and *glyoxylate* by either column or paper chromatography, and a more specific test for glyoxylate was therefore important. Secondly, the assumption has been made in the foregoing that α-ketoglutarate acts as the radioactive precursor for glutamate, and confirmation of this is desirable. Samples of freshly-harvested mycelium were incubated for 10 minutes in either [1-^{14}C]acetate or [2-^{14}C]acetate under the conditions previously described. They were then extracted in the presence of 2,4-dinitrophenylhydrazine and traces of authentic pyruvate, oxaloacetate, glyoxylate and α-ketoglutarate to act as 'traps' for labelled ketoacids. For the samples incubated in [1-^{14}C]acetate a total of 474 counts/min per 100 mg dry wt. was recovered in the hydrazone fraction; for those incubated in [2-^{14}C]acetate the figure was 443 counts/min. In both cases this value is about 5% of that recovered in the other organic acids (cf. Fig. 2). The hydrazones were chromatographed, and labelled derivatives identified by strip-scanning the developed paper. Radioactivity was confined to the oxaloacetate/α-ketoglutarate zone. With the solvent systems available, further resolution of these hydrazones was not possible. The glyoxylate derivative was, however, well separated from the others and showed no trace of radioactivity. In view of the low total radioactivity in these compounds, caution is necessary in interpreting these results. Nevertheless it seems clear that labelled glyoxylate does not form an important contaminant in the succinic acid peak discussed above. The results however do not conflict with the postulated role of α-ketoglutarate in the metabolism of acetate by this mycelium.

DISCUSSION

A variety of lines of evidence now indicates that the tricarboxylic acid cycle acts as an important metabolic pathway in fungi as in other living organisms. The presence of all or most of the necessary enzymes has been demonstrated in cell-free extracts of *Aspergillus niger* (Ramakrishnan, 1954) and *Candida albicans* (Rao, Sirsi and Ramakrishnan, 1962). Particles from yeast (Linnane and Still, 1955), *Allomyces macrogynus* (Bonner and Machlis, 1957) and *Neurospora crassa* (Boulter and Hurst, 1960) have been shown to oxidize intermediates of the cycle. These intermediates, and closely related compounds such as glutamate and aspartate, have been identified among the products of metabolism of labelled acetate or glucose in yeast (DeMoss and Swim, 1957), *Zygorrhynchus moelleri* (Moses, 1958), *Aspergillus niger* (Collins and Kornberg, 1962), and *Penicillium chrysogenum* (Goldschmidt, Yall and Koffler, 1956), and in the last case the distribution of radioactivity within the molecules of citrate and glutamate indicated their formation via the tricarboxylic acid cycle. Studies in which the kinetics of incorporation of radioactivity into cycle intermediates is determined are especially valuable, in that the sequence of labelling of these intermediates is directly shown (cf. Collins and Kornberg, 1962). This forms more convincing evidence for the operation of the tricarboxylic acid cycle than the identification of its individual reactions. Of equal importance is the fact that kinetic

studies can provide evidence for ancillary pathways of acetate utilization. Thus it has been shown that, in the utilization of acetate by *Aspergillus* (Collins and Kornberg, 1962) and by uredospores of *Uromyces phaseoli* (Staples, 1962), malic or succinic acids become labelled before citric acid. In both cases, acetate is apparently metabolized via the 'glyoxylate bypass' (Kornberg and Krebs, 1957) in conjunction with the tricarboxylic acid cycle.

The present experiments provide strong evidence for the tricarboxylic acid cycle as the most important pathway of acetate utilization in *Neocosmospora*. In the first place, organic acids are the first water-soluble components of the mycelium to acquire radioactivity, and over the first 10 minutes this activity is chiefly associated with the tricarboxylic acid cycle acids. Of these, citric acid is the first to become labelled, while succinate and malate become labelled later, which is the order of rotation of the cycle. Secondly, the chief radioactive product after 5 or 10 minutes assimilation of labelled acetate is *glutamate*, which can be derived in many fungi by the reductive amination of α-ketoglutarate, a member of the tricarboxylic acid cycle. Evidence has been presented for the possible labelling of α-ketoglutarate during [^{14}C]acetate assimilation, and the slow increase of radioactivity in succinate and malate can be explained by the heavy drainage of carbon into glutamic acid via α-ketoglutarate. Thirdly, the specific activities of citric, succinic and malic acids after 10 minutes incubation decrease in the order given: this is the order in which acetate-carbon would enter each acid by traversing the tricarboxylic acid cycle. This point is further discussed below. Finally, radioactivity is more rapidly released to respiratory CO_2 from carboxyl-labelled acetate than from methyl-labelled, in conformity with the oxidation of acetate via the cycle. From these results it is concluded that the crucial step in acetate metabolism in *Neocosmospora* is condensation with oxaloacetate to form citric acid. Alternative pathways of acetate utilization, in particular condensation to form succinate via the Thunberg–Wieland pathway (Foster *et al.*, 1949) or reaction with glyoxylic acid to form malate as in the 'glyoxylate bypass', are of minor importance. Additional evidence against the operation of the glyoxylate bypass is provided by the fact that radioactivity was not observed in glyoxylic acid.

The specific activities derived for citrate, succinate and malate (Table 3) involve two assumptions: (a) that the titration values of Fig. 3 are unchanged by acetate assimilation; and (b) that for each acid, radioactivity is distributed throughout the measured acid pool. In the present context, the second assumption means that the entire pool of each acid participates in cycle activity. With respect to the first assumption, it is clear that glutamate, and not the organic acids, forms the chief destination for acetate carbon. Changes in the amount of each acid would therefore presumably be small, and would not greatly affect the values of Table 3. A final decision on the second assumption can probably not be made without further experimentation. The existence of quiescent pools of individual acids of the tricarboxylic acid cycle, which are to some extent sequestered from the corresponding 'turnover pools', has been indicated in several higher plant tissues by MacLennan, Beevers and Harley (1963). The total pools of citrate, succinate and malate in *Neocosmospora* seem, however, modest compared with those reported by these workers, especially when the high metabolic rates characteristic of growing mycelia are considered. Furthermore, the 'compartmentation' effect reported by MacLennan *et al.* was at a minimum in the most actively growing material examined. In the absence of decisive evidence, therefore, the second assumption above is taken as essentially correct.

The rapid rate of acetate assimilation in these experiments is apparent from the fact that 2.6 μM (equivalent to one-seventh the titrable acidity of the sample) are assimilated

within 10 minutes. This indicates that acetate-carbon does not merely equilibrate with a few key metabolites in the cell. It is more likely that a net increase in one or more of these occurs, and the linear incorporation of label into *glutamate* strongly suggests that an appreciable synthesis of this compound takes place. A drainage of intermediates from the tricarboxylic acid cycle is thus implied, which must be replenished. Since the glyoxylate bypass does not appear to operate in this mycelium, it is possible that carbon is supplied to the cycle by fixation of CO_2. Reactions of this type have been shown to occur in *Aspergillus* (Cleland and Johnson, 1954), *Agaricus* (Le Roux, 1962) and beech mycorrhizas (Harley, 1964). Their importance in *Neocosmospora* remains to be investigated.

The present experiments show the tricarboxylic acid cycle to be of major importance in this organism in the utilization of exogenous acetate and, in view of the heavy labelling of glutamate, in assimilation of nitrogen also. Further experiments are required to determine its contribution to the total respiration of the mycelium. Calculation of the average specific activity of the CO_2 emitted during acetate assimilation does in fact suggest that a large part of respiration proceeds via the tricarboxylic acid cycle. A mycelial sample of 100 mg dry wt. is calculated to release 11.7 μM CO_2 in 5 minutes in the presence of acetate. Between the 5th and the 10th minutes of [1-^{14}C]acetate assimilation, 420 counts/min are released as CO_2, or 36 counts/min per μM CO_2. In the earliest stages of assimilation of [1-^{14}C]acetate via the cycle, labelled CO_2 is derived chiefly from malate. Table 3 shows that the carboxyl groups of malate have an average specific activity after 10 minutes assimilation of 162 counts/min, or 4–5 times that of the CO_2. Since malate would be only one of several sources of respiratory CO_2, this is taken to indicate that the tricarboxylic acid cycle may prove of great significance in the respiration of this organism.

ACKNOWLEDGMENTS

I wish to thank Professor J. L. Harley, F.R.S., for his interest and guidance in this work. The project was carried out during the tenure of a postdoctoral fellowship at the University of Oxford, for which I thank the Committee on I.C.I. Fellowships. Grateful acknowledgment is also made to Professor G. E. Blackman, F.R.S., for his kind provision of research facilities at the Department of Agriculture, Oxford.

REFERENCES

ARONOFF, S. (1956). *Techniques of Radiobiochemistry*. Iowa State College Press.

BONNER, B. A. & MACHLIS, L. (1957). Respiration of the mycelia and mitochondria of the filamentous watermold, *Allomyces macrogynus*. *Pl. Physiol., Lancaster*, **32**, 291.

BOULTER, D. & HURST, H. M. (1960). The use of various tissue preparations in the study of the oxidative metabolism of some fungi. *The Ecology of Soil Fungi* (Ed. by D. Parkinson and J. S. Waid), p. 277. Liverpool University Press.

BUDD, K. & HARLEY, J. L. (1962). The uptake and assimilation of ammonia by *Neocosmospora vasinfecta*. *New Phytol.*, **61**, 138.

CANVIN, D. T. & BEEVERS, H. (1961). Sucrose synthesis from acetate in the germinating castor bean; kinetics and pathway. *J. biol. Chem.*, **236**, 988.

CLELAND, W. W. & JOHNSON, M. J. (1954). Tracer experiments on the mechanism of citric acid formation by *Aspergillus niger*. *J. biol. Chem.*, **208**, 679.

COLLINS, J. F. & KORNBERG, H. L. (1962). The metabolism of C_2 compounds in micro-organisms. 4. Synthesis of cell-materials from acetate by *Aspergillus niger*. *Biochem. J.*, **77**, 430.

DEMOSS, J. A. & SWIM, H. E. (1957). Quantitative aspects of the tricarboxylic cycle in baker's yeast. *J. Bact.*, **74**, 445.

FOSTER, J. W., CARSON, S. F., ANTHONY, D. S., DAVIS, J. B., JEFFERSON, W. E. & LONG, M. V. (1949). Aerobic formation of fumaric acid in the mold *Rhizopus nigricans*: synthesis by direct C_2 condensation. *Proc. natn. Acad. Sci. U.S.A.* **35**, 663.

GOLDSCHMIDT, E. P., YALL, I. & KOFFLER, H. (1956). Biochemistry of filamentous fungi. 4. The significance of the tricarboxylic acid cycle in the oxidation of acetate by *Penicillium chrysogenum*. *J. Bact.*, **72**, 436.

HARLEY, J. L. (1964). Incorporation of carbon dioxide into excised beech mycorrhizas in the presence and absence of ammonia. *New Phytol.*, **63,** 203.
HARLEY, J. L. & BEEVERS, H. (1963). Acetate utilization by maize roots. *Pl. Physiol., Lancaster*, **38,** 117.
KORNBERG, H. L. & KREBS, H. A. (1957). Synthesis of cell constituents from C_2 units by a modified 'Krebs cycle'. *Nature, Lond.*, **179,** 988.
LINNANE, A. W. & STILL, J. L. (1955). The isolation of respiring mitochondria from baker's yeast. *Arch. Biochem. Biophys.*, **59,** 383.
LE ROUX, P. (1962). Quelques resultats concernant l'incorporation du gaz carbonique par les carpophores d'*Agaricus campestris. C. r. hebd. Séanc. Acad. Sci. Paris*, **254,** 2420.
LEVY, A. L. & CHUNG, D. (1953). Two-dimensional chromatography of amino acids on buffered papers. *Analyt. Chem.*, **25,** 396.
MACLENNAN, D. H., BEEVERS, H. & HARLEY, J. L. (1963). 'Compartmentation' of acids in plant tissues. *Biochem. J.*, **89,** 316.
MOSES, V. (1958). ^{14}C-Glucose metabolism in fungal cells. *J. gen. Microbiol.*, **20,** 184.
PALMER, J. K. (1955). Chemical investigations of the tobacco plant. 10. Determination of organic acids by ion exchange chromatography. *Bull. Conn. agric. Exp. Stn.*, **589.**
RAMAKRISHNAN, C. V. (1954). The tricarboxylic acid cycle in *Aspergillus niger*. *Enzymologia*, **17,** 169.
RAO, G. R., SIRSI, M. & RAMAKRISHNAN, T. (1962). Enzymes in *Candida albicans*. 2. Tricarboxylic acid cycle and related enzymes. *J. Bact.*, **84,** 778.
SMITH, I. (1960). *Chromatographic and Electrophoretic Techniques*. Vol. 1; *Chromatography*. Heinemann.
STAPLES, R. C. (1962). Initial products of acetate utilization by bean rust uredospores. *Contrib. Boyce Thompson Inst.*, **21,** 487.
YEMM, E. W. & FOLKES, B. F. (1954). The regulation of respiration during the assimilation of nitrogen in *Torulopsis utilis*. *Biochem. J.*, **57,** 495.