High Incorporation of Labeled Acetate into Yeast Ergosterol During Sporulation[1]

The availability of ^{13}C-NMR-spectrometers with fast Fourier transform accessories has greatly increased the possibility of obtaining detailed information on biosynthetic pathways without extensive and time-consuming chemical degradation of the metabolite of interest. Recently BURLINGAME et al.[2] incorporated [2-^{13}C]-acetate into yeast palmitoleic acid (30% ^{13}C enrichment) during aerobic growth and showed the alternate carbons to be labeled. In connection with our studies on the biosynthesis of the yeast sterol, ergosterol, from glycine and serine, we have been interested in using substrates labeled with carbon-13 as well as carbon-14. Carbon-14 has been employed as a tool for elucidating ergosterol biosynthesis[3]. However the fate of the acetate precursor in the sterol requires chemical degradation such as done by HANAHAN and WAKIL[4] on the C_{23} to C_{28} ergosterol fragment indicating the labeling pattern in one part of the molecule. If ergosterol can be biosynthesized from [1 or 2-^{13}C]-acetate, or [1, 2-^{13}C]-acetate, the fate of every acetate carbon could be learned from the ^{13}C-NMR-spectrum since the chemical shifts for ergosterol carbons are known[5-7]. Parallel experiments with ^{13}C-labeled glycine, serine and other substrates would then be simplified.

Currently 2 techniques are available for obtaining highly labeled ergosterol from yeast. KLEIN et al.[8] found that yeast grown under anaerobic conditions will accelerate biosynthesis when aerated and produced a 10-fold increase in sterols relative to anaerobically grown yeast. This procedure has recently been used by FRYBERG et al.[3] to study the intermediates in ergosterol biosynthesis by yeast. If radioactive glucose is provided during aeration, over 75% of the specific activity was found in the non-saponifiable fraction[9]. While no data was given for acetate incorporation, KLEIN indicated that acetate could be used although longer aeration time would be necessary.

We have examined the labeling of ergosterol from [2-^{14}C]-acetate in *S. cerevisiae* SK1 using KLEIN's anaerobic-aerobic procedure (Table I). While the highest yield of recovered ergosterol was for the 20 g (wet cells)/100 ml (aeration medium), the highest specific activity was for the lower sterol yielding cell densities.

The second enrichment technique depends on the observation that to supply coenzyme A to pantothenic acid deficient yeast culture spurs the de novo biosynthesis of lipids including sterols. HANAHAN and WAKIL[10] found that ^{14}C-labeled acetate supplied at the time of this accelerated biosynthesis results in the labeling of $^1/_4$ of the ergosterol carbons. KLEIN et al.[11,12] showed this procedure to yield a several 100% increase in total lipids including sterols relative to the unenriched medium.

Our results which employed a modified HANAHAN and WAKIL procedure incorporating the KLEIN anaerobic-aerobic technique are presented in Table II. These results indicate the inadequacy of this approach for our purposes.

Recent work has shown that during yeast sporulation induced by acetate, there is a dramatic rise in lipid biosynthesis[13,14]. Therefore, it appeared likely that yeast sporulation in a labeled acetate medium would yield ergosterol with a high specific activity. This result was realized as shown in Table III. Experiment B which employs anaerobic presporulation growth and aerobic sporulation produces significantly more ergosterol with a greater incorporation of label.

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Table I. Biosynthesis of labeled ergosterol from [2-^{14}C]-acetate in *S. cerevisiae* SK1 using anaerobic-aerobic procedure. Anaerobic Growth in YPD[a,b]. Aeration suspension[c] (0.1% glucose, 0.1 *M* [2-^{14}C]-acetate, 0.1 *M* phosphate, pH 7.0)

Cell concentration (g wet cells/100 ml aeration medium)	Ergosterol recovered[d] (mg)	Incorporation efficiency[e]	Overall incorporation[f]
5	4.8	10	0.08
10	3.9	13	0.20
15	4.1	5	0.08
20	6.5	5	0.11
25	3.3	3	0.04
30	3.0	4	0.04

[a] YPD is 1% yeast extract, 2% peptone, 10% dextrose (Difco). [b] 0.5 l 2-day-old innoculation of 15 l culture grown for 5 days at 30 °C with mechanical stirring and N_2 purging followed by centrifugation and washing twice with sterile distilled water. [c] Aerated for 24 h by shaking at 200 rpm at 30 °C. [d] Cells saponified, extracted with Petroleum ether, TLC on $AgNO_3$-impregnated silica gel, eluted with $CHCl_3$. [e] dpm/mole ergosterol recovered/dpm/mole acetate substrate × 1 mole ergosterol/15 mole acetate × 100 = % incorporation efficiency. [f] Total dpm of ergosterol recovered/total dpm of acetate substrate × 100 = % overall incorporation.

Table II. Biosynthesis of labeled ergosterol from [2-^{14}C]-acetate in *S. cerevisiae* SK1 using CoA-enrichment and anaerobic-aerobic procedure. Anaerobic Growth in Coenzyme-A deficient medium[a, b]. Aeration suspension[c] (0.1% glucose, 0.1 *M* [2-^{14}C]acetate, 0.1 *M* phosphate, pH 7.0, 1.0% YNB containing 25 mg CoA[d]

Cell concentration (g wet cells/100 ml aeration medium)	Ergosterol recovered[e] (mg)	Incorporation efficiency[f]	Overall incorporation[g]
20	4.8	4.5	0.08

[a] 1% Wickerham's Yeast Nitrogen Base (YNB) without pantothenic acid, 10% dextrose, and 17 mg/ml Tween 80 and 20 µg/ml tetracycline. [b] Same as (b) in Table I. [c] Same as (c) in Table I. [d] Grade II-A from Sigma. [e] Same as (d) in Table I. [f] Same as (e) in Table I. [g] Same as (f) in Table I.

Table III. Biosynthesis of labeled ergosterol from [2-^{14}C]-acetate in *S. cerevisiae* SK1 during sporulation.
Sporulation Conditions for A and B: aerobic, 1% labeled acetate, 27 °C, vigorous mechanical stirring, 4×10^7 cells/ml (1 l total volume, containing 5.5 g wet cells in 6 l flask)

Sporulation yield	(A) 99% asci in 3 days	(B) 90% asci in 5 days
Ergosterol recovered[b] (mg)	4.4	7.9
Incorporation efficiency[c]	71	76
Overall incorporation[d]	0.11	0.21

Presporulation growth conditions: (A) aerobic, YPD[a], 30 °C, 2 days 1 l from loop, shaking at 200 rpm. (B) anaerobic, YPD, 30 °C, 2 days 1 l from loop, shaking at 200 rpm. [a] Same as (a) in Table I. [b] Same as (d) in Table I. [c] Same as (e) in Table I. [d] Same as (f) in Table I.

As yeast sporulation in a liquid medium is carried out at approximately 4×10^7 cells/ml, a liter of 1% acetate is required to obtain the ergosterol yields we report. However, since the sporulation medium contains only acetate, the recovery of unincorporated label can be accomplished by a simple extraction.

Data of labeled substrate incorporation into a metabolite can be stated in various ways. We have reported firstly the overall incorporation as an indication of the metabolic magnitude of ergosterol biosynthesis from acetate. Secondly, the incorporation efficiency expresses the percentage of ergosterol carbons derived from acetate. The factor of 1/15 (cf. Table I, footnote e) in the calculation of incorporation efficiency allows for the biosynthesis of 1 mole of ergosterol from 15 moles of acetate. We have assumed that acetate contributes to all ergosterol carbons except C_{28} which arises from single carbon donors (e.g. formate) by transmethylation via methionine[15, 16].

The data in Table III clearly indicates that the sporulation technique gives a more efficient incorporation than the 2 earlier methods described. Further work on biosynthesis in yeast using ^{13}C-labeled substrates is in progress in our laboratory.

Zusammenfassung. Es werden drei Methoden für die Durchführung von Versuchen mit ^{13}C-markierten Substraten (Einbau markierter Acetate in Hefeergosterol) verglichen: 1. Anaerobisches Wachstum mit nachfolgender Belüftung und Markierung. 2. Anaerobisches Wachstum in Nährlösung mit ungenügendem CoA-Gehalt mit nachfolgender Belüftung und Markierung in einer mit CoA angereicherten Lösung. 3. Sporenbildung mit markierten Acetaten.

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A New Method for Recording Migratory Restlessness in Caged Birds

Many birds that are normally day-active migrate at night. If kept in a cage, they develop nocturnal activity during the migratory seasons which manifests itself in an intense hopping off and onto perches, a fluttering about in the cage or a vigorous beating of the wings ('whirring'). This migratory restlessness or 'Zugunruhe' has often been taken for an expression of the migratory drive in birds. Quantitatively, however, it was either only the hopping or general locomotor activity that was measured so far[1–4], but never the whirring itself which might be a more specific expression of the migratory drive. Moreover, in recording perch hopping, both day- and night-activity of a

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