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Differences in the Utilization of Glycerol and Glucose by *Mycobacterium phlei*

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A basic difference was found in the kinetics of uptake and utilization of glucose and glycerol by washed suspensions of *Mycobacterium phlei*. With glucose, the rates of uptake, respiration, and assimilation were saturated at low external substrate concentration. With glycerol, these rates were found to increase with increasing substrate concentration and did not show saturation at any concentration tested. Qualitatively similar patterns were observed for cells grown on either glycerol or glucose. Above a saturation concentration, ratios of cell ^{14}C to CO_2 ^{14}C for uniformly labeled ^{14}C-glucose were constant at a value of 0.96. Glycerol-*U*-^{14}C, on the other hand, yielded cell-$^{14}C/CO_2$-^{14}C ratios which were highest at the lowest glycerol concentration tested, and decreased with increasing substrate concentration. The distribution of the glucose and glycerol carbons assimilated into *M. phlei* were qualitatively similar. Quantitatively, however, the uptake and assimilation of glycerol was far more rapid than that of glucose for all substrate concentrations employed. These quantitative differences in the utilization of glycerol and glucose could account for the increased content of nonessential lipid and polysaccharide found in glycerol-grown *M. phlei*.

In a previous study, *Mycobacterium phlei* cells grown on glycerol medium and glucose medium were found to have identical rates of growth, as judged by protein and deoxyribonucleic acid (DNA) synthesis, yet pellicle weights on glycerol were consistently greater than on glucose (11). This increased weight of glycerol-grown cells was attributable to an increased lipid and polysaccharide content. In the present study, the uptake and utilization of glycerol and glucose by washed suspensions of *M. phlei* were compared in an attempt to account for the increased lipid and polysaccharide content of the glycerol-grown cells.

MATERIALS AND METHODS

Culture conditions. Cells of *M. pheli* strain 72 were grown as surface cultures at 37 C on media containing, per liter: asparagine, 6.0 g; potassium citrate, 2.0 g; K_2HPO_4, 5.1 g; KH_2PO_4, 2.2 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; $ZnSO_4 \cdot 7H_2O$, 0.025 g; $FeCl_3 \cdot 6H_2O$, 0.10 g; $CoCl_2 \cdot 6H_2O$, 0.002 g; $MnCl_2 \cdot 4H_2O$, 0.001 g; and $CaCl_2 \cdot 2H_2O$, 0.02 g; the *p*H was 7.0. Either 3% (w/v) glycerol or 6% (w/v) glucose was added as the major source of carbon. Media were dispensed in 100-ml amounts in 250-ml flasks. Glycerol media were autoclaved directly; glucose solutions were autoclaved separately and added to sterile basal medium.

Preparation of suspensions. Cultures (5-day) were harvested by centrifugation, washed three times with distilled water, adjusted to the desired cell concentration in distilled water, and used immediately. For dry weights, samples of the suspensions were pipetted into tared aluminum cups and were dried at 80 C to constant weight. Cells for nitrogen analyses were dried by lyophilization and were kept over P_2O_5 in a desiccator until analyzed. Since the nitrogen content of the 5-day cultures used in these experiments showed considerable variation, i.e., 5.6 to 6.8% N for glycerol-grown cells, 7.8 to 8.9% N for glucose-grown cells, nitrogen analyses were performed on each cell suspension used, and the results were recorded accordingly.

Uptake analyses. The uptake of glycerol or glucose was determined by measuring the disappearance of the substrate. Incubation mixtures, in 18 × 125 mm screw-capped tubes, consisted of 16 to 20 mg (dry weight) of bacilli (approximately 1.5 mg of cell N), 800 μmoles potassium phosphate buffer (*p*H 7.0), and the indicated substrate in a final volume of 4 ml. Tubes were prepared in duplicate and were incubated at 37 C in a tumbling apparatus. At the appropriate time intervals, reaction mixtures were centrifuged at 15,000 × *g* for 20 min at 4 C. Immediately after centrifugation, nonsedimented bacilli were removed by deproteinization with $BA(OH_2)$, $ZnSO_4$ mixtures (10). Samples of the supernatant fluid obtained after centrifugation were removed for analysis. Total uptake was calculated as the difference between the zero-time and final concentrations.

O_2 uptake. Respiratory measurements were made by use of standard Warburg techniques at 37 C (13). The system contained 1 to 2.5 mg (dry weight) of cells (approximately 0.1 mg of cell N), 600 μmoles of potassium phosphate buffer (*p*H 7.0), and the indicated substrate in a final volume of 3.3 ml. The center well contained 0.2 ml of 20% KOH. In all experiments, substrates were added directly into the main chamber of the Warburg vessel to assure complete mixing with the highly clumped bacterial cells. The respiratory rates were observed for a minimum of 90 min. Results are an average of at least three replicates.

CO_2 production. Carbon dioxide produced during the utilization of glycerol and glucose was determined by isotopic analyses. The bacilli were exposed to uniformly labeled ^{14}C-glycerol or to glucose-*U*-^{14}C (New England Nuclear Corp, Boston, Mass.) in Warburg vessels under the above conditions (*see* O_2 uptake), with the exception that 0.3 ml of 30% KOH was added to the center well. Incubations were terminated by the addition of H_2SO_4, and the cultures were shaken for an additional 45 min to trap the $^{14}CO_2$ liberated. The ^{14}C-carbonate in the center well was transferred to the main chamber of another Warburg vessel and was redistilled by acidification into hyamine-OH. The $^{14}CO_2$-hyamine solution was then assayed for radioactivity.

Chemical analyses and fractionation procedures. The nitrogen content of the lyophilized cells was determined by micro-Kjeldahl digestion and by the colorimetric Nessler procedure of Wilson and Knight (14). Glucose was determined by the method of Nelson (9); glycerol was determined by the method of Lambert and Neish (6). Lipids were extracted and estimated by the method of Winder and O'Hara (15). Lipid-extracted cells were further extracted with 10% (w/v) trichloroacetic acid for 3 hr at 0 C, then with 5% (w/v) tricholoroacetic acid for 30 min at 90 C. By these procedures, the cells were fractionated into a lipid fraction, an acid-soluble fraction, and hot trichloroacetic acid-soluble and insoluble fractions.

Isotopic analyses. Cells exposed to glycerol-*U*-^{14}C or glucose-*U*-^{14}C were washed repeatedly with water until the washes were free of radioactivity. The cells were solubilized by heating in 1.0 ml of 0.5 N KOH for 2 hr, and cell fractions were assayed for radioactivity by adding samples to the scintillation mixture of Bray (1) and counting. Quench corrections were determined by means of internal standardization.

RESULTS

The extreme differences in the kinetics of glycerol and glucose uptake by washed suspensions of *M. phlei* are shown in Fig. 1. To equate the experiments on the basis of cell numbers, results are expressed in terms of cell nitrogen (11). Glycerol disappearance was far more rapid than that of glucose at all concentrations employed. With glycerol, increases in substrate concentration up to 125 μmoles/ml resulted in marked increases in uptake rates; additional increments of glycerol in excess of 125 μmoles/ml further increased uptake rates, and the slope of the change in rates was decreased. It is of particular interest that saturation was not achieved with glycerol at any concentration tested. With glucose, on the other hand, the rates of uptake increased up to a concentration of 50 μmoles/ml; at this concentration, the maximal rate of uptake was achieved, and additional increments of glucose had no further effect on the rate of uptake. Qualitatively similar patterns were observed for cells grown on either glycerol or glucose (Fig. 1). Glycerol utilization was found to be even more rapid in cells grown on glucose. Such cells utilized glycerol from 15 to 38% more rapidly than cells grown on glycerol. In contrast, glucose uptake was the same for cells grown on either of the two media.

Uptake studies of this nature reflect both assimilation into cellular material and substrate oxidation by the cells. Since cells grown on glycerol and glucose media differ in their content of lipid and polysaccharide storage materials (11), the possibility existed that the observed differences in the rates of uptake reflected only the assimilatory and not the respiratory capacity of the cells. The relationship of the observations to the method employed in determining substrate uptake was also considered. Therefore, an effort was made to determine whether dissimilatory patterns also varied with these two substrates by studying oxygen uptake, carbon dioxide evolution, and assimilation into cellular material.

Figure 2 illustrates that the rates of oxygen uptake correlated with the rates for total substrate uptake, but it appears that oxygen uptake required lower substrate concentrations to achieve the same relative saturation levels. The difference in the magnitude of the substrate concentrations required is assumed to be the result of the different experimental conditions employed. Substrate uptake was determined in tumble tubes, whereas oxygen uptake and subsequent incubations were more highly aerated in the Warburg apparatus. Therefore, quantitative comparisons can be made only in those instances when incubation procedures are similar.

The rates of oxygen uptake (Fig. 2) increased with increasing glycerol concentration and did not show saturation; on the other hand, oxygen uptake remained constant above a glucose concentration of 5 μmoles/ml. Glycerol was oxidized most rapidly by glucose-grown cells; subtraction of the endogenous oxygen uptake did not change this relationship. When glucose was employed as the substrate, the rates of oxygen uptake at saturation were greater for glycerol-grown cells; however, the rates for glycerol-grown and glucose-grown cells were comparable when the endogenous oxygen uptake was subtracted.

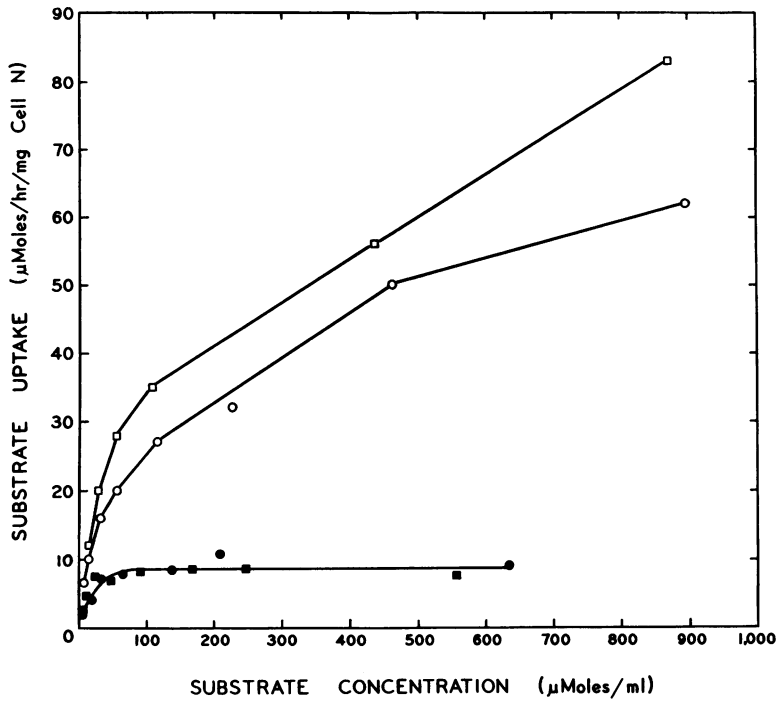

FIG. 1. *Rates of uptake of various concentrations of glycerol and glucose by Mycobacterium phlei. Washed suspensions of cells from 5-day cultures were incubated with the indicated substrate concentrations. Symbols:* ○, *glycerol, glycerol-grown cells;* □, *glycerol, glucose-grown cells;* ●, *glucose, glycerol-grown cells; and* ■, *glucose, glucose-grown cells.*

To establish relative rates of respiration and assimilation at various concentration levels, *M. phlei* was incubated with glycerol-*U*-^{14}C and glucose-*U*-^{14}*C*, respectively, and the incorporation of isotope into respiratory carbon dioxide and cellular material was compared (Table 1). Once again, both carbon dioxide formation and glycerol assimilation increased with increasing glycerol concentrations, indicating that neither respiration nor cellular assimilation were limiting. The ratio of cell C to carbon dioxide C was maximal at the lowest glycerol concentration, then decreased with increasing substrate concentration. It appears, therefore, that respiration competes more successfully for the glycerol at the higher concentrations employed, or assimilatory processes are saturated at lower concentrations than are respiratory processes. When glucose-*U*-^{14}*C* was employed as the substrate, large increases in concentration above 3 μmoles/ml had little effect upon the isotope incorporation into either cellular material or carbon dioxide. The ratios of cell C to carbon dioxide C were constant, with an average value of 0.96, at glucose concentrations above the saturation level. These findings further substantiate the kinetic differences between glycerol and glucose utilization by *M. phlei*.

The distribution of the glycerol and glucose carbon utilized by the *M. phlei* cells is shown in Table 2. Of the substrate utilized, 34 and 47% of the glycerol and glucose carbons, respectively, were assimilated. Quantitative differences were apparent in the assimilation of the two substrates, but the percentage distribution in the isolated fractions indicates a similarity in the end products of glycerol and glucose assimilation. Although, as expected from previous findings (11), a major portion of the assimilated carbons were recovered in the lipids, the even larger recovery in the hot trichloroacetic acid-soluble fractions is of particular interest. Elucidation of the active components in this fraction awaits further experimentation. Results similar to those described in Tables 1 and 2 were also obtained when incubations were carried out with glucose-grown cells.

DISCUSSION

These experiments have shown that there is a marked difference in the capacity of washed suspensions of surface cultures of *M. phlei* to take up exogenous glycerol and glucose. With

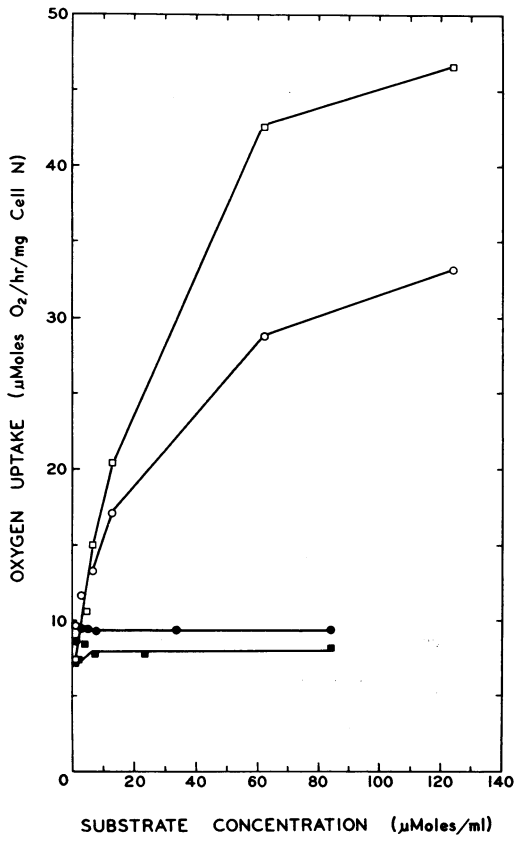

FIG. 2. *Rates of oxygen uptake by Mycobacterium phlei utilizing various concentrations of glycerol and glucose. Washed suspensions of 5-day cultures were incubated with the indicated substrate concentrations. Symbols: ○, glycerol, glycerol-grown cells; □, glycerol, glucose-grown cells; ●, glucose, glycerol-grown cells; and ■, glucose, glucose-grown cells. Oxygen uptake rates without exogenous substrate: glycerol-grown cells, 7.3 μmoles of O_2 per hr per mg of cell N; glucose-grown cells, 4.8 μmoles of O_2 per hr per mg of cell N.*

glycerol, the uptake increased with increasing substrate concentration and did not show saturation with any of the substrate concentrations tested. The rates of respiration and assimilation of the glycerol carbon also appear to be limited primarily by the external glycerol concentration, since both parameters increased directly with the external glycerol concentration. Unlike glycerol, the rates of glucose uptake were independent of the external substrate concentration and were constant above a low saturation level of substrate. Similar saturation kinetics were observed in the rates of respiration and assimilation of glucose.

These differences in substrate saturation may

TABLE 1. *Distribution of ^{14}C from various concentrations of glycerol-U-^{14}C and glucose-U-^{14}C between cells of Mycobacterium phlei and CO_2 produced*[a]

Substrate concn	Substrate-^{14}C/mg of cell N (μmoles)		Cell/CO_2 ratio
	Cells	CO_2	
μmoles/ml			
	Glycerol[b]		
7.8	8.85	12.24	0.72
15.6	12.69	20.10	0.63
31.3	15.09	26.88	0.56
62.5	16.62	33.39	0.50
125.0	17.79	39.60	0.45
	Glucose[b]		
0.3	1.14	2.28	0.50
3.0	13.46	14.15	0.95
30.0	14.45	14.99	0.96
60.0	15.30	15.90	0.96
90.0	15.59	16.21	0.96

[a] Cells from 5-day glycerol cultures exposed to the substrates for 3 hr.

[b] Substrate activity: glycerol = 12,960 disintegrations per min (dpm)/μmole; glucose = 31,150 dpm/μmole.

TABLE 2. *Incorporation of ^{14}C from glycerol-U-^{14}C and glucose-U-^{14}C into Mycobacterium phlei*[a]

Fractions	Glycerol[b]		Glucose[b]	
	^{14}C/mg of cell N	Percentage in fractions	^{14}C/mg of cell N	Percentage in fractions
	μmoles		*μmoles*	
CO_2 produced	39.96		14.64	
Unfractionated cells	20.73		13.02	
Fractions:				
Lipids	6.45	31.5	4.74	32.9
Cold trichloroacetic acid soluble	1.68	8.2	0.84	5.8
Hot trichloroacetic acid soluble	9.66	47.1	6.84	47.5
Residue	2.70	13.2	1.98	13.8
Total recovery in fractions	20.49	98.8	14.40	110.5

[a] Cells from 5-day glycerol cultures exposed to the substrates for 3 hr.

[b] Glycerol concentration = 125 μmoles/ml; specific activity, 12,960 dpm/μmole. Glucose concentration = 90 μmoles/ml; specific activity, 31,150 dpm/μmole.

provide an explanation for the increased content of nonessential lipid and polysaccharide storage materials found in glycerol-grown *M. phlei* (11). Qualitatively, similar patterns were found for the distribution of glycerol and glucose carbon assimilated in *M. phlei*. Quantitative differences in the overall lipid and polysaccharide content may then be related to the internal concentration of substrate carbon in excess of that needed for the synthesis of cell constituents essential for growth and multiplication. Presumably, the synthesis of essential constituents would be rate-limiting. It has been shown previously that the rates of protein and DNA synthesis are identical with both glycerol and glucose substrates (11). The internal concentration of glycerol in carbon-excess media could provide ample substrate for the synthesis of the basic cell constituents, and the excess internal concentration of glycerol could be diverted toward maximal lipid and polysaccharide synthesis. In glucose, however, the internal substrate concentration would be limiting, and, thus, less glucose carbon would be available for the deposition of nonessential lipid and polysaccharide.

Similar reasoning may explain the differences in weight yields of other mycobacteria grown on glycerol and glucose media. The dry weights and lipid content of *M. tuberculosis* have been found to increase progressively with the glycerol content of the medium (3, 4, 12). Although dry weights and the lipid content of these bacilli are also proportional to the amount of glucose present (4, 12), bacilli from glucose media were found to have 60 to 80% less lipid than glycerol-grown cells (3, 4, 12). The differences in substrate saturation may also account for the observations that the respiratory activity of *M. tuberculosis* (7) and other mycobacteria (2, 5) increases with increasing glycerol concentration, and that, at equivalent concentrations, glycerol stimulates respiration to a greater extent than does glucose (2, 7).

The results of this investigation do not provide an explanation for the mechanisms involved in the observed differences in substrate uptake. However, at least two possibilities are likely. Either two different systems are involved, or the same system, with different saturation constants for the respective substrates, is responsible for the uptake. Further studies are required to elucidate the mechanisms involved.

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