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Influence of Temperature Adaptation on Glucose Metabolism in a Psychrotrophic Strain of *Cytophaga johnsonae*†

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Selective enrichment of yellow-orange-pigmented, gram-negative bacteria related to *Cytophaga johnsonae* from lake sediment was dependent on low temperatures (ca. 5°C). However, this temperature effect was abolished when excessive amounts of dissolved organic carbon (10 mM *N*-acetylglucosamine) were added. A psychrotrophic freshwater isolate of *C. johnsonae* was used to study the physiological versatility of this group. Exponential growth rates were found to be dependent on the temperature to which the cells used as the inocula were acclimated. Glucose incorporation and respiration were also dependent upon the acclimation temperature of the inocula. Patterns of $^{14}CO_2$ evolution obtained from position-labeled [^{14}C]glucose indicated that glucose was predominantly metabolized via the Embden-Meyerhof-Parnas pathway, which, however, was greatly reduced at 25°C when the concentration of glucose was as low as 5 μM/liter. Transport, respiration, and incorporation of glucose (0.2- to 20,000-μM/liter concentrations) into macromolecular cellular compounds were characterized by multiple K_m values which were a function of substrate concentration and temperature. It appeared possible that these multiple K_m values reflected the changing participation of the Embden-Meyerhof-Parnas pathway in glucose metabolism. These results may provide a physiological explanation for the selective enrichment of psychrotrophic freshwater cytophagas. Moreover, they exhibit the limits of interpreting kinetic data based on conventional heterotrophic potential measurements, especially when some complications may arise from temperature and substrate adaptations of the more versatile members of the chemoorganotrophic microflora such as *C. johnsonae*.

Yellow-orange-pigmented, gram-negative bacteria which can be readily isolated from various freshwater habitats on chitin agar are often flexuous rods with gliding motility. They form spreading colonies and produce a flexirubin-type pigment. They are mostly psychrotrophs, similar to the phenotype of *Cytophaga johnsonae* described by Stanier (21). They are abundantly found in permanently or temporarily cold freshwater habitats (18). These organisms are also degraders of various α- and β-linked polysaccharides in lakes (15). The combined effect of water temperature and availability of dissolved organic carbon on their growth has been noted (15, 16).

In an attempt to explain the results obtained in enrichment experiments, the physiological versatility of a representative, psychrotrophic isolate of *C. johnsonae* strain C-21 was examined, using temperature and substrate concentration gradients. This organism was isolated from a small eutrophic lake at 5°C (15). The techniques used to determine the incorporation and respiration of glucose by cells were similar to those usually applied to determine the kinetics of the heterotrophic potential in aquatic habitats (1, 5, 6).

Physiological versatility is apparently expressed by the existence of different metabolic pathways for glucose as a function of temperature or substrate availability (2, 7, 14, 23). Modified radiorespirometry experiments were carried out with *C. johnsonae* by using ^{14}C-differentially labeled glucose.

MATERIALS AND METHODS

Enrichment experiments. A 10-g portion of Lake Tahoe sediments (400-m depth) was suspended in 400 ml of surface water enriched with colloidal chitin (200 mg/liter). After the suspension had settled for 30 min, 5-ml portions were distributed (in triplicate) into culture tubes (20 by 150 mm) which were incubated at 5 and 25°C with and without 0.05 ml of 1 M *N*-acetylglucosamine solution. Half of the tubes were sealed with

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an overlay of 5 ml of sterile mineral oil to help exclude oxygen, and the other half were placed on a rotary shaker (100 rpm).

Colony-forming units (CFU) were determined at weekly time intervals for 3 weeks, using a double layer of chitin agar. Plate count agar (10 ml; Difco Laboratories, Detroit, Mich.) was placed in the bottom of a sterile petri dish and was overlaid with chitin agar containing agar (15 g/liter), colloidal chitin (10 g/ml), yeast extract (100 mg/liter; Difco), and *N*-acetylglucosamine (100 mg/liter) (16). Serial dilutions of 0.1 ml of the sediment suspension were made in 5 mM KH_2PO_4 buffer (pH 7.0) containing Tween 80 (10 mg/liter). Spread plates (in triplicate) were made on each dilution. After 14 days of incubation at 18°C, *C. johnsonae*-like colonies were counted and identified by the formation of chitinolytic halos resulting from the bathochromic color shift due to flexirubin pigments when KOH was added (20). Microscopic verification of the presence of long flexuous rods, often showing gliding motion, was also used.

Cultural experiments. *C. johnsonae* Stanier, strain C21, was isolated from freshwater (15). Its psychrotrophic properties have recently been reported by W. Reichardt, R. Y. Morita, and R. R. Colwell (Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, N63, p. 174). The organism was cultivated in a mineral medium (MM) consisting of the following (per liter): 1.0 g of $(NH_4)_2SO_4$, 0.2 g of $MgSO_4 \cdot 7H_2O$, 3.0 mg of $CaCl_2$, and 0.3 mg of $FeCl_3$, buffered with 10 mM KH_2PO_4-$NaHPO_4$ buffer (pH 7.0; autoclaved separately) and supplemented with 10 mM membrane filter-sterilized glucose (0.10 μM).

Temperature-acclimated cells were produced by growing batch cultures of the organism at an incubation temperature of 5, 7, or 25°C in 1-liter Erlenmeyer flasks containing 500 ml of MM supplemented with 10 mM glucose, using a rotary shaker at 100 rpm. When the cultures reached their late exponential growth phase, 0.5 ml was transferred into fresh medium and the process was repeated for several months.

Growth rates. Exponential growth rates as a function of temperature were determined with a temperature gradient incubator (Scientific Industries, Mineola, N.Y.). The temperature gradient incubator was adjusted so that 30 different temperatures could be obtained at 1 to 2°C intervals. This permitted the incubation of 30 tubes (in duplicate) at 30 different temperatures simultaneously. Those tubes containing sterile medium (10 ml) were temperature equilibrated before being inoculated with 0.1 ml of acclimated log-phase culture suspensions (optical density at 500 nm [OD_{500}] = 0.50). Growth was measured by OD_{500} readings with a Bausch & Lomb Spectronic 20 spectrophotometer taken at 3- to 8-h intervals for periods up to 72 h.

Assays with D-[*U*-^{14}C]glucose. Cells were harvested by centrifugation (20 min, 10,000 × *g*) during their exponential growth phase and suspended in MM to an OD_{500} of 0.02. Sterile temperature gradient incubator tubes used with the same serum cap and bucket assembly as serum flasks for mineralization assays (5, 6) were filled with 4.9-ml portions of the cell suspension and incubated in the temperature gradient with 0.1 ml of a mixture containing 250 μmol of unlabeled glucose per liter and 0.3 μCi of D-[*U*-^{14}C]glucose (284 mCi/mmol). After an incubation period of 100 min, the assay was stopped by injecting 0.2 ml of 1 N H_2SO_4. At the same time 0.15 ml of phenethylamine (CO_2 absorbent) was injected into the bucket assembly containing fluted filter paper. The tubes were shaken for 1 h at 22°C. The fluted filter paper was removed and placed in a scintillation vial to which 10 ml of toluene-based Omnifluor was added. Radioactivity of the $^{14}CO_2$ produced was measured with a Beckman LS100 scintillation counter. CFU were determined at the initiation of each experiment. The acidified cell suspensions were kept at 2°C and within 100 min were filtered through membrane filters (0.22 μm; Millipore Corp., Bedford, Mass.) at 400 mmHg (ca. 53 kPa). The filters were rinsed three times with 5 ml of MM, dried at 50°C for 40 min, and then placed in scintillation vials each containing 10 ml of Omnifluor to measure the radioactivity of the incorporated glucose.

To determine the influence of the substrate concentration on rates of D-[*U*-^{14}C]glucose respiration and incorporation at 7 and 25°C, cells were grown at 16°C, harvested as above, resuspended in MM to an OD_{500} of 0.015, and subsequently starved for a period of 6 h at 16°C. The assays for incorporation and respiration of glucose were carried out in 60-ml serum vials with cap and bucket assembly (1, 5). A concentration gradient of glucose was produced by diluting a stock solution of filter-sterilized (0.22 μm; Millipore) unlabeled glucose (200 mM; 1:1) with distilled water. Portions, 0.5 ml, of the resulting concentration were pipetted into serum flasks (in triplicate), followed by 0.1 ml of D-[*U*-^{14}C]glucose (0.15 μCi; specific activity, 329 mCi/mmol). The assay suspension (4.4 ml) was incubated for 150 min at 7°C and 30 min at 25°C on a rotary shaker at 120 rpm. After the assay was stopped by acidification, all flasks were shaken (150 rpm) at 5°C before measuring the radioactivity of the $^{14}CO_2$ absorbed to phenethylamine and the ^{14}C incorporated in the 0.22-μm membrane-filtered cells, using the techniques described above.

To determine the influence of substrate concentrations on transport, 0.5 ml of each of 20 unlabeled glucose concentrations (see above), 0.1 ml of D-[*U*-^{14}C]glucose (0.15 μCi; specific activity, 329 mCi/mmol), and 0.4 ml of the culture suspension (OD_{500} = 0.45; grown at 16°C) were mixed in test tubes and incubated in a water bath at 7 or 25°C. Exactly 5 min after the cell suspensions had been added to the above mixture, duplicate 0.4-ml portions were filtered through Millipore membrane filters (0.22 μm), using a 400-mmHg vacuum. Samples poisoned with 2 mM dinitrophenol served as controls. The filters were rinsed three times with 5 ml of MM and subsequently treated as described above for incorporation assays.

Modified radiorespirometric assays were carried out with acclimated cells at 7 and 25°C with equimolar amounts of differentially labeled [^{14}C]glucose in 60-ml serum flasks containing either 4.8 or 1.8 ml of the cell suspension (OD_{500} = 0.100) and 0.1 ml of 250 μM or 25 mM unlabeled glucose to give final concentrations of 5 μM or 1.25 mM, respectively. Also added to the reaction mixture was 0.1 ml of a 25 mM solution of one of the following position-labeled [^{14}C]glucose preparations: D-[1-^{14}C]glucose (8.2 mCi/mmol); D-[2-^{14}C]glucose (6.0 mCi/mmol); D-[3,4-^{14}C]glucose (10.32 mCi/mmol); D-[6-^{14}C]glucose (9.0 mCi/mmol). The reaction mixture was shaken at 150 rpm. At 1-h intervals, the

TABLE 1. Enrichment of *Cytophaga*-like bacteria from Lake Tahoe sediment suspension[a]

Enrichment conditions	Without *N*-acetylglucosamine			With *N*-acetylglucosamine		
	10^6 CFU/liter	% CFU on chitin agar	% Chitinoclastic CFU	10^6 CFU/liter	% CFU on chitin agar	% Chitinoclastic CFU
5°C, aerobic	137	47	74	283	71	88
5°C, anaerobic	130	54	84	73	63	79
25°C, aerobic	<2	0	0	62	70	98
25°C, anaerobic	3	1	2	190	40	99

[a] Maximum counts occurring during 3 weeks of enrichment after 2 weeks at 25°C and after 3 weeks at 5°C; the initial number of *Cytophaga*-like bacteria was $<10^6$ CFU/liter.

reaction mixture was removed and the rates of incorporation and respiration were determined. All runs were made in triplicate. All radioactive substrates were obtained from New England Nuclear, Boston, Mass.

RESULTS AND DISCUSSION

Enrichment of *Cytophaga*-like freshwater bacteria. When colloidal chitin and *N*-acetylglucosamine were added to Lake Tahoe sediment, the numbers of bacteria resembling the phenotype of *C. johnsonae* Stanier increased regardless of the temperature of incubation (5 or 25°C) or the availability of oxygen (Table 1). Colloidal chitin enrichment for these bacteria was restricted to the lower temperature. Additional dissolved organic carbon compounds or *N*-acetylglucosamine was necessary to obtain a similar enrichment of the organism at 25°C. Under substrate conditions most likely to prevail in natural freshwater environments, a low-temperature-controlled selection for chitin-degrading cytophagas may be anticipated. Similar conclusions have been drawn from studies of *Cytophaga*-like bacteria in cold freshwater habitats (10, 15, 17). Since dissolved organic compounds of *N*-acetylglucosamine was necessary for enrichment for cytophagas at 25°C, this indicated a competitive capacity for substrate utilization by these organisms in the upper temperature range tested.

The exponential growth rates of 25°C acclimated cells (inoculum of *C. johnsonae* strain C21), when grown at different temperatures, showed a steady increase until reaching their optimum at between 27 and 30°C (Fig. 1). Slightly higher growth rates were obtained when 7°C acclimated inocula were used at the lower temperature range. The growth rates of cells acclimated to 7°C were maximal at around 25°C, but no significant differences were found over a fairly large range of near-optimal growth temperatures ranging from 15 to 30°C. It is known that the temperature characteristic (μ) can be different for cells acclimated at different temperatures (19).

Cells acclimated to 25°C kept their rates of glucose respiration at a relatively constant level between 5 and 27°C. On the other hand, cells acclimated to 5°C showed minimal respiration rates in the lower temperature range (Fig. 2). At around 10°C the respiration rates of 5°C acclimated cells started to increase. Between 18 and 31°C the respiration rates were more than twice the level observed for the 25°C acclimated cells. Glucose incorporation into cellular material was

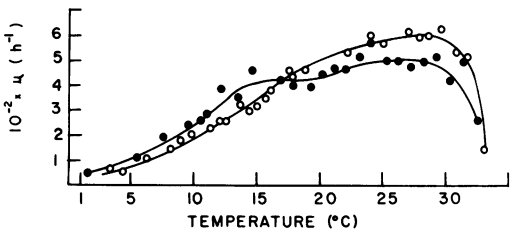

FIG. 1. Influence of temperature on exponential growth rates (μ) of psychrotrophic *C. johnsonae* strain C21, when acclimated to 7°C (○) and 25°C (●).

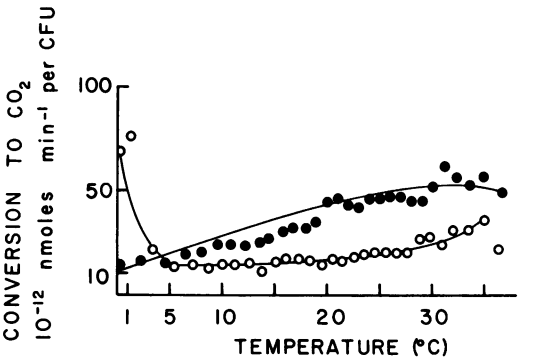

FIG. 2. Influence of temperature on specific respiration rates of glucose per CFU of *C. johnsonae* strain C21. Initial substrate concentration was 5 μM/liter after temperature acclimation to 5°C (●) and 25°C (○).

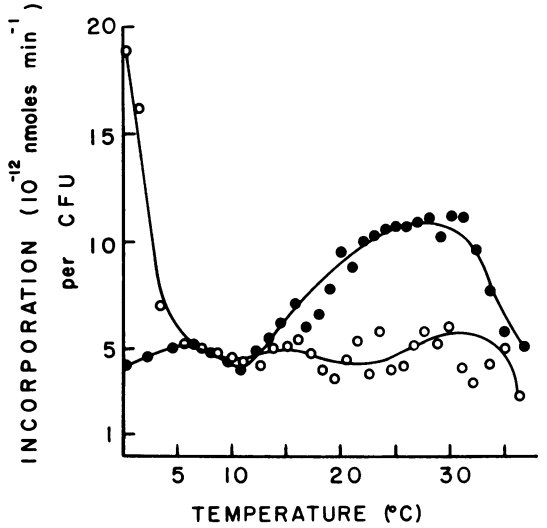

FIG. 3. Influence of temperature on specific rates of glucose incorporation into macromolecular cell compounds per CFU of *C. johnsonae* strain C21. Initial substrate concentration was 5 μM/liter after temperature acclimation to 5°C (●) and 25°C (○).

greater with 5°C acclimated cells between ca. 12 to 31°C (Fig. 3). There appears to be excessive glucose incorporation with 25°C acclimated cells when temperatures of <5°C are used. A comparison of Fig. 2 and 3 with Fig. 1 shows that the growth and incorporation rates started to decline at approximately the same temperature range (above 30°C), whereas respiration rates did so only above 35°C.

The apparent impact of temperature acclimation in growth and glucose utilization by 7 and 25°C acclimated cells was considered an indicator of extreme metabolic versatility of the organism. When 7 and 25°C acclimated cells were subjected to a sudden temperature shift, complete acclimation of the cells to the new temperature required at least two generations (data not shown). Apparently, this time lag was in contrast to the immediate response noted for exponential growth rates for *Escherichia coli* for temperature ranges with a constant-temperature characteristic (12). However, it resembled the pattern of temperature and substrate adaptation reported for psychrotrophic *Pseudomonas* spp. (8, 13).

The respiration pattern of 7 and 25°C acclimated cells at various temperatures of incubation may appear anomalous, but the 7°C acclimated cell curve may be the result of temperature affecting the fluidity of the membrane, which in turn affects transport, respiration, and incorporation. Previously, Haight and Morita (4) demonstrated that *Vibrio marinus* grown at the temperature at which it was isolated (ca. 5°C) demonstrated better oxygen uptake in the presence of glucose at 15°C (organism's optimum growth temperature) than did cells grown at

TABLE 2. Michaelis-Menten constants for transport, respiration, and incorporation of glucose at different concentration ranges by 16°C acclimated cells[a]

Concn range (μM/liter)	7°C			25°C		
	K_m (μM/liter)	r^2 [a]	V_{max} (10^{-9} nmol/min per CFU)	K_m (μM/liter)	r^2	V_{max} (10^{-9} nmol/min per CFU)
Transport (5 min)						
0.03–0.07	1.4	0.98	0.001			
5–39				82.9	0.97	0.06
156–1,250	1,212	0.92	0.5			
2,500–10,000	14,300	0.99	3.9			
Respiration (30 min)						
0.04–2.5				11.4	0.99	0.02
39–1,250	427	0.98	2.4			
156–2,500				5,118	0.91	8.1
2,500–20,000	2,273	0.95	3.0			
5,000–20,000				18,559	0.89	17.1
Incorporation (30 min)						
0.1–0.4				1.0	0.88	0.005
0.3–0.7	0.7	0.94	0.004			
2.5–20	40	0.99	0.1			
20–78				129	0.99	7.6
1,250–5,010	1,077	0.99	4.8			

[a] r^2 = Goodness of fit of linear transformation according to Eadie (3).

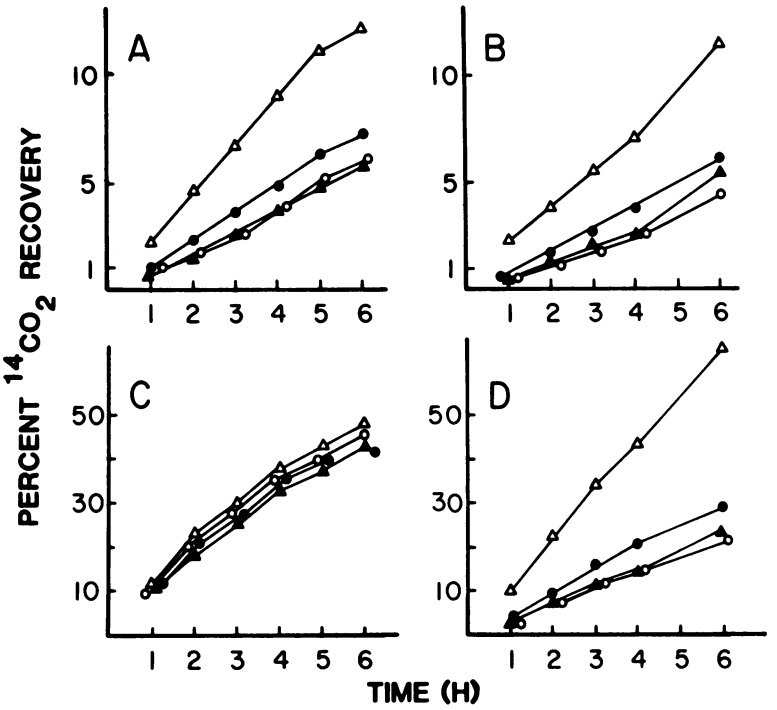

FIG. 4. Radiorespirometric pattern for *C. johnsonae* strain C21: percent cumulative recovery of $^{14}CO_2$ from equimolar concentrations of ^{14}C-differentially labeled glucose. The assay temperatures were run with acclimated cells grown at the same temperature. (A) 5 μM glucose at 7°C; (B) with 1.25 mM glucose at 7°C; (C) with 5 μM glucose at 25°C; (D) 1.25 mM glucose at 25°C. Symbols: ○, D-[1-^{14}C]glucose; ●, D-[2-^{14}C]glucose; △, D-[3,4-^{14}C]glucose; ▲, D-[6-^{14}C]glucose.

15°C. This was also true for endogenous respiration. They also demonstrated membrane differences between cells grown at two different temperatures as evidenced by the leakage of intracellular material with increased temperature. Because respiration on a substrate is better, the incorporation of glucose by 5°C acclimated cells is better (Fig. 3).

It is difficult to explain the "overshoot" of respiration and incorporation of glucose in Fig. 2 and 3 by the 25°C acclimated cells when the temperature of incubation is shifted to below 5°C. The data presented in Fig. 2 and 3 for this "overshoot" are indicated by 12 data points (each point on Fig. 2 and 3 is the average of duplicate samples). However, it should be pointed out that cold shock leads to a general increase in permeability of bacterial cells (9). Hence, the 25°C acclimated cells may undergo cold shock, whereas the 7°C acclimated cells do not. Increased permeability might be reflected in higher respiration and incorporation rates. In an effort to explain these results, studies on transport and radiorespirometric studies were initiated.

The kinetic rates of glucose transport, incorporation, and respiration were determined for a gradient of 20 different concentrations of glucose ranging from 0.2 to 20,000 μM/liter. Michaelis-Menten kinetics (3) proved applicable only within certain concentration ranges (Table 2). Both K_m and V_{max} values increased considerably towards higher concentration ranges. Ranges in which Michaelis-Menten kinetics were applicable differed as a function of the temperature at which the rates of glucose conversion were determined.

Transport kinetics at 7°C were characterized by three different K_m and V_{max} values as compared with only one pair of these parameters being determinable at 25°C in the lower temperature range.

K_m values obtained for respiration at 25°C indicated a broader range of substrate affinities than at 7°C. For incorporation, on the other hand, the maximal K_m value obtained at 25°C was considerably lower than that determined in the 7°C assay.

The variety of K_m values obtained for transport and incorporation of glucose suggests a greater physiological versatility of *C. johnsonae* at the lower temperature (7°C). This interpretation would help in understanding the selective

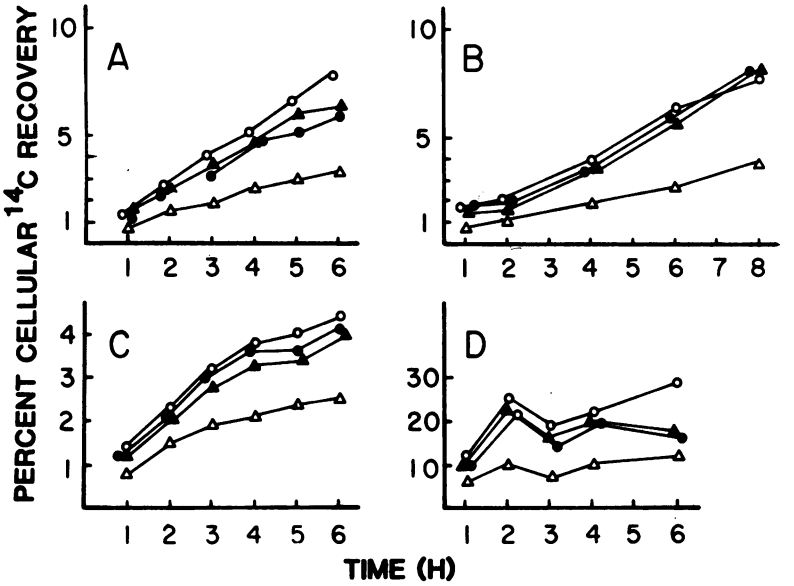

FIG. 5. Percent cumulative incorporation of ^{14}C into the macromolecular cell compounds of *C. johnsonae* strain C21 during radiorespirometric assays (see Fig. 4), using ^{14}C-position-labeled glucose. The assay temperatures were run with acclimated cells grown at the same temperature. (A) to (D) and symbols are as given in the legend to Fig. 4.

advantage of freshwater cytophagas in the lower temperature.

The patterns of sequence in which differentially labeled [^{14}C]glucose was respired or incorporated into cellular macromolecules did not reveal any significant alterations as a function of the temperature at which the cells had been acclimated and assayed (Fig. 4). At substrate concentrations corresponding to those used in the temperature gradient experiments, however, the relative predominance of D-[3,4-^{14}C] glucose as a major contributor to $^{14}CO_2$ evolution was markedly reduced at the elevated temperature (25°C). At the same time, mainly C-1 followed by C-6 and C-2 moieties were incorporated into macromolecular cell compounds irrespective of the temperatures and substrate concentrations chosen (Fig. 5).

The radiorespirometric data obtained for *C. johnsonae* C21 (Fig. 4) indicate major changes of glucose catabolism as a function of temperature. When the modified version of Wang's (23) technique was used, the maximal contribution of D-[3,4-^{14}C]glucose to the evolution of $^{14}CO_2$ indicated a predominance of the Embden-Meyerhof-Parnas pathway under all experimental conditions tested (22, 23). However, for the lower substrate concentration of 5 μM/liter, the relative conversion of D-[3,4-^{14}C]glucose to $^{14}CO_2$ was strikingly reduced at 25°C compared with the corresponding experiment at 7°C. According to Wang (22), this could indicate a shift to a less extensive participation of the Embden-Meyerhof-Parnas pathway in favor of the pentose phosphate and tricarboxylic acid cycle pathways. As far as the applicability of Wang's (22) methods is concerned, at a substrate concentration of only 5 μM/liter a possible interference by an excessive dilution of the labeled substrate in the endogenous cellular pools was least likely to occur.

Glucose catabolism via the Embden-Meyerhof-Parnas pathway seemed to be reduced only at substrate concentrations as low as 5 μM/liter but not at the much higher concentration of 1.25 μM/liter. It appears that the Embden-Meyerhof-Parnas pathway was favored at relatively high concentrations of glucose, irrespective of the temperature range chosen. This would also correspond to the observations made by Whiting et al. (24) that alternative pathways of glucose catabolism in *Pseudomonas fluorescens* were regulated by the available concentration of glucose.

The coexistence of concurrent metabolic pathways may also be indicated by multiple kinetic parameters (11). The triplicate K_m values obtained for glucose respiration by *C. johnsonae* at 25°C covered a substrate concentration range of more than three orders of magnitude. It is possible that these K_m values reflected the complex pattern of metabolic pathways resulting

from the reduced Embden-Meyerhof-Parnas participation in the upper temperature range (25°C).

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