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^{13}C NMR studies of carbon metabolism in the hyphal fungus *Aspergillus nidulans*

(polyols/mannitol/erythritol/glycolysis/[^{13}C]glucose)

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ABSTRACT **Natural-abundance high-resolution ^{13}C NMR spectra (linewidth, 10 Hz) of the hyphal fungus *Aspergillus nidulans* have been obtained after growth on glycolytic or gluconeogenic carbon sources. Various polyols, some tricarboxylic acid-cycle intermediates and amino acids, and some phospholipids and fatty acyl compounds are present. The polyols found are mannitol, arabitol, erythritol, and glycerol. The nature of the carbon source has a pronounced effect on the pool sizes of the various polyols. All are present when the fungus is grown on sucrose or sucrose/acetate under strongly aerobic conditions. When grown on acetate, both arabitol and glycerol levels are low, whereas on glycerol erythritol is also hardly detectable. The effect of oxygen is most outspoken in glycolytically grown cultures. Limited oxygenation leads to low levels of arabitol and glycerol. Strong oxygenation changes the ratio of erythritol to mannitol, favoring the C_4 polyol. An increase in oxygen supply leads to (*i*) stimulation of the fluxes through the pentose phosphate pathway and glycolysis, (*ii*) an overflow of reduced metabolites both at the pentose phosphate pathway level (erythritol and arabitol) and at the C_3 level of the glycolytic pathway (glycerol), and (*iii*) the usual accumulation of mannitol. Upon starvation, glycerol, the other three polyols, and the tricarboxylic acid-cycle intermediates and their associated amino acids disappear in this order. As in yeast, gluconeogenic substrates lead to the synthesis of trehalose, which also occurs when mycelium is grown on acetate/sucrose under limiting aeration. A transient formation of trehalose has been observed upon incubation of starved mycelium, cultured on different substrates, with [^{13}C]glucose.**

The application of high-resolution ^{13}C NMR to study carbon metabolism in intact microbial cells has been nicely demonstrated in *Escherichia coli* (1, 2) and *Saccharomyces cerevisiae* (3, 4). In these studies the time-dependent isotopic distribution over various metabolites has been investigated when specifically labeled glycolytic or gluconeogenic substrates were applied. Only a few studies using natural-abundance ^{13}C NMR have been reported thus far on filamentous fungi—namely, *Neurospora crassa* (5) and *Penicillium ochrochloron* (6, 7). In the present paper, we describe the application of ^{13}C NMR to an investigation of the carbon metabolism of *Aspergillus* species, some of which are of industrial importance—e.g., for the production of gluconic and citric acid. The genetics of carbohydrate metabolism has been studied almost exclusively in *Aspergillus nidulans* (8, 9). Therefore, this organism was taken as a model, with the advantage of having at one's disposal specific genetic disorders that block certain pathways. This study describes results with the fungal wild type grown on different carbon sources under different conditions of oxygen supply. Characteristic for filamentous fungi like *Aspergillus* is the accumulation of several polyols during active growth. There have been some reports on the presence of polyols in other systems. In *E. coli* grown on glucose, a slow accumulation of arabitol was found (10), whereas in halophilic algae glycerol accumulates (11). Finally, in the mammalian crystalline lens, ^{13}C NMR indicates the conversion of glucose into sorbitol (12).

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MATERIALS AND METHODS

Mycelium of a wild-type strain WG096 of *A. nidulans* (*yA2*, *pabaA1*) was grown under different conditions of aeration with sucrose (0.1 M), sucrose/acetate (0.1 M/0.01 M), glycerol (0.1 M), acetate/sucrose (0.1 M/0.01 M), and acetate (0.1 M) as carbon sources. Mycelium was grown by incubating conidiospores (10^6/ml) in minimal medium at 37°C containing per liter 6 g of $NaNO_3$, 1.5 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.5 g of KCl, 2 mg of *p*-aminobenzoic acid, and also some crystals of $FeSO_4$, $ZnSO_4$, and $MnCl_2$. The pH of the medium was adjusted to 6 by titration with NaOH before inoculation.

The different carbon sources were filter-sterilized. Strong aeration was effected by a large air stream distributed by a sparger and led into a 10-liter culture flask containing 7 liters of medium. Limited aeration was obtained by growing the mycelium in a Gallenkamp orbital shaker with 300 ml of medium in 1-liter flasks that were tightly closed by a screw cap.

All cultures were grown for 18 hr. Then the mycelium was harvested by filtration on a cold Büchner funnel, washed three times with cold saline, and kept at ≈0°C. The cold mycelium was subsequently divided into portions of about 1 g, which were either directly transferred into NMR sample tubes for direct measurements or resuspended under aeration at 37°C into 250-ml flasks containing 50 ml of minimal medium. No carbon source was added to starve the mycelium prior to incubation with ^{13}C-labeled carbon sources. The starvation time required to reduce the spectral amplitudes of accumulated metabolites sufficiently never exceeded 2 hr. Incubations were carried out by adding the appropriate amount of carbon source to the starved mycelium under standard growth conditions.

Randomly ^{13}C-enriched glucose was obtained from the leaves of tobacco plants grown in a $^{13}CO_2$ atmosphere. ^{13}C-enriched starch was extracted, purified, and subsequently converted to glucose by acidic hydrolysis (13). The percentage of enrichment was determined by mass spectrometry and estimated to be 12% (14). The ^{13}C NMR spectrum of the glucose prepared indicated a random labeling of all carbon atoms.

^{13}C spectra were obtained at 75.46 MHz on a Bruker CXP-300 NMR spectrometer equipped with a 10-mm ^{13}C probe operating in the Fourier-transform (FT) mode. Proton broadband decoupling was applied throughout all experiments. The measuring temperature was kept close to 0°C. The free induction decays (FIDs) were accumulated in consecutive blocks of 30 min (3600 transients) and sequentially stored on disk by using a 60° pulse (pulse time, 20 μs) and 8000 data

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points. The chemical shifts were measured by using the mannitol resonances (71.8, 70.2, and 64.4 ppm at 0°C) as the internal standard because these resonances are present under all conditions. These values for the mannitol resonances were obtained by assigning the value of 92.97 ppm to the α-carbon resonance position of endogenous nonphosphorylated glucose as observed in mycelium grown under conditions of limiting aeration; 92.97 ppm is the value used by Shulman *et al.* (1) as an internal reference in ^{13}C studies on *Escherichia coli*.

The resonance position of mannitol turned out to be temperature sensitive and, therefore, has been used for temperature control and calibration. The procedure turned out to be very reliable and gives close correspondence of the assigned resonances in mycelium to the values reported for the same metabolites in other microbial organisms (3, 4), rat liver cells (15, 16), perfused mouse livers (17, 18), and intact rat liver (19). For most of the metabolites assigned in the *in vivo* spectra, separate resonances were calibrated at 0°C against mannitol in solutions of $H_2O/^2H_2O$, 90:10 (vol/vol), containing both the particular metabolite and mannitol. Spectra of fungal extracts compare exactly to those recorded *in vivo*.

The mycelium (1 g) to be measured was transferred from growth, starvation, or incubation medium into NMR sample tubes (10-mm o.d.) after filtration and washing. The sample tubes contained 1 ml of saline solution ($H_2O/^2H_2O$, 60:40, vol/vol).

RESULTS

Growth on Various Carbon Sources Under Different Conditions of Aeration. *Glycolytic growth.* In Fig. 1 two spectral regions of interest from the natural-abundance ^{13}C spectrum of mycelium of wild-type *A. nidulans* are shown after growth on sucrose/acetate (0.1 M/0.01 M) under strong aerobic conditions, which reflect the accumulation of several primary metabolites. Remarkable is the typical lower limit of about 0.1 ppm (7.5 Hz) for the linewidth in both parts of the spectrum in spite of the high viscosity and large heterogeneity of the mycelial material. The region between 0 and 60 ppm shows mainly resonances belonging to tricarboxylic acid-cycle intermediates and associated amino acids, whereas the polyol-, sugar-, and phosphorylated sugar-resonance region is found between 60 and 110 ppm. The resonances

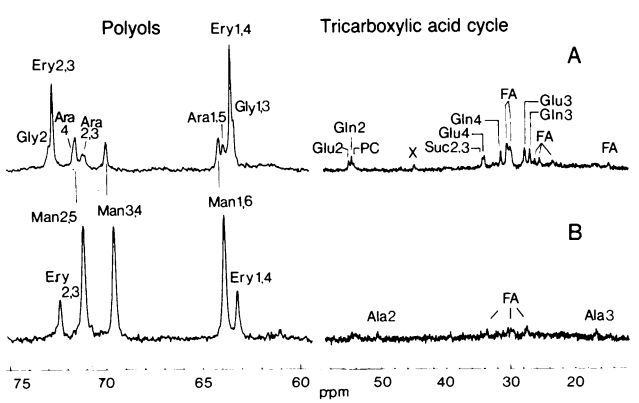

FIG. 1. (*A*) Natural-abundance ^{13}C NMR spectrum of *A. nidulans* grown on sucrose/acetate (0.1 M/0.01 M) under strongly aerobic conditions. The spectrum of the polyol- and tricarboxylic acid-cycle resonance region represents 10 blocks of 3600 transients accumulated at 0°C using 8000 data points. A line broadening (LB = 1) of 1 Hz is used, and zero-filling with 8000 data points is applied. (*B*) Glycolytic growth under conditions of limiting aeration. FA, fatty acyl chain; PC, phosphatidylcholine; PE, phosphatidylethanolamine; Man, mannitol; Gly, glycerol; Ery, erythritol; Ara, arabitol; Tre, trehalose; Suc, succinate. Other abbreviations are standard ones for amino acids.

observed in the tricarboxylic acid-cycle region belong to the methyl carbon of alanine (C-3); the methylene carbons of glutamate (C-3 and C-4), glutamine (C-3 and C-4), and succinate (C-2 and C-3); and the α-amino carbons of alanine (C-2), glutamate (C-2), and glutamine (C-2). In addition, broader methyl and methylene carbon resonances of saturated fatty acyl chains and phospholipid head groups—namely, phosphatidylcholine and phosphatidylethanolamine—were observed. These resonances became clearly visible in spectra of mycelium grown on acetate under limiting aeration conditions. These resonances also have been reported for *P. ochrochloron* (6) and interpreted as being indicative for the accumulation of fatty acids and lipids in the stationary state. They also appear in the background of mouse and rat liver spectra (17–19).

The resonances in the polyol resonance region belong to mannitol, erythritol, arabitol, and glycerol, which are known to accumulate in fungi (20). In Fig. 1*A*, the resonances of mannitol (C-1 and C-6 at 64.4, C-2 and C-5 at 71.8, and C-3 and C-4 at 70.2 ppm) and of erythritol (C-1 and C-4 at 63.7 and C-2 and C-3 at 73.0 ppm) are very obvious. Both resonances belonging to glycerol are partly resolved and appear at 63.5 (C-1 and C-3) and at 73.2 ppm (C-2) as shoulders on the erythritol resonances. Two resonances of arabitol (C-2 at 71.3 and C-3 at 71.5 ppm) are well resolved, whereas the low-field resonance at 71.9 ppm (C-4) appears as a shoulder on the low-field mannitol resonance of 71.8 ppm. The other two arabitol resonances (C-1 and C-5) nearly coincide at 64.0 ppm and 64.1 ppm.

Two narrow resonances at 128.8 and 130.5 ppm were always observed in ^{13}C spectra of freshly grown mycelium irrespective of the carbon source used for growth. They correspond to the unsaturated carbon bond resonances of mobile mono- and polyunsaturated aliphatic chains and have been assigned by Canioni *et al.* (19).

When larger line broadenings (>5 Hz) were used, some broader resonances of low amplitude became more clearly visible. They represent most likely the hydroxymethyl and hydroxymethylene carbons of polysaccharides, including the corresponding anomeric carbons occurring at 101.0 and 108.3 ppm. The resonances between 170 and 180 ppm are due to the carbons of various carboxylic acids and carboxyl groups involved in ester linkages.

When the mycelium was grown on sucrose/acetate (0.1 M/0.01 M) medium under limiting aeration conditions, a relatively low erythritol level was observed compared to mannitol, whereas the glycerol and arabitol levels were negligible. This also was the case for resonances in the tricarboxylic acid-cycle region except for alanine, which reached a relatively high level compared to aerobic growth (Fig. 1*B*).

Gluconeogenic growth. When mycelium was grown on glycerol, patterns as shown in Fig. 2*A* were obtained for the two spectral regions of interest. In the polyol resonance region, in addition to the three mannitol resonances, two glycerol resonances of high amplitude appeared as the result of substrate uptake. Superimposed on the glycerol peaks, the resonances of only a minor amount of erythritol could be observed, which became better resolved in the ^{13}C spectra of glycerol-grown mycelium upon starvation, when glycerol had disappeared (Fig. 2*B*).

Under these growth conditions, trehalose characteristically was formed in a significant amount. Of the five trehalose resonances displayed, those at 61.6 (C-6), 70.7 (C-4), and 73.6 ppm (C-3) were well resolved; the other two at 73.1 (C-5) and 72.1 ppm (C-2) were resolved only partially. The spectra of mycelium grown on glycerol with low oxygen supply were similar but indicated lower metabolite levels. The accumulation of metabolites during growth on acetate/sucrose (0.1 M/0.01 M) is shown in Fig. 3*A*. The polyol resonance region shows equal amplitudes for mannitol and erythritol

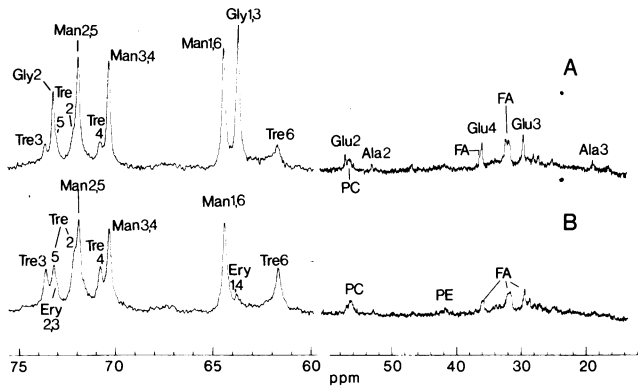

FIG. 2. (*A*) Natural-abundance ^{13}C NMR spectrum of *A. nidulans* after growth on glycerol (0.1 M) under strongly aerobic conditions; further conditions were as in Fig. 1*A*. (*B*) ^{13}C NMR spectrum of a culture grown as in *A* after starvation for 1 hr under strongly aerobic conditions; further conditions were as in Fig. 1*A*. Abbreviations are as in Fig. 1.

and minor amounts of glycerol and arabitol. Three well-resolved resonances enter into the spectrum that either correspond to amylose or to glycogen. A similar observation was reported for perfused mouse liver, where glycogen is synthesized from glycerol (18).

The accumulation in the tricarboxylic acid-cycle resonance region resembled that observed for growth on glycerol, where glutamate was observed and glutamine was absent.

When oxygenation of the culture was low, erythritol was only slightly accumulated; the amylose or glycogen disappeared (Fig. 3*B*) but trehalose was synthesized. However, the tricarboxylic acid-cycle resonance region was very poor in amino acids.

Gluconeogenic growth on acetate as sole carbon source under strong aeration resulted in accumulation patterns that closely resembled those obtained for acetate/sucrose-grown cultures under limited oxygen supply. However, the accumulation of trehalose was characteristic for growth on acetate. The presence of minor concentrations of a glycolytic substrate together with acetate led to the accumulation of amylose, instead of trehalose, at least when no limitation in oxygen supply existed (cf. Fig. 3*A*).

Starvation Under Vigorous Aeration. Prior to the incorporation of ^{13}C-enriched glucose, mycelium was starved in order to reduce the existing pools of nonenriched metabolites. The various polyol pools were exhausted with different rates. In sucrose-grown mycelium, glycerol disappeared within 1 hr. Preferential mobilization of glycerol upon starvation was still better observed in glycerol-grown mycelium, which had accumulated predominantly mannitol and glycerol (Fig. 2*B*). Within 1 hr glycerol disappeared, but simultaneously a concomitant increase of the trehalose resonances was observed. The mannitol level remained nearly constant during this time span. The other polyols showed a constant decrease in amplitude during starvation.

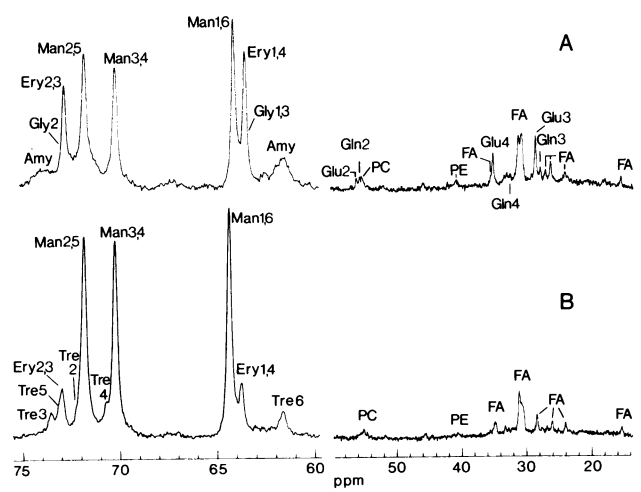

FIG. 3. (*A*) Natural-abundance ^{13}C NMR spectrum of *A. nidulans* after growth on acetate/sucrose (0.1 M/0.01 M) under strongly aerobic conditions; other conditions were as in Fig. 1*A*. (*B*) Spectrum of a similar culture grown under conditions of limiting aeration. Amy, amylose; other abbreviations are as in Fig. 1.

Incubation with ^{13}C-Enriched Glucose. *Glycolytic growth.* ^{13}C NMR spectra of *A. nidulans* mycelium grown on sucrose/acetate (0.1 M/0.01 M) under vigorous aeration are shown in Fig. 4 after starvation, when 12% randomly ^{13}C-enriched glucose was fed (30 mg per incubation of 1 g of mycelium). The label became mainly incorporated into mannitol and to some extent into arabitol and trehalose. The mannitol and arabitol resonances reached a maximum after 1–2 hr of incubation. In this time span, the transient trehalose resonances already disappeared, whereas the residual erythritol pool remaining after starvation decreased continuously with time.

The increase of mannitol and arabitol as an initial response to ^{13}C-enriched glucose feeding and the simultaneous leveling off of natural-abundance erythritol illustrates that the different polyols have characteristic turnover features.

In the tricarboxylic acid-cycle region, the glutamate pool is the first one that became labeled, whereas glutamine and alanine increased with a lag time. Ultimately the ^{13}C-labeling indicated comparable levels for glutamate and glutamine.

Incubation of mycelium grown on sucrose/acetate (0.1 M/0.01 M) with limiting amounts of oxygen and incubated with [^{13}C]glucose under the same conditions resulted in a label distribution that differed dramatically from the aerobic case (Fig. 4). Resonances belonging to nonphosphorylated glucose, which were never observed under aerobic incubation conditions, were present in this spectrum together with a small amount of trehalose. Accumulation of label was mainly restricted to mannitol, whereas the alanine resonance

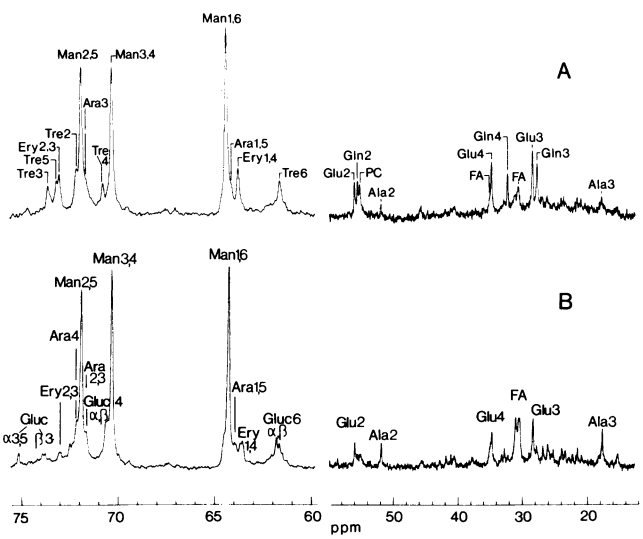

FIG. 4. (*A*) ^{13}C NMR spectrum of mycelium of *A. nidulans* after growth on sucrose/acetate, subsequent starvation (1 hr), and incubation with [^{13}C]glucose (30 mg) under strongly aerobic conditions for 1 hr; the number of transients was 14,400. Other conditions were as in Fig. 1*A*. (*B*) ^{13}C NMR spectrum of *A. nidulans* after growth on sucrose/acetate, subsequent starvation (1 hr), and 1 hr of incubation with [^{13}C]glucose, all under conditions of limiting oxygenation; the number of transients was 14,400. Gluc, glucose; other abbreviations are as in Fig. 1.

amplitudes were now comparable to glutamate, indicative for reduced respiration.

Gluconeogenic growth. Incubation with [^{13}C]glucose of acetate/sucrose-grown and subsequently starved mycelium resulted in similar patterns as described for glycolytically grown mycelium.

Also acetate-grown mycelium, which accumulated trehalose as shown above, was starved and subsequently incubated with [^{13}C]glucose. Except for erythritol, which slightly increased within 0.5 hr and then disappeared, the other polyols (mannitol and arabitol) as well as trehalose still increased after a 2-hr incubation period with [^{13}C]glucose. In the tricarboxylic acid-cycle region, only glutamate became specifically labeled.

DISCUSSION

In mycelium of *Aspergilli* and other fungi, a variety of low molecular weight soluble sugar alcohols along with trehalose have been reported to be present during active growth (21–23). These compounds are considered to contribute to the osmotic stabilization of the hyphal cells and to form endogenous reserves that are continually metabolized, also in the presence of exogenous substrates. The nature as well as the turnover of these reserves depend on the growth conditions. Therefore, the pool composition may vary during different stages of the fungal life cycle (see, e.g., ref. 20). In yeast the accumulation of trehalose, particularly in ascospores, is an example of this (24). Reserve carbohydrates also have been reported to appear sometimes in a consecutive manner, C_6 polyols being synthesized first and then C_5 polyols appearing prior to C_4 polyols (see ref. 20).

The major features of carbon metabolism in *A. nidulans* are represented in Fig. 5. The presence of high concentrations of polyols during active growth is very obvious for the natural-abundance ^{13}C NMR spectra obtained. Irrespective of whether the fungus is grown on glycolytic or gluconeogenic carbon sources, the polyol resonances dominate the sugar resonance region. Thus, spectra obtained with *Aspergillus* are quite distinct from those seen previously with *E. coli* or *S. cerevisiae* (1–4). Therefore, it was particularly important to devote this pioneer study to assign various resonances and to monitor the major metabolic events.

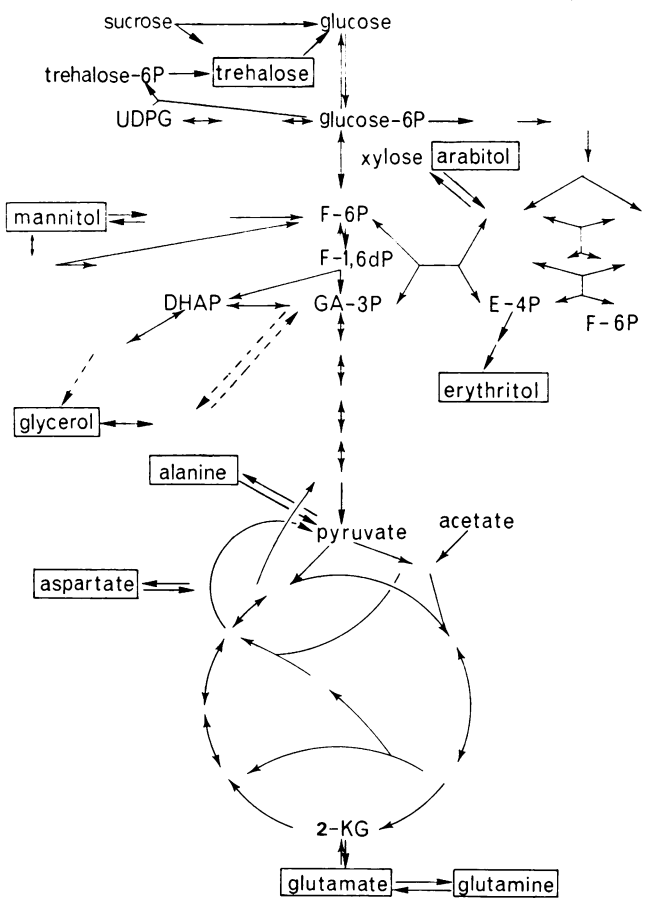

FIG. 5. Scheme of major events in carbon metabolism in *A. nidulans*. The major compounds observed by ^{13}C NMR are indicated.

The polyol pool sizes are strongly influenced by the carbon source used and by oxygen supply. The metabolic control mechanisms involved in polyol metabolism are still poorly understood. Biosynthesis of mannitol in fungi most likely occurs by reduction of fructose 6-phosphate, followed by hydrolysis of the resulting mannitol 1-phosphate by a specific phosphatase (25). Both NAD^+- and $NADP^+$-dependent mannitol-1-phosphate dehydrogenases as well as mannitol dehydrogenases have been found and sometimes simultaneously (cf. ref. 20). The regulatory features of phosphofructokinase have not been thoroughly investigated yet in Aspergilli, but this enzyme is a good candidate to control the glycolytic flux and thus to influence mannitol biosynthesis and catabolism. Thus far, no conclusive evidence has been presented for the existence of different, separatedly controlled routes for mannitol catabolism and anabolism (20).

Glycolytic growth is realized in these studies by feeding sucrose or sucrose in the presence of some acetate, the uptake of which occurs simultaneously (26). Besides mannitol accumulation, the most characteristic feature of the ^{13}C NMR spectra obtained is the accumulation of erythritol, the level of which strongly depends on a high oxygen level. Erythritol synthesis is more favored by growth under glycolytic conditions than under gluconeogenic conditions. The accumulation of appreciable levels of both arabitol and glycerol under the same growth conditions is another indication that the fluxes through both the pentose phosphate pathway and glycolysis are high and result in sufficient reduction capacity to lead to an overflow of different polyols.

The major feature of strong aerobic growth on acetate/sucrose (0.1 M/0.01 M) is that only small amounts of glycerol and arabitol are present, whereas the mannitol and erythritol pools are high and equal in size. Since the erythritol level is low in cultures solely grown on acetate, this polyol most likely originates from the sucrose present in acetate/sucrose-grown cultures.

When acetate is used as sole carbon source, a small amount of trehalose is synthesized. However, trehalose is also present in a significant amount in natural-abundance ^{13}C NMR spectra of glycerol-grown mycelium that lacks erythritol. Thus, the biosynthesis of pentose phosphate pathway-mediated polyols is not stimulated by gluconeogenic substrates. However, gluconeogenic substrates do stimulate the formation of trehalose very markedly.

Upon starvation, the polyol pools are depleted at different rates; particularly glycerol becomes rapidly exhausted and is partially converted into trehalose in glycerol-grown mycelium. In sucrose/acetate-grown mycelium, glycerol also disappears, but no conversion into trehalose has been observed with natural-abundance labeling.

Our results conncecting low levels of glycolytic substrates and trehalose synthesis are in line with observations in yeast (24). It is quite possible that regulation of the trehalase activity by glycolytic carbon sources is the dominant factor in all of the transient trehalose effects.

In the natural-abundance ^{13}C NMR spectra of the amino acid and tricarboxylic acid-cycle resonance region, pronounced glutamate and glutamine concentrations are found when the fungus is vigorously aerated. This is indicative for high tricarboxylic acid-cycle activity. The levels of these amino acids and that of alanine remain initially constant

upon starvation, only to diminish when the polyol levels become low. A limited oxygen supply results in a relatively poor spectrum in this region. The situation resembles considerably that observed for *P. ochrochloron* in the stationary phase (6) and for mouse liver (17, 18).

When adding ^{13}C-labeled glucose, a fast incorporation of label into mannitol is always observed regardless of the growth conditions or the degree of oxygen supply. In strongly aerated mycelium, a transient incorporation of ^{13}C label from glucose into trehalose is observed, whereas the incorporation into arabitol is also transient. However, glycerol and erythritol resonances still present before incubation with labeled glucose disappear at the same time, indicating their preferential metabolization.

In the tricarboxylic acid-cycle region, glutamate is the first to increase upon [^{13}C]glucose labeling, whereas, depending on the medium composition, also glutamine levels increase with a lag time. Finally a significant amount of alanine appears. The incorporation of ^{13}C-enriched glucose in mycelium grown with a low oxygen tension indicates that, as a result of this condition, glycolysis is slowed down considerably. The presence of nonphosphorylated glucose as well as a relatively high level for alanine taken together with the absence of tricarboxylic acid-cycle-related compounds are obvious signs of this.

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