Volume 13, number 2 MOLECULAR & CELLULAR BIOCHEMISTRY November 30, 1976

DETERMINATION OF INTERMEDIARY METABOLITES IN YEAST. CRITICAL EXAMINATION OF THE EFFECT OF SAMPLING CONDITIONS AND RECOMMENDATIONS FOR OBTAINING TRUE LEVELS

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(Received February 20, 1976)

Summary

The effect of sampling conditions on the levels of adenine nucleotides, pyridine nucleotides, glycolytic intermediates and related metabolites in yeast has been studied. A systematic examination of the conditions for harvesting has shown that it can be best accomplished by rapid filtration. Delays in the handling for removal of the medium, as is usual in the process of obtaining a number of data reported in the literature, lead to important changes in some of the metabolites examined. It is also shown that when a washing is imperative it can be carried out with a methanol-water mixture (50/50, v/v) cooled at −40° without loss of intracellular concentrations of non-readily diffusible metabolites.

On the basis of this experience the outline of a generally applicable procedure is presented.

Introduction

Knowledge of the concentration of intermediary metabolites is of prime interest in the study of metabolic processes and their regulation. However, the experimental values may considerably differ from those prevailing *in vivo* if precautions are not taken to stop cellular metabolism rapidly. In the case of microorganisms, owing to the relatively low cellular densities in the culture medium, determination of metabolites generally requires a concentration step. Filtration[1,2,3] centrifugation[4,5] or centrifugation followed by resuspension in a small volume of fresh medium[6] have been used to circumvent this difficulty. A critical examination of these procedures and their influence on the values of metabolites has been made by Gancedo and Gancedo[6].

Since many conflicting values found in literature for some metabolites are probably due to differences in the sampling conditions used, we have undertaken a critical appraisal of the sampling process and studied the influence of each partial step in the final values obtained for different metabolites. The results indicate that a note of caution on the concentration of yeast metabolites reported in the literature should be kept in mind, since the requirements for satisfactory sampling have not been met in most of the cases. We present here a procedure for sampling which is rapid, does not require special equipment and makes it possible to obtain values which most nearly represent the actual concentration of the metabolites in growing yeast cells.

Material and Methods

Reagents

Enzymes, pyridine nucleotides and adenine nucleotides were obtained from Boehringer. All other reagents were of A.R. grade.

Organism and growth conditions
S. cerevisiae (strain 13-Gal) was grown with vigorous shaking at 30 ° in a minimal medium as described in[7] except that sodium citrate was replaced by 0.25 g/l sodium chloride. 2% Glucose as carbon and energy source was used.

Determination of yeast wet weight
Aliquots of 50 ml of a culture at different times of incubation were filtered in vacuum through Millipore. The yeast was resuspended in 50 ml of water, filtered through Millipore and immediately weighted. A calibration curve relating wet weight to optical density of the culture was drawn in this form.

Sampling of the yeast
The yeast was grown to a cellular density of 8 to 10 mg/ml (wet weight). A volume of culture containing ca. 1.5 g of wet yeast was divided into aliquots which were allowed to grow for 15 more minutes. Each aliquot was filtered in vacuum through a Millipore filter 0.45 μm pore size and 47 mm diameter (filtration step). The yeast pellicle was immediately gathered by means of a spatula and immersed in liquid nitrogen (gathering step). The sample was kept in liquid nitrogen until extraction. All the aliquots corresponding to the initial volume were extracted together as described below. Since some yeast remained on the filter, a correction factor should be introduced. This factor was calculated by washing the filter with water and measuring the optical density of the resulting suspension against a calibrated curve. The correction factor oscillates between 2 and 10%. In some cases, where indicated, the filter with the yeast pellicle was directly poured into the liquid nitrogen.

Washing of the yeast
When washing was appropriate, immediately after the filtration step was concluded, 5 ml of water at 0 or 30°, or methanol-water (50/50, v/v) at −40° were passed through the yeast pellicle. Thereafter the yeast was gathered and treated as described above.

Preparation of acid extracts
The gathered yeast corresponding to the sample under study were mixed with 3 ml of 3 M $HClO_4$ and 0.4 ml of 0.4 M Tris in a mortar previously cooled with liquid nitrogen. The mixture was ground to powder in the presence of liquid nitrogen, transferred to a test tube and kept for 30 min at −10° with frequent vigorous shaking. The resulting liquid was frozen in liquid nitrogen and then allowed to thaw. This process was repeated twice to liberate quantitatively the cell metabolites[8]. The extract obtained was centrifuged in the cold for 10 min at 10,000 g and the supernatant adjusted at pH 6.5 with 10 N KOH at 0°. After 15 min at 0° the precipitated $KClO_4$ was centrifuged off. The supernatant was utilized for the determination of acid stable metabolites.

Preparation of alkaline extracts
For the determination of reduced pyridine nucleotides the corresponding aliquots were ground to powder under liquid nitrogen in a precooled mortar with 3 ml of 0.1 N KOH. The mixture was transferred to a tube, allowed to stand 20 min at −10° and then heated at 90° for 1 min. After cooling at 0° the extract was neutralized with 0.15 ml of 2 M $KHCO_3$. After centrifugation at 10,000 g for 10 min in the cold the supernatant was used for the various determinations.

Assay of metabolites
The methods used for the determinations were essentially as described in BERGMEYER[9]. Measurements were made with a Gilford 2400 spectrophotometer. Pyridine nucleotides were measured with an oxygen electrode (Clark type) following the method described by GREENBAUM *et al.*[10]. For the calculation of concentrations it has been assumed that 1.67 g wet yeast contain 1 ml cell sap[11].

Results

The effect of the length of the time of the filtration step on the concentration of metabolites
Of the methods reported in the literature for the concentration of microorganisms with minor alterations in their metabolite levels, vacuum filtration through Millipore seems to be the most suitable method[1,2,3]. However, the time which elapses during filtration of an adequate amount of culture with standard equipment, 30 to 120 seconds, is not negligible. In order to

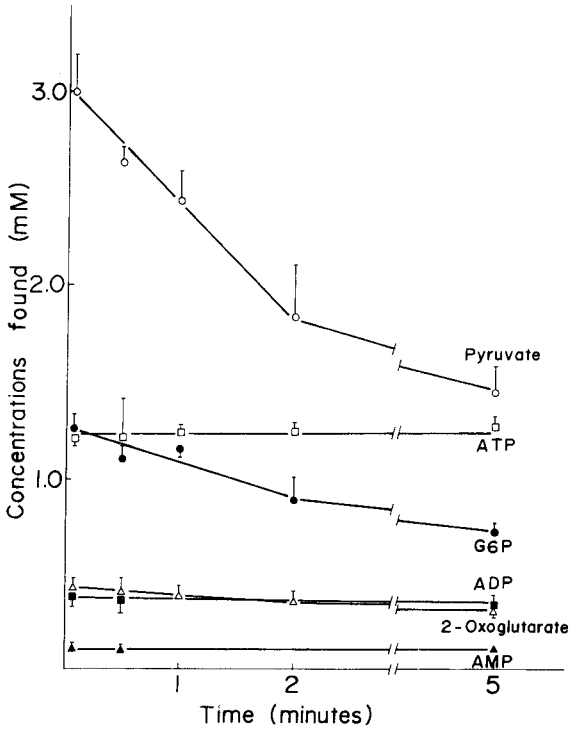

Fig. 1. *The effect of the length of time of the filtration step on the concentration of metabolites.* Aliquots of a culture containing ca. 1.5 g wet weight were divided into several portions of appropriate volumes required to attain the times of filtration indicated. These portions were treated as indicated in Methods. The time spent in the "gathering step" was in all cases 3 to 4 seconds. Mean values and standard deviation of four experiments are shown. (Abbreviation used in the graphs: G6P, glucose-6-phosphate).

minimize this time the sample was divided into aliquots which were filtered independently, their harvests being pooled afterwards for extraction. The effect of increasing the time employed in the filtration step is shown in Figure 1. To modify the filtration time, culture samples of 1.5 g yeast (wet weight) were divided into aliquots of appropriate volumes whose filtration lasted for the times indicated. As can be seen although the values for adenine nucleotides did not change even when the filtration time was prolonged to 5 min, substantial decreases of pyruvate and glucose-6-phosphate were observed. This fact indicates that the filtration step should be performed as a general rule in the shortest possible time.

Instability of metabolite levels in unfrozen pellets

After filtration the cells remain on the filter without culture medium and partially under anaerobic conditions, therefore large changes in the concentration of metabolites may occur before freezing. The effect of prolonging the time that the yeast remains on the the filter was examined with different metabolites and the results can be seen in Figure 2. Pyruvate and glucose-6-phosphate rapidly changed from their initial value. After 30 seconds the level of pyruvate was less than 70% of that found after 4 seconds. The decrease continued and after 10 min only 10% was recovered (Fig. 2A). The decrease of glucose-6-phosphate was smaller than that of pyruvate but by no means negligible (Fig. 2A). NAD^+ and NADH did not appreciably change in 10 min (Fig. 2B) while $NADP^+$ decreased significantly. Also NADPH seemed to decrease (Fig. 2B). NH_4^+ and 2-oxoglutarate decreased with time, while glutamate did not change even after 10 min (Fig. 2C). Adenine nucleotides did not vary during the first 20 seconds but after this time serious changes occurred. After 2 minutes AMP and ADP increased by a factor of 5 and 2 respectively while ATP decreased to 30% of its original value. These effects produced important changes in the value of the energy charge[12] (Fig. 2D).

Extrapolation to zero time of the results of Figure 2 indicates that no significant artefact was introduced at this step when the interval from the end of filtration to freezing lasted 4 seconds.

The effect of washing yeast on the concentration of metabolites

Washing of the organism may be necessary when a compound to be measured intracellularly is present at high concentration in the culture medium, inasmuch as filtration alone may not be sufficient for a complete removal of the medium. Since washing may alter the concentration of some metabolites[1,2] the influence of washing at different temperatures was investigated. Water was used at 0 and 30° and a 50/50 (v/v) mixture of methanol-water at −40°. The results are presented in Figure 3. The majority of metabolites tested remained constant when washing was performed at −40°. Only a small but statistically significant decrease of ca. 20% was found for pyruvate. NH_4^+ however decreased greatly (Fig. 3B) and this decrease was most likely related to the removal of that

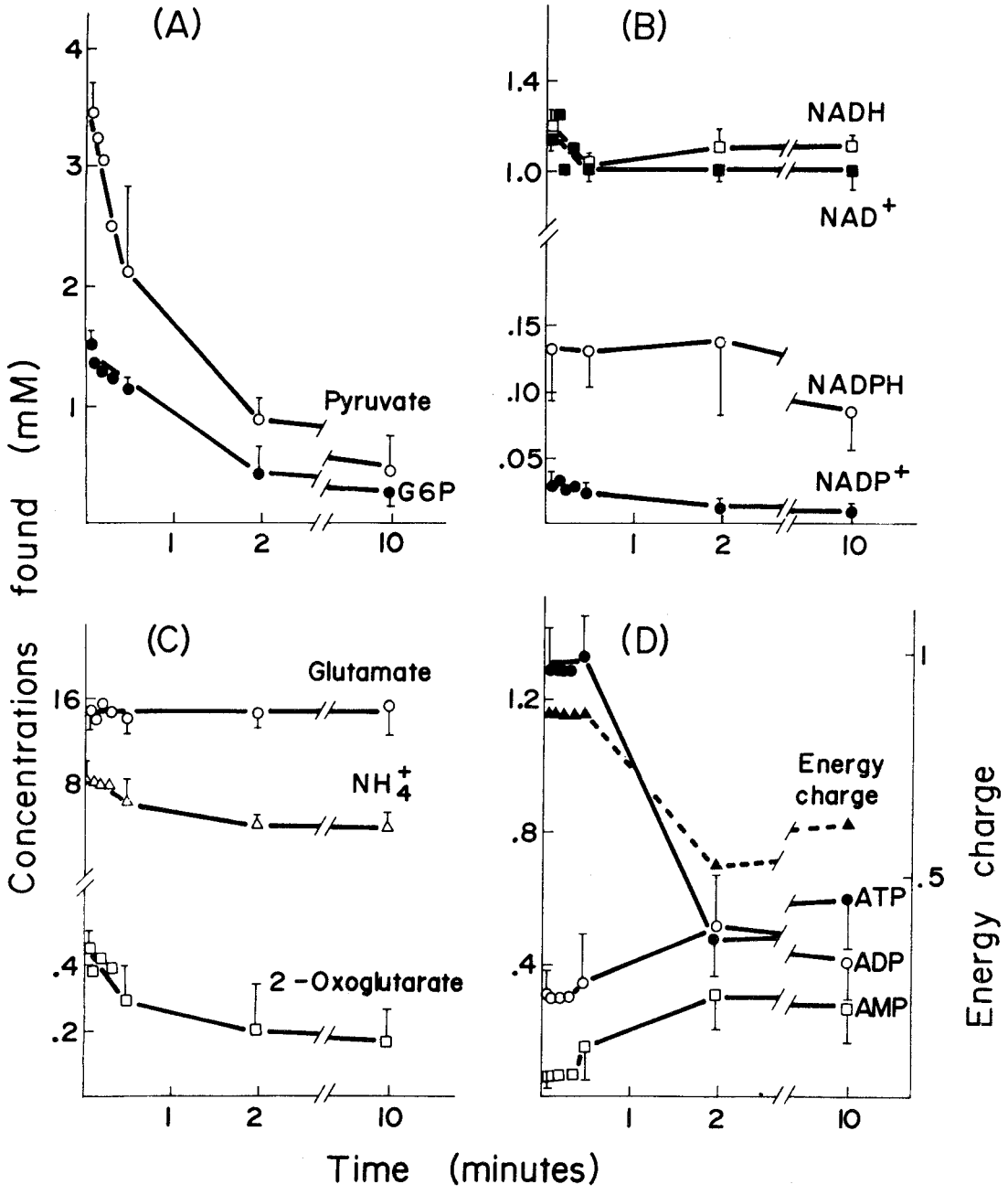

Fig. 2. *The effect of the delay in transferring the pellet to liquid nitrogen on the concentration of metabolites.* Aliquots of cultures containing ca. 1.5 g wet weight were divided into 10 portions which were treated as indicated in Methods. The filtration of each portion lasted 4 to 6 seconds. The delay in transferring the pellet to liquid nitrogen varied from 4 seconds to 10 minutes as indicated. The points and their standard deviations are derived from four experiments.

present in the culture medium. The NH_4^+ concentration of the culture medium was 50 mM and that found in the yeast cake ca. 8 mM. Assuming that the value found after washing, ca. 4.5 mM, is the actual concentration inside the cells it can be readily calculated that about 5% of the volume of the yeast cake corresponds to the intercellular space. This value is in good agreement with that found by CONWAY and DOWNEY by a completely different approach[11]. Moreover, similar values of NH_4^+ were found in yeast after washing with greater volumes of

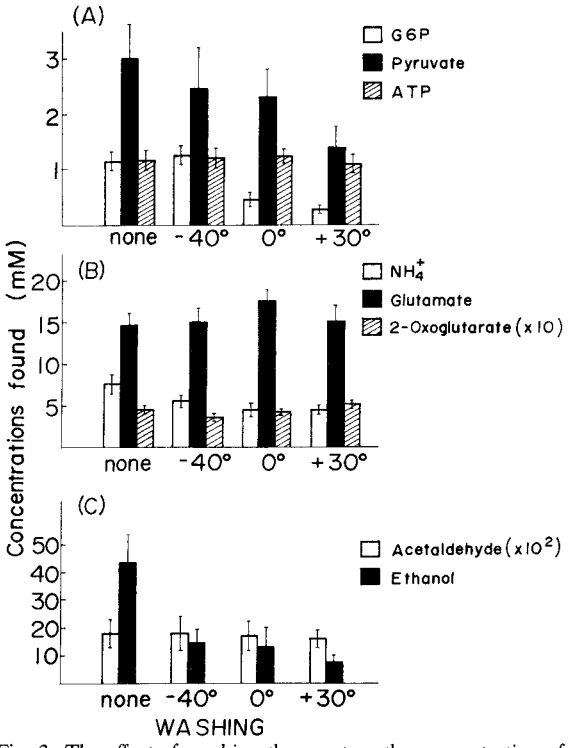

Fig. 3. *The effect of washing the yeast on the concentration of metabolites.* Aliquots of cultures containing ca. 1.5 g wet weight were divided into 10 portions which were treated and washed as indicated in Methods. Mean values and standard deviations corresponding to six experiments are shown. At −40° only the decrease in pyruvate (A) NH_4^+ (B) and ethanol (C) are statistically significant ($P < 0.05$).

methanol-water, a fact that supports the conclusion that the value found after washing at −40° represents the real situation *in vivo*.

Ethanol is removed from the cells even at −40° (Fig. 3C) a fact that confirms its free permeation through the cell membrane[13,14]. The large decrease in ethanol concentration cannot be ascribed to removal of medium since the concentration in the latter was found to be the same as that in the yeast. Acetaldehyde, which has been also reported as freely permeable did not show any significant variation during washing.

It can be concluded that washing at −40° should be used for determining NH_4^+, gives low values for ethanol and to a lesser extent for pyruvate, and does not affect the levels of other metabolites.

Discussion

The results reported in this paper clearly demonstrate that yeast metabolites can suffer important changes in a very short period of time. Consequently, for the estimation of the physiological concentrations of intermediates all manipulations must be performed very rapidly. Accordingly, the disparity between the values found by different workers[6] is most likely due to the fact that in most of the cases the requirements for satisfactory sampling were not met. During sampling of yeast by filtration certain metabolites may change substantially (Fig. 1). Similarly, sampling by centrifugation requires a prolonged period of time during which sedimented cells remain in the absence of culture medium and oxygen. This situation has been mimicked in experiments in which the interval between rapid filtration and freezing the sample varied in length. Figure 2 demonstrates that changes in the concentration of most of intermediates fall precipitously as soon as yeast is deprived of culture medium and that therefore sampling by centrifugation should be avoided. Only adenine nucleotide concentrations remained constant during filtration (Fig. 1) and in cells deprived of culture medium during at least 20 seconds (Fig. 2). In this respect, it seems interesting to point out that the constancy of ATP during sampling is not an indication of the behavior of other intermediates as has been assumed[1,2]. In fact, the decrease in ATP is most likely the last step of a succession of events. It is the prior decrease of intermediates in pathways related to ATP production that finally results in a decrease in the production of ATP. This is in agreement with our findings that changes of intermediates of catabolic pathways occur prior to changes in the adenine nucleotides concentration (Figs. 1 and 2).

Filtration and washing the yeast pellicle with a mixture of methanol-water at −40° eliminates the culture medium remaining in the intercellular space. Since no metabolic transformation occurs at this low temperature (Fig. 3) intracellular intermediates also present at high concentration in the medium can be accurately estimated under these conditions except those which freely permeate the cellular membrane (Fig. 3).

Outline of recommended procedure

On the basis of the results reported in this article, the following sampling procedure for the estimation of metabolites in yeast is recommended: a volume of culture corresponding to

1.5 g (wet weight) is divided into three aliquots and are allowed to grow for another 15 min. This amount of yeast allows the measurement of metabolites present at concentrations within the range of those studied here. A proportionally higher or smaller number of aliquots should be filtered for estimation of intermediates present at other concentrations. The aliquots are filtered in vacuum through Millipore filters of 0.45 μm pore size and 47 mm diameter. After filtration, the yeast may be washed if appropriate (for metabolites added to the medium) with 5 ml of a methanol-water mixture (50/50, v/v) cooled at $-40°$. The filter and the yeast are immediately immersed in liquid nitrogen and allowed to remain there until extraction. The three aliquots are extracted together under liquid nitrogen in a mortar under appropriate conditions described in this article.

Acknowledgments

We are very grateful to Dr. C. GANCEDO for many stimulating discussions and help during the preparation of this manuscript, and to Drs. G. DELAFUENTE, H. HOLZER and A. SOLS for their criticisms. We also deeply appreciate the excellent technical assistance of Miss E. MORENO.

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