S-ADENOSYLMETHIONINE IN THE VACUOLE OF *CANDIDA UTILIS*

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Recently, it has been demonstrated by ultraviolet microscopy that *S*-adenosylmethionine is accumulated in the vacuole of *Candida utilis* (Svihla and Schlenk, 1959). The exceptional concentration of this compound under certain cultural conditions, and the availability of simple analytical procedures permit a correlation of optical and biochemical studies. The opportunity of observing a well-defined chemical compound in the vacuole of viable cells without staining or pretreatment should yield information on the structure and function of this part of the yeast cell, especially during the growth cycle. The present report deals with some of these problems.

MATERIALS AND METHODS

Candida utilis strain ATCC 9950 was used, and the technical details of culture and analysis were the same as outlined earlier (Schlenk and DePalma, 1957; Svihla and Schlenk, 1959). Yields of yeast are reported on a dry weight basis. The culture medium had the following composition per L: KH_2PO_4, 2 g; K_2HPO_4, 1 g; $(NH_4)_2SO_4$, 2 g; trisodium citrate, 0.3 g; $MgCl_2 \cdot 6H_2O$, 0.1 g; $MnSO_4 \cdot 7H_2O$, 0.1 g; $CaCl_2$, 0.1 g; $ZnSO_4 \cdot 7\ H_2O$, 0.1 g; glucose (sterilized separately), 15 g. Various supplements were added as indicated in the experiments. This medium is termed "nitrogen-containing medium," whereas "nitrogen-free medium" had the same composition except for substitution of K_2SO_4 for $(NH_4)_2SO_4$.

In previous experiments (Svihla and Schlenk, 1959), L-methionine or a combination of L-homocysteine and L-methylmethionine was used as the organic sulfur supplement to stimulate the production of *S*-adenosylmethionine. In the present experiments, a combination of all three compounds was used. The concentration of *S*-adenosylmethionine attained by this procedure is higher than that reported in our earlier experiments. A concentration of 5 μmoles per ml of each sulfur amino acid was tolerated without undue decrease of cell yield. Apparently, the organic sulfur compounds enter the yeast cells without mutual interference or competition, whereas the equivalent supplement of 15 μmoles of L-methionine per ml causes serious inhibition of growth. L-Homocysteine was prepared from commercial L-homocysteine thiolactone hydrochloride by dissolving the latter in 1.5 ml of 1 N NaOH per mmole; after 5 min at 25 C, one adds 3.0 ml of 0.5 M KH_2PO_4 for neutralization.

The presence of L-homocysteine in the yeast culture medium leads to the formation of *S*-adenosylhomocysteine under special circumstances (Schlenk, *unpublished data*). This substance has the same ultraviolet absorption spectrum as *S*-adenosylmethionine, and it would be difficult to distinguish the two compounds in ultraviolet microscopy. Chromatography of perchloric acid extracts of the yeast used in this study did not reveal more than 1 μmole of *S*-adenosylhomocysteine per gram of cells; therefore, it does not interfere in the experiments reported here.

S-Adenosylmethionine and 5′-methylthioadenosine, used as supplements, were produced by incubation of commercial, dried *Saccharomyces cerevisiae* in large quantities of the medium specified above with a supplement of 5 μmoles per ml of L-methionine (Schlenk *et al.* 1959). *S*-Adenosyl-L-homocysteine was synthesized from adenosine and L-homocysteine by liver enzyme (de la Haba and Cantoni, 1959). These compounds were used in highly purified form.

Microscopy with the UV91 color-translating ultraviolet microscope was the same as described earlier (Svihla and Schlenk, 1959) with the following exceptions: A 10× Bausch and Lomb ocular replaced the 6× ocular. The wave lengths used were changed from 260, 350, and 280 mμ to 275, 350, and 265 mμ. This does not change the contrast of the resulting picture noticeably but does simplify operation. Energy from the A-H6 lamp is greater at 265 mμ than at 260 mμ, and

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by lowering the operating voltage from 600 v to about 550 v the line emission at 265 mμ is increased as observed on the photocell exposure control meter. Utilization of the 265 mμ line made wave length calibration checks easy and reduced exposure time. The 275 mμ wave length gave approximately the same energy as the 265 mμ line as shown by the control meter. Figure 1 illustrates the results obtained at the different wave lengths with control material and enriched cells.

A Radio Corporation of America image converter tube no. 7404 in a suitable case, used in conjunction with the microscope, permitted direct observation of the living cells under illumination with any wave length of light produced by the lamp, passed by the microscope, and received by the tube. The useful range was 235 to 620 mμ.

RESULTS

Stability of S-adenosylmethionine in the vacuole. Earlier experiments (Svihla and Schlenk, 1959) have shown that *S*-adenosylmethionine is formed rapidly, and that its concentration remains almost unchanged after the maximum level has been attained. It is not utilized after the nutrients of the culture medium are exhausted, despite starvation of the cells by continued aeration. Further information on the persistence of *S*-adenosylmethionine was desired as a prerequisite to ultraviolet optical studies. For this purpose, cells containing *S*-adenosylmethionine were transferred into nitrogen-free medium and aerated at 30 C by agitation in the presence of glucose (table 1). An increase in the amount of yeast was observed which corresponded to about the maximum expected if all *S*-adenosylmethionine in the vacuoles had been used as a source of nitrogen. However, extraction of the cells, chromatography, and spectrophotometric analysis showed that the sulfonium compound was nearly untouched. Nitrogen reserves other than *S*-adenosylmethionine must have been used for the limited growth of the culture.

The possibility remained that the utilization of *S*-adenosylmethionine is linked with growth and metabolism of a type or intensity not occurring under the conditions of the experiment described in table 1. To test this possibility, cells containing *S*-adenosylmethionine were transferred into medium containing ammonium salt and glucose. Table 2 shows that growth of the *S*-adenosylmethionine-containing cells resembles that of control cells. The total amount of sulfonium compound, although declining per unit of weight, stays rather constant as calculated for the entire culture. By repeated transfer into medium devoid of organic sulfur supplement, the low level of endogenous *S*-adenosylmethionine content of the control cultures is approached eventually. It is apparent from these experiments that the sulfur adenine compound is catabolized very slowly. This observation offered a basis for further ulraviolet optical studies and for extended biochemical research.

Optical experiments. The persistence of *S*-adenosylmethionine permits optical studies of its distribution in growing cultures. Obviously, there are the following alternatives: the mother cells may retain all of the sulfonium compound and produce buds, leading to daughter cells whose vacuoles do not contain any of the substance and are translucent to ultraviolet light at 265 mμ. Mother and daugher cells should be easily distinguished on this basis. On the other hand, *S*-adenosylmethionine may be shared by the daughter cells. This would result in a cell population with diminished but rather uniform ultraviolet density of the vacuoles.

These alternatives were put to a test by ultraviolet photomicrography of consecutive samples from a culture of cells that contained *S*-adenosylmethionine. The experimental conditions corresponded closely to those listed in table 2. The cells were transferred for continued growth into medium which contained ammonium salt, but no organic sulfur supplement. Thus, further synthesis of *S*-adenosylmethionine remained at the low endogenous level, and the ultraviolet-absorbing material visualized in figure 2, *A* to *C*, was largely derived from the initial enriched parent cells. Each row, *A* to *C*, represents selected typical cells from one sample of the culture. The material was withdrawn from the culture flask at time intervals that, as closely as possible, provided for doubling the cells. For comparison, row *D* shows corresponding material from a control culture. It is apparent from figure 2, *A* to *C*, that the mother cells share the vacuolar *S*-adenosylmethionine with the daughter cells. As the cells multiply, the concentration of the compound is gradually reduced to a level at which distinction from control cultures is no longer possible by our present technique.

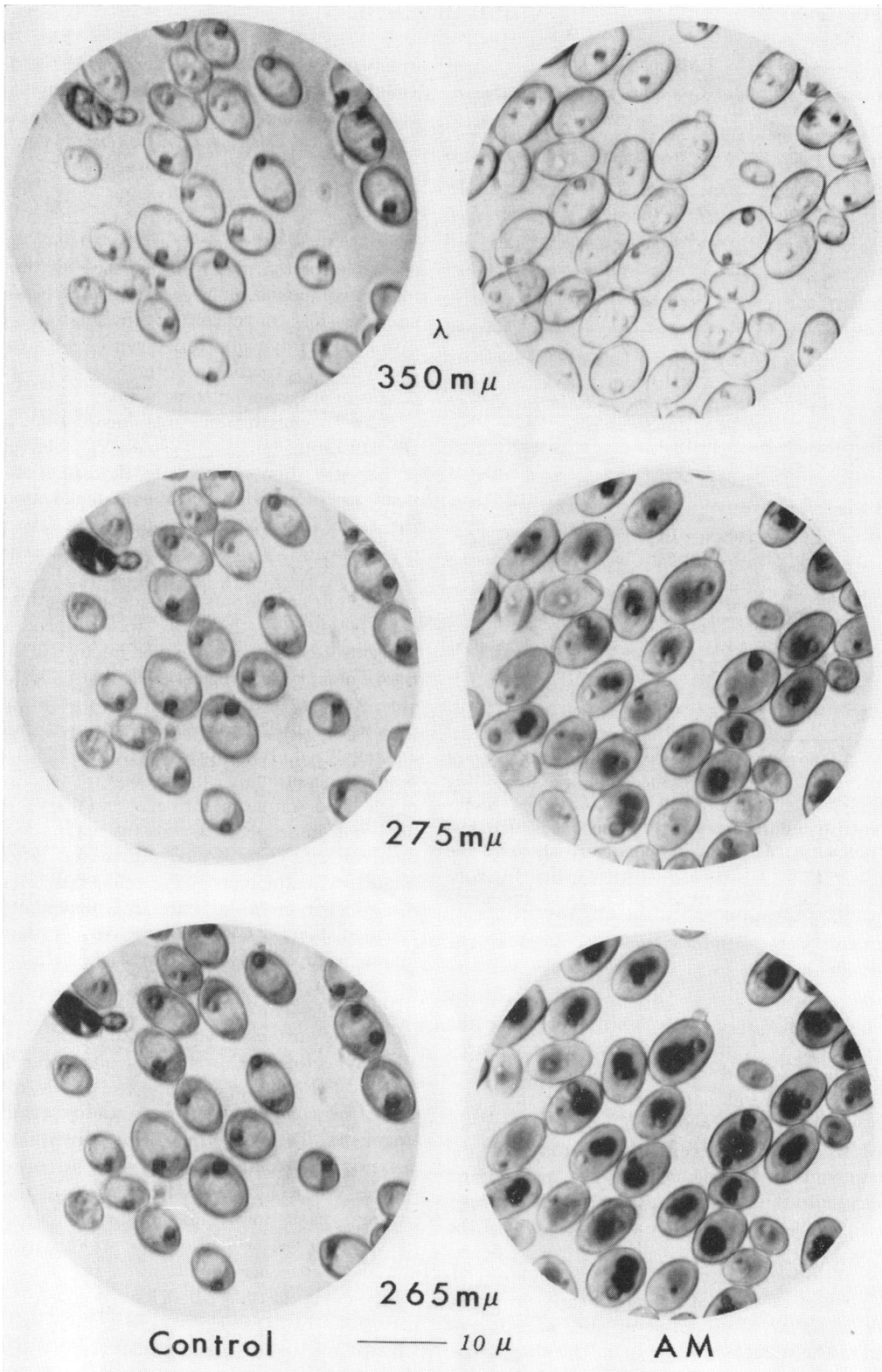

Figure 1. Candida utilis, photographed with different wave lengths of ultraviolet light. *Left side*, control cells; *right side*, cells containing *S*-adenosylmethionine.

TABLE 1

Growth limitation of Candida utilis in the absence of available nitrogen

Time of Culture	Yeast Present in Medium	Total *S*-Adenosylmethionine
hr	*g/100 ml*	*μmoles*
0	0.40	23.0
4.5	0.44	20.2
7.0	0.58	21.2
21.5	0.56	19.1

TABLE 2

Fate of intravacuolar S-adenosylmethionine during growth of Candida utilis

Time of Culture	*S*-Adenosylmethionine-containing Cells			Control Cells	
	Amt of cells in culture medium	Conc of *S*-Adenosylmethionine		Amt of cells in culture medium	Conc of *S*-adenosylmethionine
hr	*g/100 ml*	*μmoles/g yeast*	*total/100 ml culture*	*g/100 ml*	*μmoles/g yeast*
0	0.10	83.0	8.3	0.10	2.1 ± 0.8
3	0.22	43.8	8.7	0.21	2.1 ± 0.8
5	0.41	19.4	7.9	0.41	2.1 ± 0.8
12	0.60	9.6	5.8	0.58	2.1 ± 0.8

S-Adenosylmethionine-containing cells were cultivated in the presence of organic sulfur supplement; control cells were obtained under identical conditions except for the omission of the supplement. After 24 hr of culture at 30 C, the cells were centrifuged and resuspended for continued growth without organic sulfur supplement in medium containing ammonium salt and glucose. Samples were withdrawn and analyzed at the specified times.

This result raises questions concerning the mechanism of transfer of the vacuolar material into the bud and daughter cells. Again, two possibilities are at hand: the vacuole may produce a temporary extension into the bud for partial transfer of its contents. Alternatively, the compounds of the vacuole may be released and spread into the cytoplasm at the time of budding. After transfer into the bud and separation of the latter from the mother cell, they would be reassembled in a new vacuole of the daughter cell.

Our best pictures of a selected series from one culture (figure 3) do not show signs of a protrusion of the vacuole into the bud. The alternative, release of the material from the vacuole into the cytoplasm, finds occasional support in the ultraviolet photographs of some cells taken at the onset of budding.

Biochemical experiments. The observation that *S*-adenosylmethionine in the vacuole of *C. utilis* persists through several divisions raises further questions. It would be important to find out whether the confinement in the vacuole is responsible for the metabolic inertness of the sulfonium compound, or whether *C. utilis* lacks the enzymes for conversion of the material into nitrogenous fragments which can be assimilated. Biochemical experiments were carried out to obtain information on these points.

S-Adenosylmethionine was incorporated into the culture medium as the sole exogenous source of nitrogen. For comparison, the same supplement was provided in a medium which contained the usual amount of ammonium salt. The concentration of the latter in the medium was 30 μmoles of NH_4^+ per ml. Assuming that all of the six nitrogen atoms of *S*-adenosylmethionine may be utilized, the latter was supplied in a concentration of 5 μmoles per ml to match the total amount of nitrogen which is usually available. After a culture period of 26 hr at 30 C, the cells were centrifuged, washed twice with water, and analyzed. It is apparent from table 3 that *S*-adenosylmethionine in the culture medium is not a good source of nitrogen, but its presence does not interfere with the utilization of ammonium salt. There is moderate accumulation of *S*-adenosylmethionine in the cells; this is somewhat higher in the absence of ammonium salt. In all instances, 60 to 80 per cent of the sulfonium compound was found to be left in the medium after the culture period.

It would seem possible that the cell wall and membrane are obstacles to the uptake of *S*-adenosylmethionine from the culture medium, or that the cellular enzymes are unable to degrade the compound to more readily available fragments. To clarify this point, the ability of the principal components to serve as nitrogen sources was tested (table 4). Analysis of the resulting yeast for *S*-adenosylmethionine was included to see which of the structural units stimulates the accumulation of the sulfonium compound in the cells. There have been earlier experiments by DiCarlo *et al.* (1951) and by Chantrenne and Devreux (1956) on the utilization of adenine and some of its derivatives.

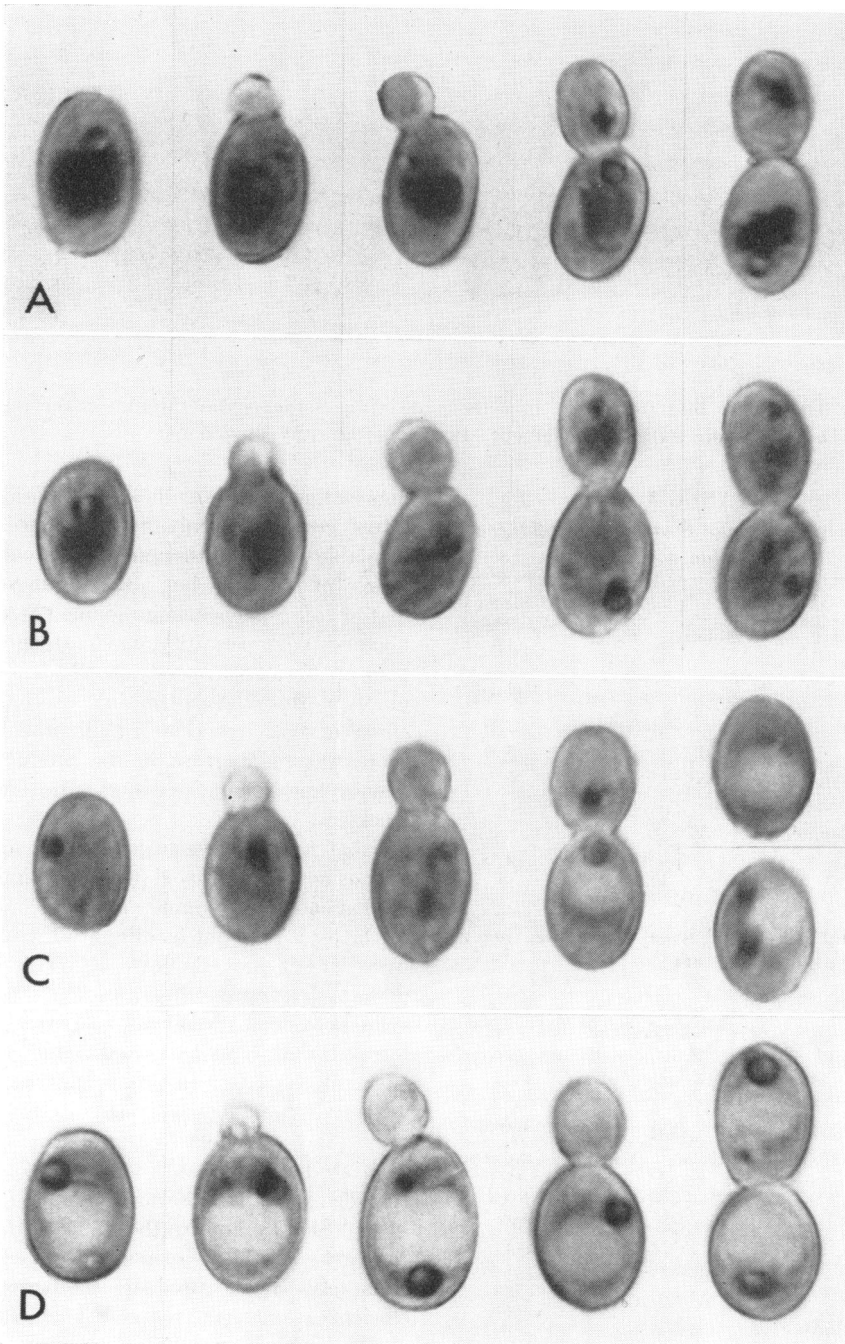

Figure 2. Distribution of *S*-adenosylmethionine in growing cells of *Candida utilis*, photographed at 265 mμ. The experimental conditions are explained in the text.

Schultz and Pomper (1948) have examined methionine as a nitrogen source for *C. utilis*. Our results are in agreement; differences in the degree of response may be explained by the experimental conditions. In our experiments, adenine and adenosine as well as ammonium salt were used as nitrogen sources, while 5′-methylthioadenosine, L methionine, and DL-

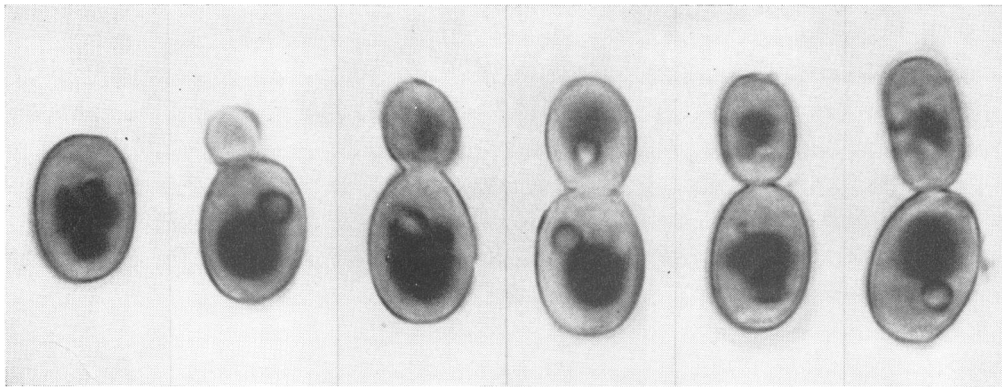

Figure 3. Details of bud formation in cells containing *S*-adenosylmethionine after transfer into medium without organic sulfur supplement. Photomicrography at 265 mμ.

TABLE 3

Utilization of exogenous S-adenosylmethionine by Candida utilis

Nitrogen Supplement in Medium	Conc in Medium	Yield of Cells	*S*-Adenosylmethionine Extracted from Cells
	μmoles/ml	*g/100 ml*	*μmoles/g*
None		0.20	2.5
NH_4^+	30	0.86	6.0
S-Adenosylmethionine	5	0.37	30.5
S-Adenosylmethionine + NH_4^+	5 30	0.98	12.0

The initial amount of yeast was 0.15 g; the culture period was 26 hr at 30 C.

TABLE 4

Fragments of S-adenosylmethionine as nitrogen sources for Candida utilis

Nitrogen Source	Conc in Medium	Yield of Yeast	*S*-Adenosylmethionine Content of Yeast
	μmoles/ml	*g/100 ml*	*μmoles/g*
None		0.17	<3
Adenine	6	0.62	<3
Adenosine	6	0.66	<3
5′-Methylthioadenosine	6	0.34	5.6
L-Methionine	30	0.39	53.0
S-Adenosyl-L-homocysteine	5	0.45	36.0
DL-Homoserine	30	0.49	<3
NH_4^+	30	0.67	<3

The initial amount of yeast was 0.15 g; the culture period was 40 hr at 30 C.

homoserine were significantly inferior. Of the latter compound only the racemic form was available to us. Methionine not only served as a source of nitrogen, but also stimulated the formation of *S*-adenosylmethionine. *S*-Adenosyl-L-homocysteine permitted moderate growth, and part of it was converted into *S*-adenosylmethionine. 5′-Methylthioadenosine exerted a weak effect in both respects. In all other instances, there was no increase in the production of *S*-adenosylmethionine beyond the endogenous level.

From the effective utilization of adenine and adenosine as contrasted with the limited effect of *S*-adenosylmethionine one may conclude that the latter is not split readily into these fragments by yeast enzymes. Removal of the methyl group, perhaps by transmethylation, leads to *S*-adenosylhomocysteine which is a better source of nitrogen than *S*-adenosylmethionine. The limited effect of the latter in promoting the growth of yeast cells may be explained in this way. The only other enzymatic degradation which has been studied in some detail leads to 5′-methylthioadenosine, homoserine lactone, and homoserine (Shapiro and Mather, 1958; Mudd, 1959). In yeast cells this is a slow reaction and, as seen from table 4, the products themselves are not efficient as nitrogen sources. The irreversibility of the biosynthesis of the sulfonium compound from adenosine triphosphate and methionine had been noted before (Mudd and Cantoni, 1958).

DISCUSSION

The limited ability of the enzymes of *C. utilis* to degrade *S*-adenosylmethionine may account

in part for the persistence of this compound in the cells. In addition, storage in the vacuole may be suspected of being responsible for the slow catabolism. However, the present experiments show that this confinement is not the main reason, because an extracellular supply of the sulfonium compound likewise is not utilized to any appreciable extent. Regardless of the reasons, it is clear that *S*-adenosylmethionine, formed in excessive amounts in the presence of methionine, is of little avail to the yeast cell as a source of nitrogen. To the biochemist, overproduction and accumulation open an easy preparative route to this still scarce substance. From a cytological viewpoint, special interest is stimulated by the accumulation and permanence of the compound in the vacuole. Presumably, the present experiments constitute the first instance of demonstrating a well-defined chemical substance in the vacuole without fixing, staining, or other preparatory manipulation of the cells. Thanks to the slow catabolism of the compound, its whereabouts can be followed through two or three divisions of the cells. The present ultraviolet optical experiments furnish proof that *S*-adenosylmethionine is transferred into the bud and concentrated in a new vacuole which becomes visible even before the bud parts from the mother cell.

Despite obvious advantages of *S*-adenosylmethionine as an "optical tracer substance," it has not yet been possible to explain the mechanism of this transfer. Yuasa and Lindegren (5959) state: "The first step (in budding) is the formation of a long tube leading from the vacuole to the periphery of the cell." Our present technique has not permitted observation of a connecting structure between the vacuoles of the mother and daughter cells, but such detail may be beyond the resolution of our system. Nor have we been able to confirm the other extreme, reported, for example, by Narayana (1956), that on transfer to fresh medium the vacuoles disappear temporarily with the onset of budding. Observation of a great many cells by ultraviolet photomicrography in the course of our work has shown the vacuole to be present constantly in *C. utilis*. It varies somewhat in shape and size; occasionally it is lobed. A few cells have been seen in an early stage of budding which appeared to lack a vacuole and the cytoplasm showed somewhat greater ultraviolet absorption than that of other cells in the field. However, most cells in the same stage of budding show a well-defined vacuole. Abolition of the vacuole would have to be a transitory process of very short duration as judged by the infrequency of cells in which the ultraviolet density appears to be continuous. It is possible and probable, however, that at this stage of cellular development part of the vacuolar *S*-adenosylmethionine is disseminated into the cytoplasm and thence finds its way into the bud. This process would involve great dilution. An approximate calculation, considering for simplicity both the cell and the vacuole as spherical bodies, with a diameter of 6 μ and 2 μ, respectively, shows that uniform distribution of the material from the vacuole into all parts of the cell would result in 27-fold dilution. Even with a much larger vacuole, the dilution would be such that the material could barely be detected by our technique. Furthermore, it appears from our ultraviolet photomicrographs that a concentration gradient between the vacuole and its surroundings is nearly always maintained. It is not surprising, therefore, that the transfer into the bud is not visible.

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SUMMARY

S-Adenosylmethionine can be accumulated in the vacuoles of *Candida utilis* by culture in a simple medium supplemented with L-methionine and related sulfur amino acids. Continued culture in the absence of sulfur supplement shows that *S*-adenosylmethionine in the vacuole is not readily catabolized. Ultraviolet photomicrography reveals that part of the material is transferred from the vacuoles of the mother cells into the vacuoles of the buds and daughter cells. The possible mechanism of this transfer and some properties of the vacuole are discussed.

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