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# Uptake of Cystine by the Yeast Phase of *Histoplasma capsulatum*

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This report deals with factors affecting the uptake of cystine by the yeast phase of *Histoplasma capsulatum*. The kinetics of uptake showed a saturation at 70 $\mu$M and an average $K_m$ value of $3 \times 10^{-5}$ M. The optimal *p*H and temperature for transport of cystine were 6.5 and 37 C, respectively. The energy of activation was 14.1 kcal/mole, and the temperature coefficient value was 2.1. A requirement for energy supplied by metabolic activity was demonstrated by the inhibition of incorporation of the amino acid by cells preincubated with either 2,4-dinitrophenol or sodium azide. Although uptake was not inhibited by any single amino acid, a combination of amino acids did cause a decrease in uptake. Thus, the data show that the uptake of cystine by yeast cells of *H. capsulatum* has the characteristics of a system of transport that requires the expenditure of energy by the cells.

*Histoplasma capsulatum* exists in two morphological forms: a parasitic form consisting of blastospores and a saprophytic form consisting of a mycelium upon which are borne both micro- and macroconidia. Both morphological forms may be cultured in the laboratory under the proper environmental conditions (4). This dimorphic behavior is primarily controlled by the temperature of incubation, but certain nutritional factors are also known to play a role. Among such factors, the sulfur-containing amino acids seem to occupy a place of central importance.

In 1949, Salvin (11) studied the effect of cysteine and related compounds on the growth of yeast cells in a liquid medium at 37 C. With a basal medium of glucose, salts, and vitamins, he showed that only with the addition of cysteine or cystine as the sole amino acid would the cells remain yeastlike and not germinate. From this work, he concluded that a reduced sulfur group in the form of a small, organic molecule (preferably an amino acid) was necessary for the establishment of the yeast phase of growth by the fungus.

Pine later showed that —SH groups must be present in a growth medium to initiate blastospore growth at 37 C (8). That the requirement for cysteine was not for —SH groups alone was supported by the fact that the cysteine could not be replaced with glutathione (8). Still later Scherr (12) studied the mycelium to yeast phase conversion of *H. capsulatum* and concluded that the concentration of —SH groups (or cysteine) was a more critical factor in maintenance of yeast cell growth than a temperature of 37 C. More recent work by McVeigh and Morton (7), using a synthetic basal growth medium incubated at 37 C, showed that of the two strains tested each grew equally well as blastospores with the addition of cystine or cysteine, less well with the addition of methionine, and very poorly with sodium thioglycolate or glutathione.

Thus, the importance of cystine or cysteine in initiation and maintenance of yeast-phase growth of *H. capsulatum* has been repeatedly emphasized. Factors which affect the uptake of cystine by *H. capsalatum* were studied by observing the transport of radioactive amino acid into yeast cells of the fungus. The results of these studies are presented in this report.

## MATERIALS AND METHODS

**Fungus.** The yeast phase of the fungus *H. capsulatum* was used in these experiments. This isolate was originally obtained by Charlotte C. Campbell from a clinical case of histoplasmosis and was designated by her as no. 6624. This isolate has been used for several years in our laboratory (strain no. 505). Stock cultures of the yeast phase of the fungus were stored at 4 C on blood-glucose-cysteine-agar slants (4) and were transferred monthly.

**Growth and preparation of the fungal cells.** A loopful of cells from the refrigerated stock cultures was inoculated into 100 ml of Salvin's medium (11) which was incubated at 37 C for 48 to 72 hr on a gyratory shaker (New Brunswick Scientific Co., New Brunswick, N.J.). At the end of the incubation period, the cell density [optical density $(OD)_{660nm}$] of this "starter culture" was determined. A flask of 250 ml of Salvin's medium contained in a 500-ml boiling flask was inoculated with $1.25 \times 10^9$ cells to give a final concentration of $5 \times 10^6$ yeast cells/ml of medium. The flasks were incubated for 72 hr at 37 C on the gyratory shaker. At the time of harvest, these cells were in the late exponential phase of growth. The yeast cells were collected by centrifugation at 1,100 × *g* for 2 to 5 min, washed three times with distilled water, and resuspended in a buffer of low salt concentration (STM). The STM contained the following ingredients: 0.01 M KCl; 0.0024 M $CaCl_2$. 0.0025 M $MgCl_2$; and 0.05 M tris(hydroxymethyl)-aminomethane (Tris) base. This solution was titrated to *p*H 7.4 at 37 C with 6 N HCl.

A dry weight versus OD standard was obtained by filtering 10 ml of a distilled water suspension of yeast cells of a known OD through a membrane filter (Millipore Corp., Bedford, Mass.). The filter pad (HA, 0.45 μm) was placed in a hot-air oven overnight at 100 C and weighed. The concentration of cells used in all of the experiments was 1 mg (dry weight)/ml of pulsing medium (STM).

**Isotope labeling.** The concentration of $^{14}$C-cystine varied with the individual experiment depending on the specific activity required. In all cases, additional unlabeled cystine was added to obtain the same concentration of amino acid. The unlabeled L-cystine was purchased from Calbiochem (Los Angeles, Calif.), and the amount of D-cystine present was calculated from the optical rotation data. The cystine was found to be 93.2% pure L form.

D,L-Cystine-*3-$^{14}$C* was obtained from two sources: Nuclear-Chicago, with a specific activity of 39.5 mc/mmole, and Schwarz BioResearch, Inc., with a specific activity of 17.0 mc/mmole.

The method for pulsing the yeast cells was as follows. A 30-mg (dry weight) amount of cells was suspended in 20 ml of STM (*p*H 7.3) in a 125-ml flask. The suspensions were equilibrated to 37 C (unless otherwise stated). Labeled cystine in 10 ml of STM was rapidly added to the 125-ml flask, and the flask was shaken throughout the experiment. At the appropriate time, a 1-ml sample was removed with a Cornwall pipette. The radioactivity was determined by filtering the samples on a filter pad (Millipore Corp.), washing the pad with 15 ml of cold distilled water, placing the pad which contained the yeast cells into 10 ml of scintillation fluid, and counting in a liquid scintillation counter (Nuclear-Chicago, model Mark I). The scintillation fluid contained the following chemicals, per liter of *p*-dioxane: naphthalene, 70 g; 2,5-diphenyloxazole, 7 g; and 1,4-bis-2-(5-phenyloxazolyl)-benzene, 0.05 g. The vials were then counted for 10 min or at least 5,000 counts/min, and the values obtained were corrected for background.

## RESULTS

**Uptake as a function of substrate concentration.** The total uptake of cystine was directly proportional to the initial extracellular concentration (Fig. 1). As the concentration of cystine increased, there was an increase in the initial velocity of uptake up to a concentration of 80 μM. A plot of the initial velocity after 1 hr of incorporation as a function of the substrate concentration showed that saturation occurred at a concentration of 70 μM (Fig. 2). A Lineweaver-Burk plot (6) was constructed, and the average value for the concentration of cystine per milligram (dry weight) of cells giving half-maximal velocity $(K_m)$ was $3 \times 10^{-5}$ M (Fig. 2).

**Effect of pH.** To determine the influence of hydrogen ion concentration on cystine uptake, cells were suspended in one of the following buffers: 0.1 M sodium acetate-acetic acid at *p*H 4.6 and 5.5; 0.05 M Tris-hydrochloride at *p*H 6.5, 7.3, 7.8, and 8.6. The dependency of uptake on the *p*H is demonstrated in Fig. 3. The optimal *p*H was 6.5, with a 17% decrease in uptake at *p*H 7.3 and 7.8. A decrease in uptake

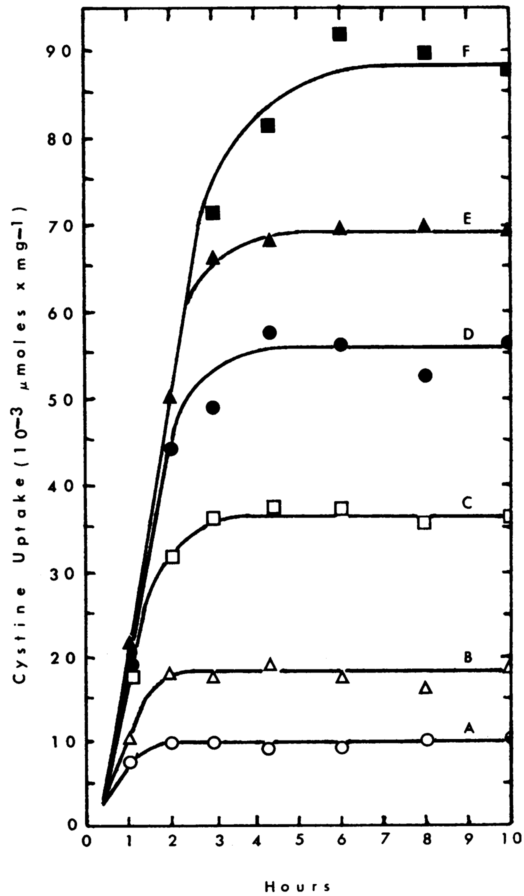

FIG. 1. *Effect of external substrate concentration on uptake of cystine. Concentration of cystine* (μM)*: A, 10; B, 20; C, 40; D, 60; E, 80; F, 100.*

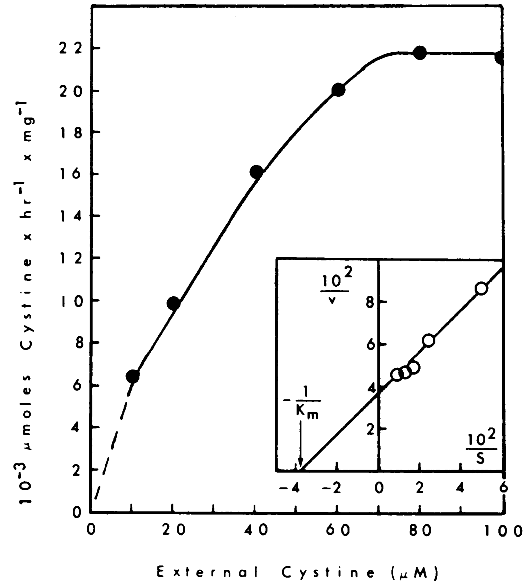

FIG. 2. *Effect of external cystine concentration on the rate of cystine accumulation at 37 C. Inset shows data as a Lineweaver-Burk plot. S, cystine expressed as micromolar concentration; v, rate of cystine uptake, $10^{-3}$ μmoles per hr per mg.*

of about 78% was observed at *p*H 5.5 and 8.6.

**Effect of temperature.** The influence of temperature on the uptake of cystine was examined. A cell suspension was allowed to equilibrate for 10 min at each of the following temperatures: 22.5, 30, 37, and 45 C. The labeled cystine was then added. Figure 4 shows that the optimal temperature for uptake was 37 C, whereas there were 40 and 70% decreases in uptake at 45 and 22.5 C, respectively. Since there was a linear response between uptake and temperature between 22.5 and 37 C, an energy of activation ($E_a$) was calculated from the equation of Crockford and Knight (2). The $E_a$ was 14.1 kcal/mole, with the values at 22.5 and 37 C.

The temperature coefficient, $Q_{10}$, which expresses the ratio of the velocity of a reaction at a temperature ($t + 10$ degrees) to that at temperature ($t$) was also calculated. The $Q_{10}$ value for the temperature span of 25 to 35 C was 2.1.

**Energy requirements.** The requirement of energy for cystine uptake by the cells of *H. capsulatum* was examined by use of 2,4-dinitrophenol (2,4-DNP) and sodium azide. The data in Table 1 show that, at saturating concentrations of cystine, there was 50 and 86% inhibition of uptake of the amino acid with 2,4-DNP at concentrations of $10^{-3}$ and $10^{-2}$ M, respectively. Furthermore, Table 1 shows that sodium azide was more effective as an inhibitor in that there was 35, 83, and 90% inhibition of uptake at concentrations of $10^{-4}$, $10^{-3}$, and $10^{-2}$ M, respectively.

**Effect of other amino acids.** The influence of other amino acids on the uptake of cystine was tested at concentrations of $10^{-3}$ and $10^{-4}$ M. Although Table 2 shows only the results at a $10^{-4}$ M concentration of amino acids, the values at the 10-fold higher concentrations ($10^{-3}$ M) were equivalent. These data show that individually none of the amino acids inhibited cystine uptake to any significant degree. However, if a combination of amino acids such as that found in Salvin's medium was used, there was an inhibition of cystine uptake. Salvin's medium without cysteine and diluted to 1:10 (SMD) was adjusted to 80 μM L-cystine. When this diluted medium was used as the pulsing medium, there was a 35% decrease in the transport of the label (Table 2). Thus, a combination of several amino

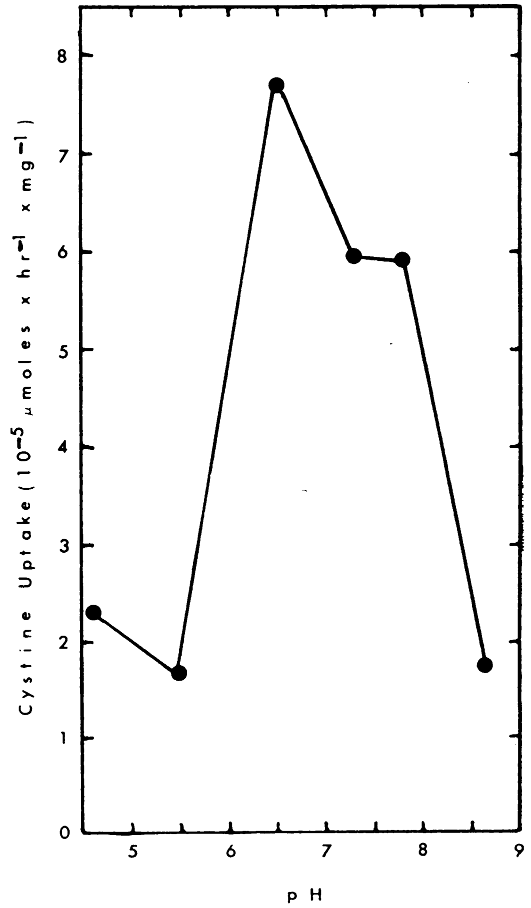

FIG. 3. *Effect of pH on the uptake of cystine at 37 C.*

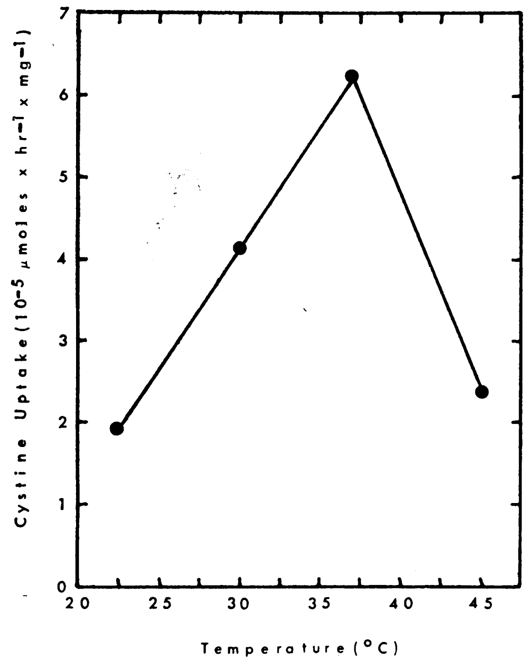

FIG. 4. *Effect of temperature on the uptake of cystine at pH 7.3.*

TABLE 1. *Effect of energy uncouplers on uptake of cystine in STM*[a]

| Concn of inhibitor | Uptake (nanomoles per hr per mg of cells) | Per cent of control |
|---|---|---|
| M | | |
| 2,4-Dinitrophenol | | |
| 0 | 23.8 | 100 |
| $10^{-5}$ | 21.6 | 91 |
| $10^{-4}$ | 23.6 | 99 |
| $10^{-3}$ | 11.8 | 50 |
| $10^{-2}$ | 3.4 | 14 |
| Sodium azide | | |
| 0 | 17.8 | 100 |
| $10^{-5}$ | 16.5 | 93 |
| $10^{-4}$ | 11.5 | 65 |
| $10^{-3}$ | 3.1 | 17 |
| $10^{-2}$ | 1.8 | 10 |

[a] Concentration of cystine, 80 μM. STM contains 0.01 M KCl, 0.0024 M $CaCl_2$, 0.0025 M $MgCl_2$, and 0.05 M Tris.

TABLE 2. *Effect of various compounds on the uptake of cystine*

| Compound added | Uptake nanomoles per hr per mg) | Per cent of control[a] |
|---|---|---|
| Leucine[b] | 17.8 | 116 |
| Isoleucine | 17.4 | 113 |
| Histidine | 14.2 | 92 |
| Arginine | 16.2 | 105 |
| Lysine | 16.1 | 104 |
| Glycine | 16.7 | 108 |
| Serine | 13.2 | 86 |
| Threonine | 16.9 | 110 |
| Aspartic acid | 13.9 | 90 |
| Phenylalanine | 13.4 | 87 |
| Tyrosine | 17.1 | 111 |
| Methionine | 14.7 | 95 |
| Alanine | 14.8 | 96 |
| Valine | 16.6 | 108 |
| SMD[c] | 10.0 | 65 |
| Yeast extract (0.3%, w/v) | 10.5 | 68 |
| Yeast extract (0.03%, w/v) | 13.8 | 90 |

[a] Control: uptake of cystine (80 μM), 15.4 nmoles per hr per mg.

[b] Amino acids, $10^{-4}$ M, L form.

[c] Salvin's medium minus cysteine and diluted 1:10. Cystine concentration, 80 μM.

acids did modify cystine uptake. Since the concentration of amino acids in yeast extract (a component of Salvin's medium) was unknown, the extract was tested to determine whether it was the ingredient which was responsible for the inhibitory effect of SMD. As seen in Table 2, although yeast extract could cause a 32% decrease in uptake, when diluted 1:10 (the concentration in SMD) there was only a 10% reduction in cystine uptake. Thus, the amino acid content of yeast extract could only partially account for the inhibitory effect of SMD on cystine uptake.

## DISCUSSION

The role of cysteine or cystine in initiation and maintenance of the yeast phase of growth of *H. capsulatum* has been of interest to many researchers. Although Scherr (12) suggested that these amino acids may act by lowering the oxidation-reduction (O-R) potential of the medium, it appears that a nutritional role may also be involved, because other compounds which are capable of lowering the O-R potential (glutathione or thioglycolate) are not effective in maintaining yeast-phase growth (7, 11). Nevertheless, more recent work has reemphasized the critical role of the O-R potential of a medium in the yeast-mold dimorphism of *H. capsulatum* (10). To examine the utilization of cystine as a nutrient by *H. capsulatum*, we decided to study the characteristics of cystine transport. The distribution of the labeled cystine within cells of *H. capsula*-

*tum* is the subject of a second report which is in preparation.

The present work shows that *H. capsulatum* possesses a transport system for cystine which exhibits enzyme-like properties. Thus, as the extracellular concentration of cystine increased, there was an increase in the initial velocity of incorporation until a point of saturation was reached. This saturation phenomenon indicated that the number of available sites at the cell surface capable of binding with the cystine was limited. Such limitation is characteristic of an enzyme-mediated reaction (14). The $K_m$ was similar to those values calculated for valine uptake by *Arthrobotrys conoides* (*5a*) and for tryptophan uptake by *Neurospora crassa* (15).

The *p*H of the medium was important for the maximum rate of cystine uptake. The optimal *p*H of 6.5 was lower than that (7.4) found optimal for growth of the yeast cells (J. P. Garcia, Ph.D. Thesis, Univ. of California, Los Angeles, 1968). This disparity may indicate that at the higher *p*H an optimal equilibrium between the transport of all of the necessary amino acids, glucose, and other compounds is reached. However, even at this higher *p*H, there was still 75% of the optimal uptake of cystine. Whether the hydrogen ion concentration was affecting the charge on the cystine, the membrane proteins, or both, is unknown. At *p*H 6.5, most of the cystine molecules have a net positive charge (1).

The requirement for energy or metabolic activity to transport cystine across the membrane, i.e., active transport, was indicated by the calculated value for the activation energy and temperature coefficient and by the inhibition of uptake by 2,4-DNP and sodium azide. Since free diffusion and other physical-chemical phenomena have energy of activation and $Q_{10}$ values on the order of only 1,000 cal/mole and 1.5, respectively (13), it appears that the cystine uptake system of *H. capsulatum* has catalytic or enzyme-like properties. The need for metabolic activity was demonstrated by the inhibitory effects of 2,4-DNP and sodium azide, both of which are known to deprive cells of metabolic energy.

As opposed to the report of Gupta and Pramer (*5a*) which showed that most of the amino acids competitively inhibited L-valine uptake in *A. conoides*, the uptake of cystine was not inhibited by any of the amino acids tested. Furthermore, several workers have reported specific transport systems for uptake of a family group of amino acids (3, 5, 9, 14) and that competition is greatest within each family. Since cystine is a sulfur-containing amino acid and is a dimer as compared to the other amino acids, one might expect a specific site for its transport. The results reported here support this conclusion, since no inhibition of cystine uptake by other amino acids was observed when present individually. This site might be specific for the disulfide form of the amino acid or might be associated with a "reductase" which would allow the sulfhydryl form to cross the membrane. In either case, the other amino acids tested would not be expected to interact with such a site.

Although the free amino acid pool has been shown to be large and expandable, it does have a definite size limit (3). This could have been a reason for decreased uptake of cystine in the presence of a mixture of amino acids. It has been shown that, in the presence of all of the amino acids tested, there was an increase in the cold trichloroacetic acid-insoluble material (high-molecular-weight molecules) as compared with the soluble material (low-molecular-weight molecules), whereas the total uptake of cystine decreased (B. E. Gilbert, *unpublished data*).

This report has dealt with the initial uptake of cystine. It has been shown that the amino acid did enter the cell, that it did so by a catalytic-like reaction, and that metabolic activity was necessary for transport.

## ACKNOWLEDGMENTS

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