PERMEABILITY AND METABOLISM OF LACTOSE IN *NEUROSPORA CRASSA*

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ABSTRACT

LESTER, G. (Worcester Foundation for Experimental Biology, Shrewsbury, Mass.), D. AZZENA, AND O. HECHTER. Permeability and metabolism of lactose in *Neurospora crassa*. J. Bacteriol. **84**:217–227. 1962.—Germinated conidia of *Neurospora crassa* suspended in buffer take up lactose, and this uptake can be attributed to the intracellular accumulation and to the metabolism of lactose. The former process predominates initially, and the latter after a few hours of incubation. The accumulation of lactose appears to be mediated by a very specific transport system, which can bring about intracellular concentrations that greatly exceed the external concentration of lactose. Both the influx and efflux of lactose appear to be dependent on metabolic energy, since azide and low temperature inhibit these events. The steroid hormone 11-deoxycorticosterone also reduces the uptake and prevents the accumulation of lactose. The metabolism of lactose was clearly demonstrated, but the pathway(s) of metabolism was not defined. The amount of β-galactosidase activity extractable from the cells was insufficient to be solely responsible for the initial step in lactose metabolism. The present studies are discussed in relationship to ion permeability and lactose metabolism in *N. crassa*, and to galactoside permeability in *Escherichia coli*.

Previous studies (Lester and Hechter, 1958, 1959) on the permeability of monovalent cations in *Neurospora crassa* have indicated the operation of a diphasic system. One phase is a nonselective, energy-dependent entry process for sodium, potassium, or rubidium; the other phase is the specific accumulation of potassium or rubidium against a concentration gradient, and is inhibited by 11-deoxycorticosterone (DOC). The uniqueness of this system, implying nonspecificity in entry, intracellular binding of ions, and its regulation by a mammalian steroid hormone, made it of interest to determine whether other substances followed the same permeability pattern. As a contrast to the ions, it seemed desirable to examine the permeability of a nonelectrolyte. Lactose was chosen for the present study, since other work (Landman, 1951; Franklin, 1954) on the utilization of lactose by *N. crassa* indicated that it was metabolized slowly. This suggested the possibility of observing an intracellular accumulation of lactose before it was metabolized. Also, recent physiological and genetic studies on the permeability of galactosides in bacteria (Cohen and Monod, 1957) provide a background for the comparison of permeability processes in diverse microorganisms. In some respects the following work complements those with ions in *N. crassa* and galactosides in bacteria, but important differences have been observed, too. These studies also put to question the role of β-galactosidase in the metabolism of lactose by *N. crassa*.

MATERIALS AND METHODS

Organism. The St. Lawrence strain 74A of *N. crassa* was used. The cultural conditions for the production of conidia and their germination are described in detail elsewhere (Lester, 1961). Briefly, conidia were germinated in a salts medium with sucrose as the carbon source. The germinated conidia were washed with 0.02 M NaH_2PO_4 in 0.05 M NaCl, pH 5.8, and resuspended in the same buffer. Unless otherwise noted, this buffered-saline solution was used as the medium in the experiments to be described.

General protocol. Suspensions of germinated conidia containing lactose, and other additions, were incubated at 30 C with agitation in a reciprocating water bath; usually the initial volume was 20 to 35 ml, in a 125-ml Erlenmeyer flask. At

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intervals, measured samples (4 to 10 ml) were filtered through paper on small Hirsch funnels, with care taken to stop filtration before drying of the cell cake could occur. The dry weight of cells, determined with duplicate samples, usually ranged from 4 to 12 mg/ml. Uptake of lactose was estimated from the decrease in lactose content of the filtrates. The cell cake was weighed immediately, resuspended in water, and extracted by heating in a boiling-water bath for 3 min. The heated suspension was made up to a known volume (3 to 7 ml) and filtered. Intracellular lactose was calculated as the total lactose of the extract minus that estimated for the extracellular medium in the cell cake. Based on previous (Lester and Hechter, 1959) and repeated determinations, the intracellular water was estimated as 71% of the cell weight; thus, 1.0 mg dry weight represented 2.45 mg (or μliters) of intracellular water. The difference between the weight of the wet cell cake and the dry weight was taken as the total water, and the extracellular water of the cell cake was calculated as the difference between the estimated intracellular water and the total water. It was assumed that the concentration of lactose in the extracellular water of the cell cake was the same as in the medium.

Lactose assay. The basic procedure for this assay involved the hydrolysis of lactose by β-galactosidase and the determination of the glucose released with a glucose oxidase-peroxidase preparation.

Samples of medium containing 0.05 to 0.5 μmoles of lactose were made up to 1.5 ml with water, and 0.5 ml of purified β-galactosidase was added. [β-D-Galactosidase from *Escherichia coli* was purified according to the procedure of Lester (1952), with the additional step of precipitating the 50% ammonium sulfate fraction at 40% ammonium sulfate. The amount of enzyme used in the assay was equivalent to that required for the hydrolysis of 0.15 mmoles of *o*-nitrophenyl-β-D-galactoside per hr at 37 C (Rickenberg, Yanofsky, and Bonner, 1953); this was about three times that required for the complete hydrolysis of lactose under the conditions described. The β-D-galactosidase was made up in 0.2 M K_2HPO_4 (pH 7.0) containing 0.25 μmole of glutathione per ml.] After 1.0 hr of incubation at 37 C, 2.0 ml of water and 1.5 ml of Glucostat (Worthington Biochemical Corp.; reagent made up according to accompanying directions for the "micromethod") were added, and the reaction mixture was incubated for 1.0 hr at room temperature. The reaction was stopped and the color developed by adding 0.5 ml of 0.8 N HCl. The color was measured in a Klett-Summerson colorimeter with a 420-mμ filter. With these conditions, equimolar amounts of lactose and glucose gave the same readings.

Extracts from cells not exposed to lactose gave high values and interfered with the assay. Possibly, glucose-containing polysaccharides were present and were hydrolyzed by polysaccharidases in the Glucostat preparation. This interference was eliminated by prior incubation of the samples with the enzyme component of Glucostat. Samples of extracts were made up to 1.0 ml with water, and 0.5 ml of double-strength Glucostat enzyme (without chromogen) was added. After 3 hr of incubation at 37 C with agitation, the enzymes were inactivated by heating in a boiling-water bath for 5 min. Then the assay was carried out as described above for medium samples, except that a duplicate set of samples was assayed without β-galactosidase, to correct for the small interference sometimes remaining after prior treatment with Glucostat enzyme.

Alternatively, interfering materials in cell extracts were eliminated by chromotographing the extracts prior to assay; this procedure also served to identify the materials assayed as lactose. The cell extracts were desalted with a mixed ion exchange resin (Rohm and Haas, MB-3; 0.5 g/10 ml of extract), and then condensed to 0.50 ml by evaporation under a stream of warm nitrogen. Insoluble material was removed by filtration through paper. Measured samples (0.05 to 0.10 ml) were applied to Whatman no. 1 paper, and the chromatogram (ascending) was developed in isopropanol–pyridine–acetic acid–water (8:8:1:4; Gordon, Thornburg, and Werum, 1956) for about 10 hr at 25 C. The position of lactose in standards and sample duplicates was visualized with a *p*-anisidine reagent (Mukherjee and Srivastava, 1952). Unsprayed sample areas corresponding to the position of lactose were cut out and eluted with 0.05 M K_2HPO_4 (pH 7.0), and the eluates were assayed for lactose as described above for cell extracts (after prior treatment).

The uptake of lactose-1-C^{14} (National Bureau of Standards) was also examined. This compound

was purified to a constant specific activity by chromatography with the system described above, and with *n*-butanol–pyridine–water (45:25:40; Opienska-Blauth, Madecka-Borkowska, and Borkowski, 1952). Radioactivity was measured in a gas-flow counter; duplicate samples of 0.05 or 0.1 ml of medium or cell extract were dried on aluminum planchets and counted three times for a total of at least 6,000 counts.

Reconstruction experiments with cell extracts and known amounts of lactose indicated that these techniques were accurate to about ± 7%. The results obtained with and without chromatography showed good agreement.

Assay for o-nitrophenyl-β-D-galactoside (ONPG). In some experiments the uptake of ONPG was examined, using the procedures described in *General protocol.* Samples of medium or cell extract containing about 0.5 μmole of ONPG were made up to 4.0 ml with water, 1.0 ml of 1.2 N NaOH was added, and hydrolysis was achieved by heating in a boiling-water bath for 15 to 20 min. The *o*-nitrophenol (ONP) released was determined colorimetrically. To determine the extent of hydrolysis of ONPG by cells, 1.0 M K_2CO_3 was added instead of NaOH, and the color of ONP was measured, without heating.

RESULTS

Effect of cell concentration and time on lactose uptake. The rate of lactose uptake is directly proportional to the concentration of cells (Table 1) and the time of incubation (Table 2). The rate of uptake varied somewhat between different batches of germinated conidia, ranging from about 0.075 to 0.125 μmoles per mg dry wt per hr, but replicates of a single batch of cells gave very similar results. Table 2 also shows the β-galactosidase activity extractable from germinated conidia in terms of ONPG hydrolysis, which proceeds at only about 15% of the rate of lactose uptake. Since lactose has a much lower affinity for *Neurospora* β-galactosidase than ONPG (Landman and Bonner, 1952), it seems reasonable to assume that the larger part of lactose uptake observed cannot be attributed to its metabolism via β-galactosidase.

Uptake and recovery of lactose-1-C^{14}. Table 3 presents two experiments in which the disposition of the lactose taken up was examined. The disappearance of lactose and radioactivity from the medium correspond closely, as indicated by the similar specific activities at the beginning and end of the incubation. This does not preclude the possibility that volatile products of lactose metabolism are present in the medium and are evaporated during planchetting of medium samples.

About 35 to 40% of the lactose taken up is found in the cells, and, similarly, about 50 to 60% of the radioactivity is recovered, indicating that lactose is metabolized. The increased specific activity of extracts suggests that some of the product(s) remains in the cells, but that 40 to 50% of the radioactivity is associated with nonextractable, volatile, or gaseous products of lactose metabolism. In experiment 2, similar values for lactose were obtained whether or not

TABLE 1. *Effect of cell concentration on lactose uptake*

Dry wt cells	Lactose uptake[a]	
mg/ml	*μmoles/ml*	*μmoles/mg cells*
1.5	0.44	0.29
3.0	0.89	0.30
5.7	1.62	0.28

[a] The initial concentration of lactose was 5.0 μmoles/ml; the uptake values are for a 3.0-hr incubation period.

TABLE 2. *Time course of lactose uptake and hydrolytic activity*

Incubation time	Lactose uptake[a]		ONPG hydrolysis[b]
hr	*μmoles/ml*	*μmoles/mg cells*	*μmoles/mg cells*
1.5	1.45	0.18	0.027
3.0	2.85	0.36	0.053

[a] The initial concentration of lactose was 5.0 μmoles/ml; the cell concentration was 8.0 mg (dry wt) per ml.

[b] Washed germinated conidia were ground in a Ten Broeck mill, and the debris removed by centrifugation at 18,000 × *g* for 30 min. The extract contained protein (Lowry et al., 1951) equivalent to 24% of the dry wt of cells extracted. The β-galactosidase activity of the extract was assayed in 1.5 ml of 0.1 M phosphate buffer (pH 6) containing 7.5 μmoles of ONPG. The reaction was stopped and the color of the liberated *o*-nitrophenol was developed by the addition of 3.5 ml of 0.5 M K_2CO_3. The values are given in terms of the cell dry wt equivalent of the extracts used.

TABLE 3. *Uptake and recovery of lactose-1-C^{14} in cells*

Expt	Incubation time	Medium			Uptake		Intracellular[a]		
		Lactose	Radioactivity	Specific activity	Lactose	Radioactivity	Lactose	Radioactivity	Specific activity
	hr	*μmoles/ml*	*counts per min per ml*	*counts per min per μmole*	*μmoles/ml*	*counts per min per ml*	*μmoles*	*count/min*	*count per min per μmole*
1	0	10.8	29,380	2,710			—[b]	—	
	3	9.0	24,180	2,690	1.8	5,200	0.69[c]	2,720	3,940
2	0	4.91	25,300	5,160			—	—	
	3	2.81	14,700	5,250	2.1	10,500	0.84[c]	6,550	7,800
	3						0.89[d]	4,570	5,130

[a] Intracellular values are for the cells (8.0 mg dry wt) in 1.0 ml of suspension.
[b] Not determined.
[c] Lactose and radioactivity determined in crude extracts.
[d] Lactose and radioactivity determined after chromatography of extract.

the extracts were chromatographed. The values for radioactivity differed, but after chromatography the specific activity of the lactose assayed was the same as that originally used. These results indicate the validity of the lactose assay in crude extracts, and permit the assumption that the difference between lactose taken up and recovered from the cells represents the lactose metabolized.

In a third experiment, germinated conidia were incubated with a lower concentration of lactose-1-C^{14} to insure the metabolism of a large portion of the lactose taken up; the specific radioactivity of the lactose was also increased. During incubation, a stream of CO_2-free air was passed into the flask and then bubbled through a saturated solution of $Ba(OH)_2$. The precipitate formed was washed and its radioactivity determined. The cells were harvested and extracted with hot water for the usual determination of intracellular lactose and radioactivity. The extracted cells were washed several times with water, ground to a fine suspension, and their radioactivity determined. The radioactivity in the barium precipitates and the extracted cells was estimated by extrapolating the values obtained with samples of various sizes to one of infinite thinness. Over 75% of the radioactivity taken up was recovered, and the distribution of radioactivity indicates a variety of metabolic products. Of 1.82 μmoles of lactose disappearing from the medium, only 0.32 μmole was recovered from the cells (Table 4). However, the radioactivity found in the hot-water extracts gave a greater apparent specific activity for lactose than was originally introduced, suggesting the presence of radioactive products of lactose metabolism. The radioactivity of the barium precipitate indicates that a sizeable fraction of the lactose taken up was extensively degraded, presumably to carbon dioxide. The radioactivity remaining in the extracted cells suggests that another portion of the lactose taken up was converted to materials which are insoluble in hot water.

TABLE 4. *Uptake and distribution of lactose-1-C^{14}*

Medium	Lactose	Radioactivity	Specific activity
	μmoles/ml	*count per min per ml*	*count per min per μmole*
0 hr	2.09	106,200	50,800
3 hr	0.27	14,600	53,300
Uptake	1.82	91,600	50,500
Recovery (3 hr)[a]			
Hot-water extract[b]	0.32	38,400	120,000
Barium precipitate		13,000	
Extracted cells		19,500	
Total recovery		70,900	

[a] The recovery values were calculated to represent 1.0 ml of suspension. The cell concentration was 7.9 mg (dry wt) per ml.
[b] This fraction is equivalent to the intracellular fraction of Table 3 (and subsequent tables).

Effect of external lactose concentration. The rate of lactose uptake is dependent on the external concentration, since at the lowest initial concentration (3.0 μmoles/ml) this rate diminishes as lactose disappears from the medium (Table 5).

TABLE 5. *Effect of concentration of lactose in the medium on its uptake and intracellular accumulation*

Incubation time	Lactose						
	Medium	Uptake		Intracellular		Metabolized	μmoles/ml cell water / μmoles/ml medium
hr	*μmoles/ml*	*μmoles/ml*	*μmoles/mg cells*	*μmoles/mg cells*	*μmoles/ml cell water*	*μmoles/mg cells*	
0	3.00			nil	nil		0.3
1.5	2.10	0.90	0.15[a]	0.08	33[b]	0.07[c]	16
3	1.29	1.71	0.29	0.11	45	0.18	35
5	0.72	2.28	0.38	0.10	41	0.28	57
7	0.20	2.80	0.47	0.07	29	0.40	145
0	25.0			0.01	4		0.16
1.5	—[d]			0.08	33		
3	22.7	2.3	0.38	0.13	53	0.25	2.3
5	21.5	3.5	0.58	0.16	65	0.42	3.0
7	20.0	5.0	0.83	0.17	69	0.66	3.4
0	49.5			0.01	4		0.08
1.5	—			0.09	37		
3	47.4	2.1	0.35	0.13	53	0.22	1.1
5	—			0.16	65		
7	44.5	5.0	0.83	0.16	65	0.67	1.5

[a] The cell concentration was 6.0 mg (dry wt) per ml.
[b] Calculated from the estimation of cell water as 2.45 μliters/mg cell dry wt.
[c] Difference between lactose uptake and intracellular lactose.
[d] Not determined.

At high concentrations of lactose, a linear rate of uptake is observed which is the same with initial concentrations of 25 or 50 μmoles/ml, and these rates are not much higher than the initial rate at 3.0 μmoles/ml. These results indicate that a maximal rate of lactose uptake occurs with an external lactose concentration somewhat above 3.0 μmoles/ml (about 5.0 μmoles/ml in our experience).

The intracellular accumulation of lactose during the first 3 hr is nearly similar over the range of external lactose concentrations employed. With the lowest lactose concentration, internal lactose decreases with time, probably owing to a rate of metabolism which exceeds the diminishing rate of uptake. At the higher external lactose concentrations, a maximal intracellular concentration is attained between 3 and 5 hr and then remains constant. Also, a constant rate of metabolism is then attained. These data suggest that the intracellular concentrations of lactose attained are the result of concentrating and metabolic processes.

The data of Table 5, especially those with the lowest concentration of lactose, show that germinated conidia are capable of accumulating lactose intracellularly to concentrations far higher than that in the external medium. At the intermediate external lactose concentration (25 μmoles/ml), there is an accumulation to the extent of almost 50 μmoles/ml in excess of that expected for a passive diffusion process. Since similar intracellular concentrations are obtained at the highest external lactose concentration (50 μmoles/ml), it seems likely that a similar concentration mechanism is operative even though the internal lactose concentrations are not much greater than those in the medium. Thus, it is reasonable to assume that the entry of lactose into germinated conidia depends on a system other than passive diffusion.

Effect of pH and phosphate. Germinated conidia were washed and resuspended in the buffer to be employed, and were incubated for 0.5 hr before adding lactose. Samples were taken for lactose analysis and pH determination at 4.0 hr; the pH was also determined when lactose was added. The data (Table 6) show no difference in lactose uptake, intracellular lactose, or lactose metabolized when the pH was between 5.7 and 7.0. With a

TABLE 6. *Effect of pH and buffer composition on the uptake and intracellular accumulation of lactose*

Buffer[b]	pH		Lactose[a]		
	0 hr	4 hr	Uptake	Intracellular	Metabolized
			μmole/mg cells	*μmole/mg cells*	*μmole/mg cells*
Sodium phosphate	5.7	5.8	0.37	0.18	0.19
Sodium phosphate	6.9	6.8	0.36	0.18	0.18
Tris-chloride	6.9	6.7	0.37	0.18	0.19
Tris-phosphate	7.0	6.9	0.36	0.16	0.20
Sodium phosphate	7.8	7.4	0.11	—[c]	
Tris-chloride	8.3	8.1	0.10	—	
Tris-phosphate	8.3	8.0	0.10	—	

[a] The initial concentration of lactose was 3.0 μmoles/ml. The cell concentration was 4.0 mg dry wt/ml. The values given are for a 4.0-hr incubation period.

[b] Sodium phosphate buffers were 0.05 M with respect to phosphate, and the pH was adjusted with NaOH or HCl. Tris (2-amino-2-hydroxymethylpropane-1:3-diol) buffers were 0.05 M with respect to tris, and the pH was adjusted with HCl or H_3PO_4.

[c] Not determined.

TABLE 7. *Effect of inhibitors on the uptake and intracellular accumulation of lactose*

Addition[b]	Concn	Lactose[a]		
		Uptake	Intracellular	Metabolized
	M	*μmole/mg cells*	*μmole/mg cells*	*μmole/mg cells*
None		0.34	0.18	0.16
Fluoride	5×10^{-3}	0.31	0.17	0.14
Cyanide	1×10^{-3}	0.33	0.19	0.14
Arsenate	1×10^{-2}	0.20	0.12	0.08
2,4-Dinitrophenol	2.5×10^{-5}	0.19	0.13	0.06
Azide	3×10^{-4}	0.02	—[c]	
p-Chloromercuribenzoate	1×10^{-3}	0.02	—	
Iodoacetate	3×10^{-3}	0.01	—	

[a] The initial concentration of lactose was 3.0 μmoles/ml. The cell concentration was 4.0 mg (dry wt)/ml, and the cells were suspended in tris chloride buffer (pH 7.0). The values given are for a 4.0-hr incubation period

[b] The sodium salts of the inhibitors were used, and the cells were incubated for 30 min in the presence of the inhibitors prior to the introduction of lactose.

[c] Not determined.

pH of about 8.0, the uptake was reduced by about 70%. These effects appear to be independent of the presence or absence of an external source of phosphate or inorganic cation.

Effect of inhibitors. The data in Table 7 were obtained with germinated conidia suspended in tris (hydroxymethyl) aminomethane (tris) chloride buffer (pH 7.0), to avoid possible competition of phosphate with arsenate. The cells were incubated for 0.5 hr with inhibitor before adding lactose. Samples were taken at 2 and 4 hr, and a linear uptake of lactose was observed; intracellular lactose was determined with 4-hr samples. In general, there was no marked difference between the effects of inhibitors on uptake and on intracellular accumulation, and consequently on the estimated metabolism of lactose. Arsenate was moderately inhibitory, indicating a possible involvement of phosphate in uptake and accumulation of lactose, which is also suggested by the inhibitory effect of 2,4-dinitrophenol. Azide and the sulfhydryl inhibitors, *p*-chloromercuribenzoate and iodoacetate, almost completely blocked lactose uptake.

Since DOC inhibited the accumulation of ions but not their entry (Lester and Hechter, 1958, 1959), the possibility of a similar action of this steroid on lactose uptake was examined. The

TABLE 8. *Effect of 11-deoxycorticosterone (DOC) and azide (NaN_3) on the uptake and intracellular accumulation of lactose*

Addition[b]	Lactose[a]				
	Initial concn	Uptake	Intracellular	Metabolized	$\frac{\mu moles/ml\ cell\ water}{\mu moles/ml\ medium}$
	μmoles/ml	*μmoles/mg cells*	*μmoles/mg cells*	*μmoles/mg cells*	
None	5	0.36	0.11	0.25	22
	25	0.37	0.12	0.25	2.5
DOC	5	0.13	nil	0.13	<0.3
	25	0.15	nil	0.15	<0.3
NaN_3	5	nil	nil	nil	<0.3
	25	nil	nil	nil	<0.3

[a] The cell concentration was 7.5 mg (dry wt)/ml. The values given are for a 3.0-hr incubation period.

[b] Before introducing lactose, the cells were incubated for 2.0 hr with DOC (0.25 mg/ml), and for 30 min with NaN_3 (3.3×10^{-4} M).

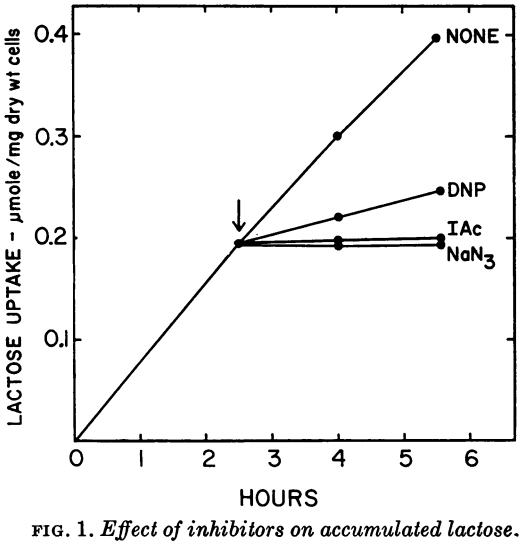

FIG. 1. *Effect of inhibitors on accumulated lactose. At the point indicated by the arrow, the inhibitors were added to give the following concentrations: 2,4-dinitrophenol (DNP), 4×10^{-4} M; iodoacetate (IAc), 4×10^{-3} M; NaN_3, 4×10^{-4} M.*

effect of DOC was examined with two concentrations of lactose, and compared with the effect of azide. Table 8 shows that DOC causes about a 60% inhibition of lactose uptake, and seems to prevent the intracellular accumulation of lactose. The uptake that does occur is not influenced by the concentration of lactose in the medium. Thus, DOC appears to permit the entry of lactose, whereas azide is completely inhibitory, as observed with ions.

The effects of the more potent inhibitors on the lactose accumulated by germinated conidia are indicated in Fig. 1. When the inhibitors were added during the course of incubation, the subsequent uptake of lactose corresponded to that described in Table 7. A low rate of uptake persisted with 2,4-dinitrophenol, and in the

TABLE 9 *Effect of temperature on the uptake and intracellular accumulation of lactose*

Incubation		Lactose[a]		
Temp[b]	Time	Uptake	Intracellular	Metabolized
C	*hr*	*μmole/mg cells*	*μmole/mg cells*	*μmole/mg cells*
0	3	nil	—[c]	
	6	nil	nil	
15	3	0.06	—	
	6	0.13	0.08	0.05
30	3	0.25	0.13	0.12
	6	0.44	0.14	0.30
30 for 3 hr		0.26	0.13	0.13
then 0 to 6 hr		0.27	0.13	0.14

[a] The initial concentration of lactose was 3 μmoles/ml The cell concentration was 6.0 mg (dry wt)/ml.

[b] The cells were incubated at the indicated temperature for 30 min prior to the introduction of lactose, unless otherwise indicated.

[c] Not determined.

TABLE 10. *Effect of other galactosides on the uptake and intracellular accumulation of lactose*

Additions[b]	Lactose[a]		
	Uptake	Intracellular	Metabolized
	μmole/mg cells	*μmole/mg cells*	*μmole/mg cells*
None	0.18	0.06	0.12
D-Galactose	0.08	0.03	0.05
Methyl-β-D-galactoside	0.18	0.06	0.12
ONPG	0.17	0.06	0.11

[a] The initial lactose concentration was 5.0 μmoles/ml. The cell concentration was 9.8 mg (dry wt)/ml. The values given are for a 3.0-hr incubation period.

[b] The galactosides (25 μmoles/ml) were introduced simultaneously with the lactose.

presence of azide or iodoacetate no further uptake was observed. It is of particular interest that the uptake values did not decrease, indicating that lactose accumulated by these cells did not return to the medium. This could suggest that azide and iodoacetate, especially, inhibit the exit as well as the entry of lactose.

Effect of temperature. Table 9 shows that at 15 C lactose uptake is only about 25% that at 30 C, and at 0 C uptake ceases. Since no significant amount of lactose entered the cells at 0 C, it would appear that entry is not a simple diffusion process, but probably one linked to cellular metabolism. This conclusion is also indicated by the effects of certain metabolic inhibitors, described above. The last two lines of Table 9 show that when cells are transferred from 30 to 0 C uptake and metabolism of lactose stop, and the level of intracellular lactose remains constant even though the intracellular concentration is about 35 times higher than that in the medium. This observation again suggests that the efflux of lactose might be dependent on cellular metabolism.

TABLE 11. *Impermeability of germinated conidia to ONPG*

Addition or treatment[b]	Incubation time	ONPG[a]			
		Medium	Hydrolysis	Intracellular	μmoles/ml cell water / μmoles/ml medium
	hr	*μmoles/ml*	*μmoles/mg cells*	*μmoles/ml cell water*	
None	0	3.00		nil	nil
	2	2.90	0.014	nil	nil
	4	2.90	0.025	0.55	0.18
DOC	0	2.95		0.55	0.18
	2	2.90	0.015	0.13	0.04
	4	2.90	0.026	0.50	0.17
NaN_3	0	2.95		0.30	0.10
	2	2.92	0.010	0.30	0.10
	4	2.92	0.020	0.10	0.03
Heat	0	3.14		(3.3)[c]	(1.1)
	2	3.12	0	(3.6)	(1.2)
	4	3.12	0	(3.5)	(1.2)

[a] The initial concentration of ONPG was 3.0 μmoles/ml. The cell concentration was 11 mg (dry wt)/ml.

[b] The cells were incubated for 2 hr with DOC (0.33 mg/ml) and 30 min with NaN_3 (3×10^{-4} M) before the introduction of ONPG. The heat treatment consisted of a 3-min immersion of the flask in a boiling-water bath and quickly cooling, just before the addition of ONPG.

[c] The values in parentheses represent the concentration of ONPG in the *total* water of the cell cake, and the ratio of these values with the concentration of ONPG in the medium.

Effect of other galactosides on lactose uptake. The specificity of lactose uptake and accumulation was examined by testing the effects of other galactosides on these processes. Of the three galactosides tested, only galactose was significantly inhibitory (Table 10). In this experiment the ratio of the other galactosides to lactose was initially 5:1, and at ratios below two or three to one even an effect of galactose was difficult to discern. ONPG has been examined at ratios to lactose of as much as 20:1 and found to be without effect; the lack of effect of ONPG was particularly surprising since its affinity for *Neurospora* β-galactosidase is many times that of lactose (Landman and Bonner, 1952). The lack of effect of ONPG was not due to its rapid metabolism since, under these conditions, no more than about 2% of the ONPG added was ever cleaved. It would appear that lactose uptake and accumulation is an extremely specific process.

The apparently anomalous lack of effect of ONPG on lactose uptake was clarified when the uptake of ONPG itself was examined. As shown in Table 11, no significant disappearance of ONPG from the medium could be measured, which coincided with the low rate of ONPG hydrolysis. The determination of intracellular ONPG yielded very low values, and the ratios of intracellular to medium concentration of ONPG of less than 0.2 indicated that ONPG was excluded from more than 80% of the cell water. The barrier to the penetration of ONPG was not appreciably affected by DOC or NaN_3; a brief heat treatment made the cells permeable to ONPG. The failure of ONPG to penetrate the cells would account for its inability to inhibit lactose accumulation or metabolism.

DISCUSSION

These studies have shown that the uptake of lactose by germinated conidia of *N. crassa* can be attributed to the metabolism of this sugar and its intracellular accumulation against an apparent concentration gradient. To achieve the observed intracellular concentrations of lactose, its rate of entry or uptake must exceed, initially, its rate of metabolism. With an adequate external

supply of lactose, a linear rate of uptake occurs for several hours. The constancy of this rate indicates that it represents the maximal rate of lactose entry, and that exposure of the cells to lactose does not alter this capacity for lactose uptake. The rate of accumulation of lactose in the cells is not linear, and a maximal and stable intracellular concentration is reached in about 3 to 5 hr. Subsequent uptake must be accounted for as lactose metabolized, and, thus, the rate of uptake also indicates the maximal rate of lactose metabolism, with the conditions employed. It appears, then, that when a certain intracellular concentration is achieved (about 0.16 μmole/mg cell dry wt) the rate of metabolism and uptake are equal (about 0.1 μmole per mg cell dry wt per hr). Whether or not these values represent the maximal capacity of these cells for the accumulation or metabolism of lactose cannot be determined from these data, although it is reasonable to assume that they are minimal values. The resolution of this question would depend on finding conditions or mutants in which lactose metabolism is blocked, or which would afford an increase in the concentration of intracellular lactose, permitting, respectively, an assessment of the maximal capacities for accumulation and metabolism. None of the conditions employed in these studies (pH, temperature, inhibitors) caused a significant dissociation of accumulation from the metabolism of lactose, with the possible exception of DOC.

Although the accumulation and metabolism of lactose are closely associated processes, they do have distinctive features permitting their separate discussion. The entry of lactose appears to be dependent on metabolic energy, since azide or low temperature (0 C) prevents lactose from entering the cells, and a similar dependency appears to be operative in the efflux of accumulated lactose. These characteristics are similar to those observed in the uptake of ions. However, the lack of specificity in the entry of ions does not seem to hold for lactose uptake, which is inhibited by galactose. The effect of galactose appears to be directed primarily at the entry of lactose rather than its metabolism; if galactose inhibited only lactose metabolism, a smaller portion of the lactose taken up should have been metabolized, resulting in an *increased* accumulation, and this is not observed. The specificity of lactose entry is also indicated by the exclusion of ONPG. Thus, in contradistinction to the entry of ions, the entry of lactose appears to be a highly specific process.

In the presence of DOC the rate of uptake of lactose is reduced to about 40 to 50% of the control rate, but no lactose is found in the cells. This could indicate that DOC does not grossly affect metabolism or entry and does inhibit accumulation, as with ions. However, similar results would obtain if the entry and accumulation of lactose were due to a single mechanism; its inhibition by DOC would reduce lactose uptake so that the rate of metabolism would exceed the rate of uptake, and no net gain of intracellular lactose would be observed.

Perhaps the most cogent argument against the operation of a diphasic, ionlike system for lactose uptake is derived from an examination of the influence of the external concentration on the intracellular accumulation of lactose. As the external concentration of rubidium or potassium was increased, the ratio of intracellular to extracellular concentration decreased and approached 1.0 (Lester and Hechter, 1958). This suggested a distribution of these ions in the cell water equivalent to that in the medium and their binding to a constant number of sites. This situation does not hold in the case of lactose (*see* Table 5). The over-all rate of uptake is directly influenced by the external concentration, reaching a maximum at about 5 μmoles of lactose/ml; a further increase of the external concentration does not affect the amount of lactose taken up per unit time, as with ions. Also, varying the external concentrations of lactose, above 5 μmoles/ml, does not alter the maximal intracellular concentrations achieved, 65 to 75 μmoles/ml cell water. Thus, when the external concentration is less than this level, the ratio of intracellular to extracellular concentration will be more than 1.0, and with higher external concentrations this ratio will be less than 1.0. The latter situation has been observed in some experiments where initial concentrations of 100 to 200 μmoles of lactose/ml were used. These observations are consonant with the possibility that lactose uptake and accumulation are primarily dependent on an energy-dependent, specific transport system.

It is of interest to compare the present studies with those of galactoside (Rickenberg et al., 1956), or galactose permeability (Horecker, Thomas,

and Monod, 1960) in *E. coli*. Both systems appear to involve a specific transport mechanism which is dependent on metabolic energy. The intracellular concentrations of lactose achieved by *N. crassa* are equivalent to about 6% (w/w) of the dry weight of the cells, or about 2% (w/v) in the cell water, which approximate the values observed in the inducible galactoside or constitutive galactose systems in *E. coli*. It has yet to be determined whether the system for lactose uptake in *N. crassa* is inducible, but the high levels accumulated suggest that lactose uptake is probably a constitutive characteristic. The uptake of lactose by *N. crassa* appears to differ from the bacterial systems by exhibiting a more rigid specificity. Neither ONPG nor methyl-β-D-galactoside inhibit lactose uptake by *N. crassa*, and it has been demonstrated that ONPG does not penetrate these cells to a significant extent. Another point of difference is that certain inhibitors, such as azide, do not seem to effect a release of accumulated lactose, whereas these agents cause a rapid efflux of galactoside or galactose from *E. coli*. Further study, especially of the *Neurospora* system, will be required to determine whether these differences represent fundamentally different systems in these organisms.

It is clear that after the first 3 hr of incubation the uptake of lactose by germinated conidia is due mainly to its metabolism, but the nature of this metabolism is obscure. The superficial analysis of the distribution of radioactivity from lactose-1-C^{14} indicates at least three types of products: (i) materials extracted from the cells by hot water; (ii) materials not extractable by water and remaining in the cells; and (iii) volatile materials, probably mainly carbon dioxide, precipitated as barium salts. The first and second accounted for 25 to 30%, and the third at least 15% of the lactose metabolized. These results indicate an extensive and possibly varied degradation of lactose, and raise the question of the nature of the first step(s) in lactose metabolism by *N. crassa*.

Landman (1951) and Franklin (1954) suggested that the major primary step in the metabolism of lactose and its utilization for the growth of *N. crassa* is hydrolytic cleavage by β-galactosidase. This hypothesis is based mainly on two types of observations: (i) mutants characterized by poor growth on lactose usually showed a reduced capacity for β-galactosidase production, and (ii) the onset of mycelial growth, after a long lag period, was preceded by, or coincident with, a rapid production of β-galactosidase. However, Franklin (1954) found some exceptions to these situations, and she cautioned against correlating the metabolism of lactose and its utilization for growth with β-galactosidase activities. The experiments presented here also raise some question as to the role of β-galactosidase in the metabolism of lactose, since the β-galactosidase activity extractable from germinated conidia could account for only a very small fraction of the lactose metabolized. Although the strain and cell preparations used in the present work differed from those used by the workers cited above, it was found that the amount of extractable β-galactosidase activity of germinated conidia was similar to that from mycelial pads grown on sucrose. Landman (1954) demonstrated the formation of large amounts of β-galactosidase activity in mycelial pads suspended in buffer containing an inducer, such as lactose, after a lag period of some 20 hr. A similar, but more rapid development of β-galactosidase might have accounted for the lactose metabolized by the germinated conidia. However, a comparison of β-galactosidase activities before and after 3 or 4 hr of incubation of germinated conidia in the presence of lactose showed only a small *decrease* in extractable activities.

The above considerations are based on estimations of extracted β-galactosidase activities, measured in terms of ONPG hydrolysis; these might not be applicable to the problem of the metabolism of lactose by intact cells. Thus, it might be speculated that *N. crassa* produced one molecular species of β-galactosidase whose intracellular conformation endows it with much greater activity than when extracted from the cell. Also, a directive action of the transport system might deliver lactose to intracellular β-galactosidase more effectively than when both components are in free solution. The poor growth of certain mutants on lactose, despite their capacity to produce normal amounts of β-galactosidase (Franklin, 1954), might be attributed to aberrancies in lactose transport. A third possibility is that *N. crassa* produces more than one species of β-galactosidase and these have different affinities for lactose and ONPG (resembling

the specificity of the lactose transport system). Lastly, the metabolism of lactose by sucrose-germinated conidia might not depend significantly on hydrolysis as the initial step, but some other process, such as the direct oxidation of lactose observed in certain bacteria by Bernaerts and DeLey (1957, 1958). Which, if any, of these possibilities approximate fact has yet to be determined.

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