AN ABSTRACT OF THE THESIS OF

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Title: STUDIES INVOLVING SUBSTRATE BINDING, UPTAKE, AND RESPIRATION NEAR THE MINIMUM GROWTH TEMPERATURE OF ESCHERICHIA COLI

Abstract approved: Redacted for Privacy
Richard Y. Morita

Escherichia coli K12, UC41 (an auxotroph) and UC175 (a wild type) were used to determine possible relationships between binding, uptake, and respiration and minimum growth temperature.

Both E. coli strains had a minimum growth temperature of 8.0 C in glucose minimal medium when cells were previously grown at 15 C. UC41 showed a greater uptake rate of the amino acids than did UC175.

A nearly constant uptake rate of L-methionine by UC41 below the minimum growth temperature was seen regardless of the wash treatment used during filtration. At and above the minimum growth temperature, uptake increased with increasing temperature indicating a direct relationship between binding and uptake and the minimum growth temperature. Cells of UC41 which were osmotically shocked to remove the histidine-binding protein were unable to resynthesize new binding protein below 8 C. The auxotroph also had a smaller uptake constant for arginine at and above the minimum growth temperature than below.

STUDIES INVOLVING SUBSTRATE BINDING, UPTAKE, AND RESPIRATION NEAR THE MINIMUM GROWTH TEMPERATURE OF ESCHERICHIA COLI

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INTRODUCTION

The common terms "minimum temperature of growth" and "minimal growth temperature" have been used in bacteriology for many decades. Most systematic and taxonomic texts, reports, and summaries include them in the description of a bacterium's cardinal temperatures. The minimum growth temperature is that temperature below which an organism is not able to reproduce and has only a limited capacity of growth.

Only a few workers have investigated the "mechanism" which governs a bacterium's minimum growth temperature and most of this work has been done with Escherichia coli. Correlations between minimum growth temperature and fatty acid composition (21, 33) and macromolecular synthesis (7, 9, 32, 34) have not shown promise as factors controlling the minimum growth temperature; however, the findings of Das and Goldstein (7) and Friedman et al. (9) have generated the theory that the inability of ribosomal subunits to bind to mRNA below the minimum temperature of growth may be the limiting factor.

Another possible explanation of the "mechanism" has been offered by Baxter and Gibbons (4) working with the eukaryote, Candida lipolytica. They have suggested that the transport of substrate into the yeast is temperature-dependent and could limit the organism's minimum growth temperature.

The possibility that this same mechanism may exist in prokaryotes is the focus of this study. Two strains of E. coli, a wild type and an auxotroph, were employed to determine the effect of the minimum

growth temperature in relation to binding, uptake (transport) and respiration.

LITERATURE REVIEW

The mechanism which allows microorganisms to grow and multiply at one temperature but not at a lower temperature is not completely understood. Shaw et al. (35) have arbitrarily defined minimal temperature of growth as "that temperature below which sustained, balanced growth does not occur".

Ingraham (14) found that E. coli K12 had a minimum growth temperature of about 8 C when grown in glucose minimal medium; that is, the cells would grown and reproduce at 8 C but not at temperatures tested below 8 C. Shaw et al. (35) and Hoffman (12) employing more precise measurements found the minimum growth temperature in glucose minimal medium was above 7.5 C. Growth was recorded at 7.8 C and 8.0 C, respectively, but not at 7.5 C.

Some of the first investigations into the mechanism governing the minimum growth temperature involved fatty acid analyses. Patching and Rose (27) showed that E. coli grown at 37 C had less lipid (8.1%) than cells grown at 15 C (8.9%). Marr and Ingraham (21) looked at the effect of growth temperature on fatty acid composition in E. coli. They found that with decreasing temperature the fatty acid composition changed from predominantly saturated fatty acids to unsaturated fatty acids. Later, the role of fatty acids in governing the minimum growth temperature was studied by Shaw and Ingraham (33). They concluded that the fatty acid composition did not determine the minimum growth temperature.

Shaw and Ingraham (34) found that by reducing the growth temperature from 37 C down to 10 C, there was an accompanying lag in growth and macromolecular synthesis. They observed lags of four, six, seven,

and eleven hours in the synthesis of proteins, the synthesis of RNA, the synthesis of DNA, and synchronous division, respectively. Later, Shaw (32) showed that macromolecular synthesis was carried out at 6 C. The cells were not able to divide, but filamentous forms of the bacterium were evident indicating that limited growth without cell division was possible below the minimum growth temperature.

Das and Goldstein (7) found that protein synthesis slowed and eventually halted at 0 C in E. coli K12. They observed that free ribosomes were unable to attach to the native mRNA at this temperature. Friedman et al. (9) further investigated this phenomenon with similar results. They have showed that free ribosomal subunits began to build up in E. coli cells at 8 C. This effectively blocked protein synthesis since no ribosomal-mRNA complexes were formed; however, all protein synthesis in progress at the time the temperature was lowered continued until completion.

Concomitantly Baxter and Gibbons (4) were investigating the minimal growth temperature of the yeast Candida lipolytica. While studying the uptake of glucosamine into the yeast, they found very little uptake below the minimum growth temperature. They suggested (no data) that a temperature-dependent transport mechanism may determine the minimum growth temperature.

METHODS AND MATERIALS

Bacterial strains.

UC175 (Hfr Ra-2) was chosen as a K12 prototroph. Another K12 strain, UC41 (leu2, lacI, gal, his1, argG-6, met, mal, xyl, str^r, λ^r), was chosen for its amino acid auxotrophic markers. Both organisms were obtained from Dr. Lyle R. Brown, Department of Microbiology, Oregon State University, Corvallis, Oregon.

Media and wash solutions.

Minimal medium (MM) was composed of ammonium chloride, 5.0 g; ammonium nitrate, 1.0 g; sodium sulfate, 2.0 g; potassium phosphate (dibasic), 3.0 g; potassium phosphate (monobasic), 1.0 g; magnesium sulfate ($MgSO_4 \cdot 7H_2O$), 0.1 g; and distilled water, one liter (8). Nutrient broth (Difco) was prepared in normal strength (8.0 g in one liter of distilled water) for culture growth, and also in dilute strength (0.5 g/l) for use as an uptake medium. Nutrient agar was used for all spread plates. All media were adjusted to pH 7.3 prior to autoclaving.

A twenty per cent solution (w/v) of D-glucose was filter sterilized and stored in sterile 100-ml prescription bottles. Similarly L-leucine, L-arginine, L-histidine, and L-methionine were filter sterilized and the solutions (two mg/ml) were stored in separate sterile prescription bottles. Both the glucose and the amino acid solutions were adjusted to pH 7.0 prior to filtering.

Acidic wash solutions were prepared by adding concentrated sulfuric

acid to (i) potassium phosphate (dibasic), 3.0 g; potassium phosphate (monobasic), 1.0 g; and distilled water, one liter, and (ii) sodium chloride, 10.0 g; and distilled water, one liter. The final pH was 1.5. Two sodium chloride solutions were also prepared in distilled water (i) 10.0 g/l and (ii) 0.5 g/l.

Radioactive compounds.

Radioactive-labelled amino acids C-14-L-leucine-UL (311 μCi/μM), C-14-L-arginine-UL (311 μCi/μM), C-14-L-histidine-UL (252 μCi/μM), C-14-L-methionine-UL (235 μCi/μM), C-14-L-histidine-C2 (five μCi/μM), and C-14-methyl-L-methionine (15 μCi/μM) were obtained from New England Nuclear Corporation. The original solutions (50 μCi/0.5 ml) were diluted to five ml with distilled water. Each solution was filter sterilized, transferred to a sterile vial, and frozen until used. This concentration was diluted with distilled water to approximately one μCi/ml for the various experiments.

Preparation of cells.

A five per cent inoculum of each UC175 and UC41 from nutrient broth culture was added aseptically to 500-ml Erlenmeyer flasks containing 200 ml of normal strength nutrient broth. Aseptic conditions were maintained throughout all experiments. The flasks were incubated on a rotary shaker for 18 hours at 37 C or for 24 hours at 15 C. The cultures were centrifuged for ten minutes at 13,200 x g in a Sorvall RC2-B refrigerated centrifuge (Ivan Sorvall, Inc. Norwalk, Connecticut) at 0 C. The supernatant was decanted off and the cells resuspended in

ten ml of MM. The new suspension was centrifuged at 13,200 x g for three minutes. The resulting supernatant was decanted off and the cells resuspended. The three-minute centrifugation and wash process was repeated twice more. Finally the cells were suspended in ten ml of MM (or other test media) and stored on ice until used. New cell suspensions were prepared for each experiment.

Minimum temperature of growth determination.

The freshly washed cells were added to 400 ml of MM containing five ml of glucose solution and ten ml of each amino acid solution to give a final optical density (OD) of 0.05 (approximately 5 X 10^7 cells/ml). Five ml of the cell suspension was placed into sterile test tubes (18 x 175 mm). A polythermostat was set to produce a temperature range of 0 C to 20 C in 1.0 C to 1.5 C increments. Duplicate tubes were made for each organism for each temperature.

OD readings were made on each tube at time zero and subsequently at each 24-hour interval in a Bauch and Lomb Spectronic 20 colorimeter at 600 nm. Spread plates were made at time zero and at the conclusion of the experiment.

Uptake and respiration studies.

The cells were prepared as above, except they were suspended in MM+ (MM containing glucose and the three amino acids other than the one being tested), MM, or 0.5 g/l nutrient broth. Five ml of the new suspension were transferred to duplicate tubes at each temperature. The tubes were allowed to equilibrate to temperature for 15 minutes prior

to the addition of the labelled amino acid.

Employing a Pipetman Model P200 adjustable microliter pipette (Rainin Instrument Co., Inc. Boston, Massachusetts), 100 µl of the labelled amino acid solution (one uCi/ml) was added to each tube to give a final concentration of 0.02 ± 0.005 µCi/ml. The cells were incubated for either one hour or 24 hours.

A duplicate set of tubes at each temperature was used solely for cellular uptake. An identical set of tubes was used to determine the amount of labelled carbon dioxide respired during the forementioned incubation periods.

Cellular uptake was determined as follows. After the incubation period, samples were immediately filtered through a 23 mm diameter HA Millipore filter with a pore size of 0.45 um (Millipore Corp. Bedford, Massachusetts). The cells were washed with either the phosphate-buffered-acidic wash or the sodium chloride-acidic wash (ten ml) to remove excess labelled material. This was followed by a 0.5 g/l sodium chloride wash (five ml) to prevent cell lysis due to acid contact. (Acidified controls were used to determine background levels of uptake.) The filters were dried in an oven at 80 C for 10 to 15 minutes after which time they were placed into five ml of toluene fluor (Omnifluor. New England Nuclear Corp. Boston, Massachusetts; four g/l). The vials were counted for four minutes in a Mark I Nuclear-Chicago liquid scintillation counter (Nuclear-Chicago. Des Plaines, Illinois).

The procedure of Harrison et al. (10) was followed for the collection of carbon dioxide except that the stoppers were fixed to test

tubes instead of serum bottles. After the reaction was stopped by acidification, the tubes were transferred to a reciprocating shaker at room temperature (23 to 24 C). The tubes were allowed to shake for one hour to facilitate CO_2 absorption. The fluted filter paper was removed and placed into a scintillation vial containing ten ml of the toluene fluor. The vials were counted for four minutes.

The efficiency of CO_2 recovery by the above procedure was determined by use of C-14-sodium bicarbonate. Per cent efficiency of recovery was determined by adding known levels of the bicarbonate to duplicate tubes containing the test medium. All respiration values were corrected to the 100% level.

Radioactivity of the amino acids was determined by pipeting 100 µl of the solution into a mixture of toluene fluor and Triton-X-100 (2:1).

Calculations.

The channels ratio method of determining counting efficiency was employed. This required the establishment of a standard quench curve (5, 6) which converts channels ratio directly to counting efficiency. These were determined for each samples. Counts per minute (cpm) was then converted to the more useable and meaningful disintegrations per minute (dpm).

Osmotic shock.

Cells of UC41 were grown in nutrient broth at 15 C and centrifuged at 13,200 x g for ten minutes. The osmotic shock treatment of Neu and Heppel (24) was followed. After osmotic shock the cells were suspended

in 0.333 M Tris (hydroxymethyl) aminomethane buffer (pH 7.5) giving a final concentration of 5 X 10^7 cells/ml. Five ml of this suspension were added to duplicate sets of tubes at each temperature and allowed to equilibrate for 15 minutes. After equilibration 100 µl of labelled histidine were added to each of the tubes at the time indicated. The cells were filtered and washed with 15 ml of 0.01 M Tris-0.15 M NaCl-0.5 uM $MgCl_2$ (pH 7.5) wash. The filters were dried and counted as above.

Kinetic analysis.

The procedures of Wright and Hobbie (41) and Hobbie and Crawford (11) were employed to determine the kinetics of C-14-arginine uptake by UC41. MM+ was used as the test medium for cells previously grown at 15 C.

RESULTS

Minimum temperature of growth.

Cultures of UC41 and UC175 were grown at 15 C and at 37 C prior to the minimum temperature of growth determination. When cells were pre-incubated at 37 C, both OD determinations and viable counts showed UC41 to have a minimum growth temperature of 10.5 C and UC175 to have a minimum growth temperature of 12.5 C (Table 1). This difference was not seen when both organisms were grown at 15 C. At this temperature, both UC41 and UC175 had a minimum growth temperature greater than 7.5 C (Table 2). Both organisms grew at 8 C, but cell division was not evident in the 7.5 C tubes after 168 hours.

Amino acid uptake followed by acidic wash.

The total uptake of the four amino acids by UC175 and UC41 grown at 37 C is presented in Figures 1 and 2. When the temperature was increased, there was relatively little increased uptake of C-14-arginine, C-14-histidine, or C-14-methionine. The total uptake of C-14-leucine, however, showed a rapid increase in uptake capacity with increasing temperature after one hour of incubation. In the same samples, CO_2 data indicated that little respiration had occurred in any of the samples (Tables 3 and 4).

Cells grown at 15 C showed varying degrees of increased uptake with increased temperature. Uptake of labelled leucine by UC175 showed the most rapid increase (Figure 3) while C-14-histidine and C-14-methionine had a much reduced uptake activity. Uptake of C-14-arginine

TABLE 1. MINIMUM GROWTH TEMPERATURE DETERMINATION FOR *E. COLI* CELLS PREVIOUSLY GROWN AT 37 C.

		Optical Density Readings				Spread Plate Counts	
	Time (hr)	0	48	144	216	0	216
Strain	Temp (C)						
UC41	9.0	.001	.001	.001	.001	1.6×10^6	1.6×10^6
	10.5	.001	.001	.001	.010	1.6×10^6	8.0×10^6
	12.0	.001	.001	.010	.050	1.6×10^6	6.0×10^7
	14.5	.001	.001	.385	.392	1.6×10^6	TNTC[a]
UC175	10.5	.020	.019	.019	.020	3.8×10^6	3.5×10^6
	12.5	.020	.018	.021	.068	3.4×10^6	9.3×10^6
	15.0	.020	.018	.058	.302	3.9×10^6	3.2×10^8

[a]TNTC - Too numerous to count

TABLE 2. MINIMUM GROWTH TEMPERATURE DETERMINATION FOR *E. COLI* CELLS PREVIOUSLY GROWN AT 15 C.

		Optical Density Readings				Spread Plate Counts	
	Time (hr)	0	72	120	168	0	168
Strain	Temp (C)						
UC41	7.5	.040	.035	.040	.048	1.3×10^7	8.8×10^6
	8.0	.030	.045	.055	.070	1.3×10^7	2.5×10^7
	9.0	.040	.063	.090	.130	1.3×10^7	2.6×10^7
UC175	7.5	.040	.032	.033	.033	4.8×10^7	4.8×10^7
	8.0	.040	.032	.045	.058	4.8×10^7	6.4×10^7
	9.0	.030	.037	.049	.059	4.8×10^7	5.9×10^7
	10.0	.030	.042	.063	.071	4.8×10^7	8.7×10^7

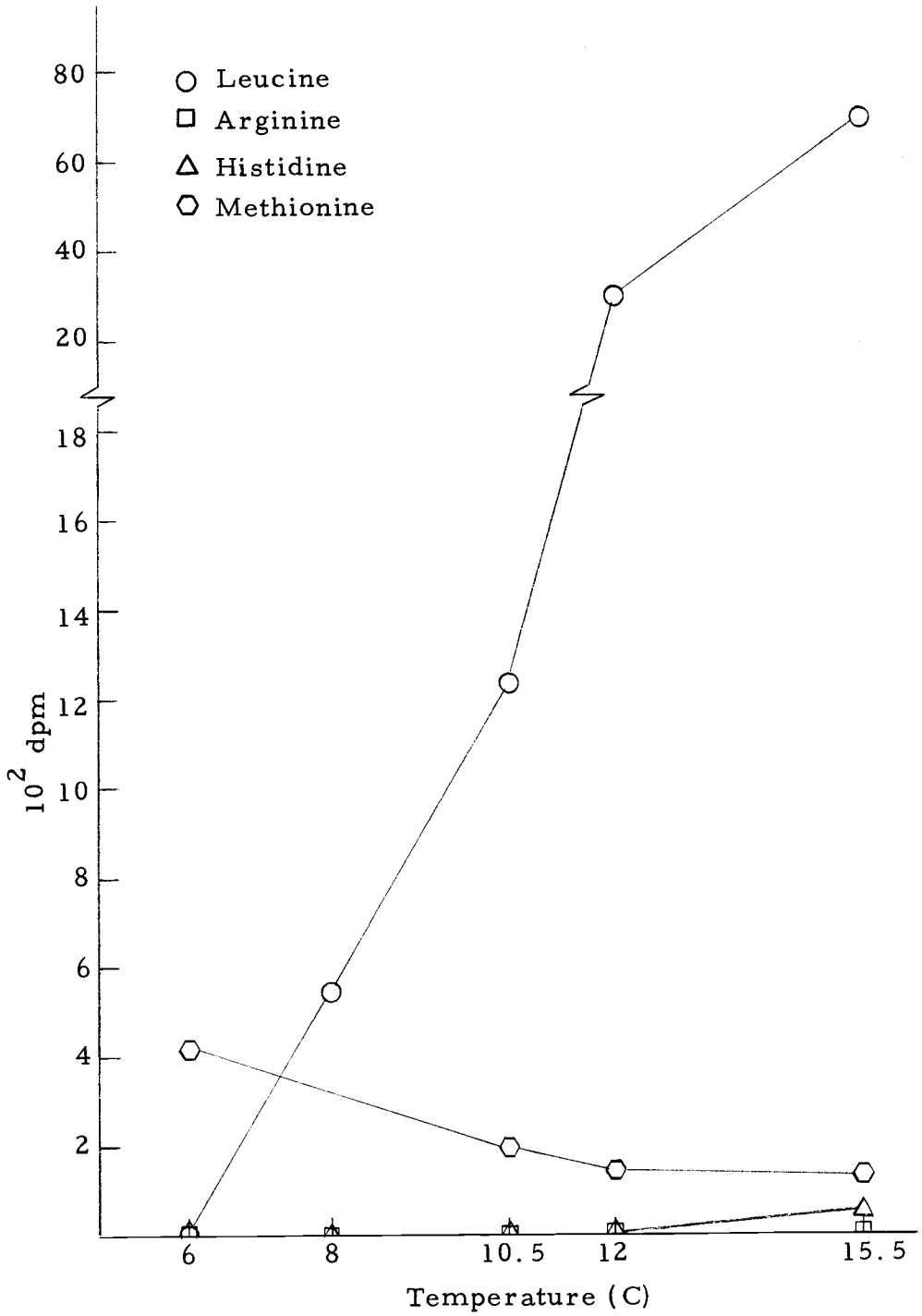

Figure 1. Uptake profile by UC175 previously grown at 37 C. One-hour uptake of each amino acid was examined in MM+.

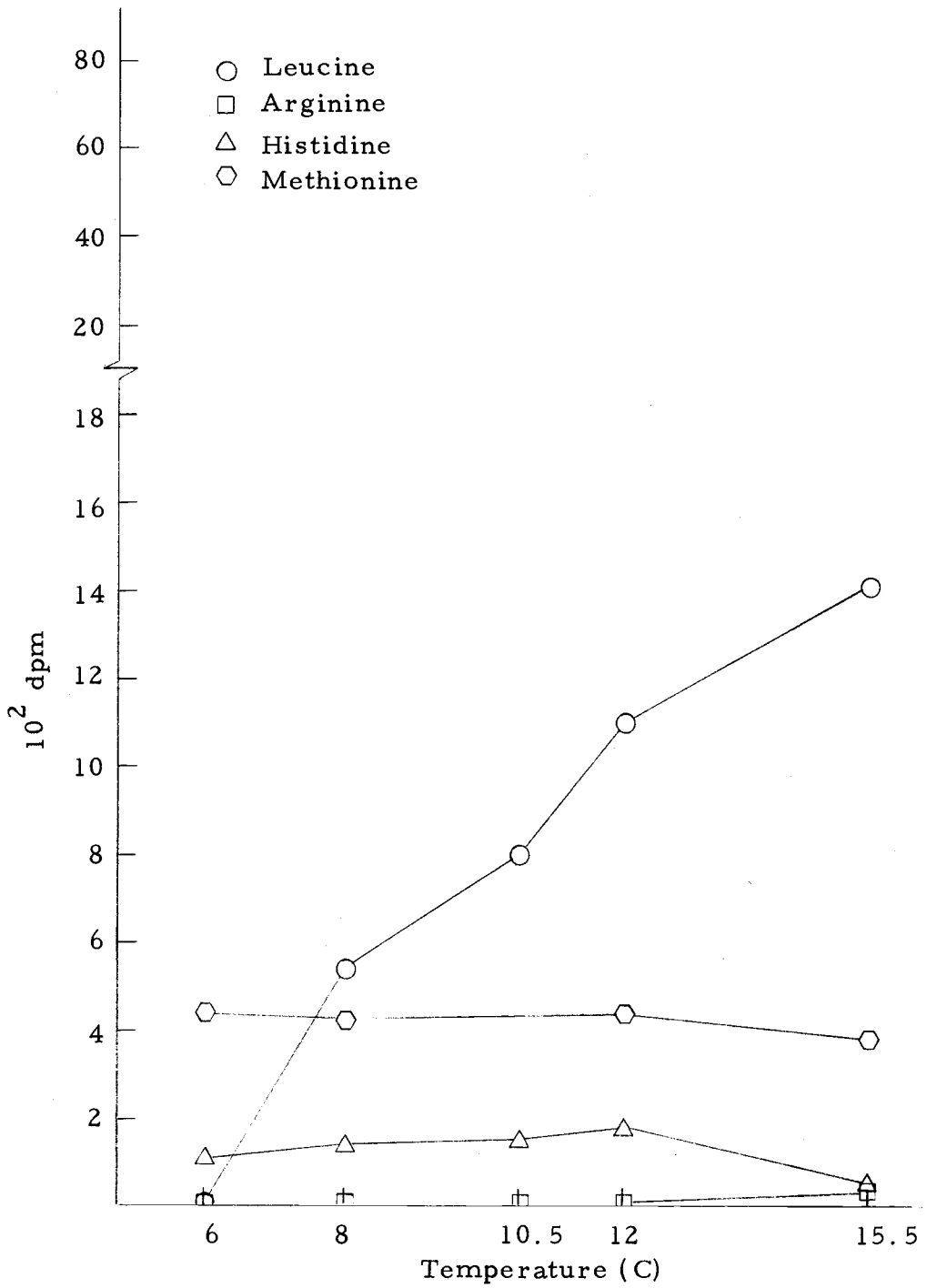

Figure 2. Uptake profile by UC41 previously grown at 37 C. One-hour uptake of each amino acid was examined in MM+.

TABLE 3. RESPIRATION BY UC175 PREVIOUSLY GROWN AT 37 C.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	6.0	0	0	0.0
	8.0	547	0	0.0
	10.5	1,217	0	0.0
	12.0	2,916	0	0.0
	15.5	6,994	0	0.0
arginine	6.0	0	0	0.0
	8.0	0	0	0.0
	10.5	0	0	0.0
	12.0	0	0	0.0
	15.5	86	0	0.0
histidine	6.0	0	0	0.0
	8.0	0	0	0.0
	10.5	0	0	0.0
	12.0	0	0	0.0
	15.5	0	0	0.0
methionine	6.0	0	0	0.0
	8.0	0	0	0.0
	10.5	0	0	0.0
	12.0	102	0	0.0
	15.5	112	0	0.0

TABLE 4. RESPIRATION BY UC41 PREVIOUSLY GROWN AT 37 C.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	6.0	146	0	0.0
	8.0	517	0	0.0
	10.5	768	0	0.0
	12.0	1,102	0	0.0
	15.5	1,407	0	0.0
arginine	6.0	0	0	0.0
	8.0	0	0	0.0
	10.5	0	0	0.0
	12.0	0	0	0.0
	15.5	0	0	0.0
histidine	6.0	101	0	0.0
	8.0	144	0	0.0
	10.5	147	0	0.0
	12.0	173	0	0.0
	15.5	*	0	-
methionine	6.0	432	108	25.0
	8.0	422	63	15.0
	10.5	361	*	-
	12.0	366	92	25.0
	15.5	365	108	19.0

* lab accident

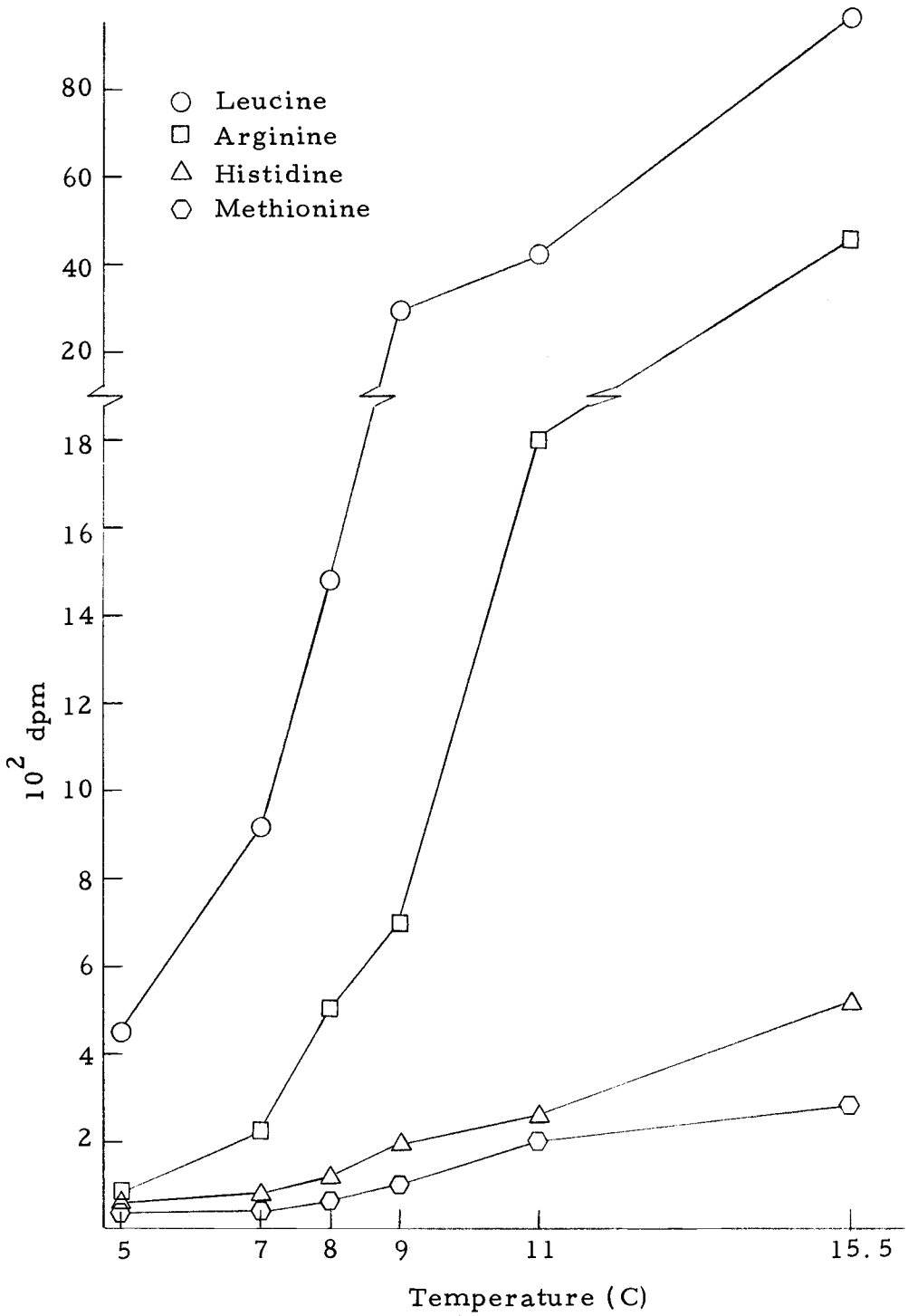

Figure 3. Uptake profile by UC175 previously grown at 15 C. One-hour uptake of each amino acid was examined in MM+.

was intermediary between the leucine and the histidine and methionine uptakes. The auxotroph also gave the same relative patterns with the same amino acids (Figure 4).

The per cent respiration of the labelled amino acids taken up by each organism was different for each amino acid with neither organism respiring C-14-leucine (Tables 5 and 6). The per cent of C-14-arginine respired by both organisms over the temperature range 8 C to 15 C was about 10%. C-14-histidine was respired in decreasing percentage with increasing temperature. Respiration of C-14-methionine differed, however, between the two organisms. UC41 did not respire any methionine from 4 C to 8 C, at 9 C about 11% was respired. UC175 did not respire methionine over the temperature range tested.

Effect of test medium on uptake (acidic wash).

The relative uptake capacity of both UC41 and UC175 in different media was determined in two experiments. MM+ was compared with MM and with 0.5 g/l nutrient broth as uptake media using 15 C cells.

Total uptake of C-14-leucine and C-14-histidine was similar in both MM and MM+ for UC41 (Figures 5 and 6). Labelled arginine showed an enhanced uptake in MM+ over the uptake in MM (Figure 5). C-14-methionine showed an inverse of the enhancement seen with arginine. Total uptake by UC41 in 0.5 g/l nutrient broth yielded greatly reduced levels of uptake of labelled leucine and arginine with arginine being the most affected (Figure 7). UC41 showed a greater uptake of C-14-methionine and C-14-histidine than was shown in either MM or MM+.

Respiration of the labelled amino acids in each medium was also

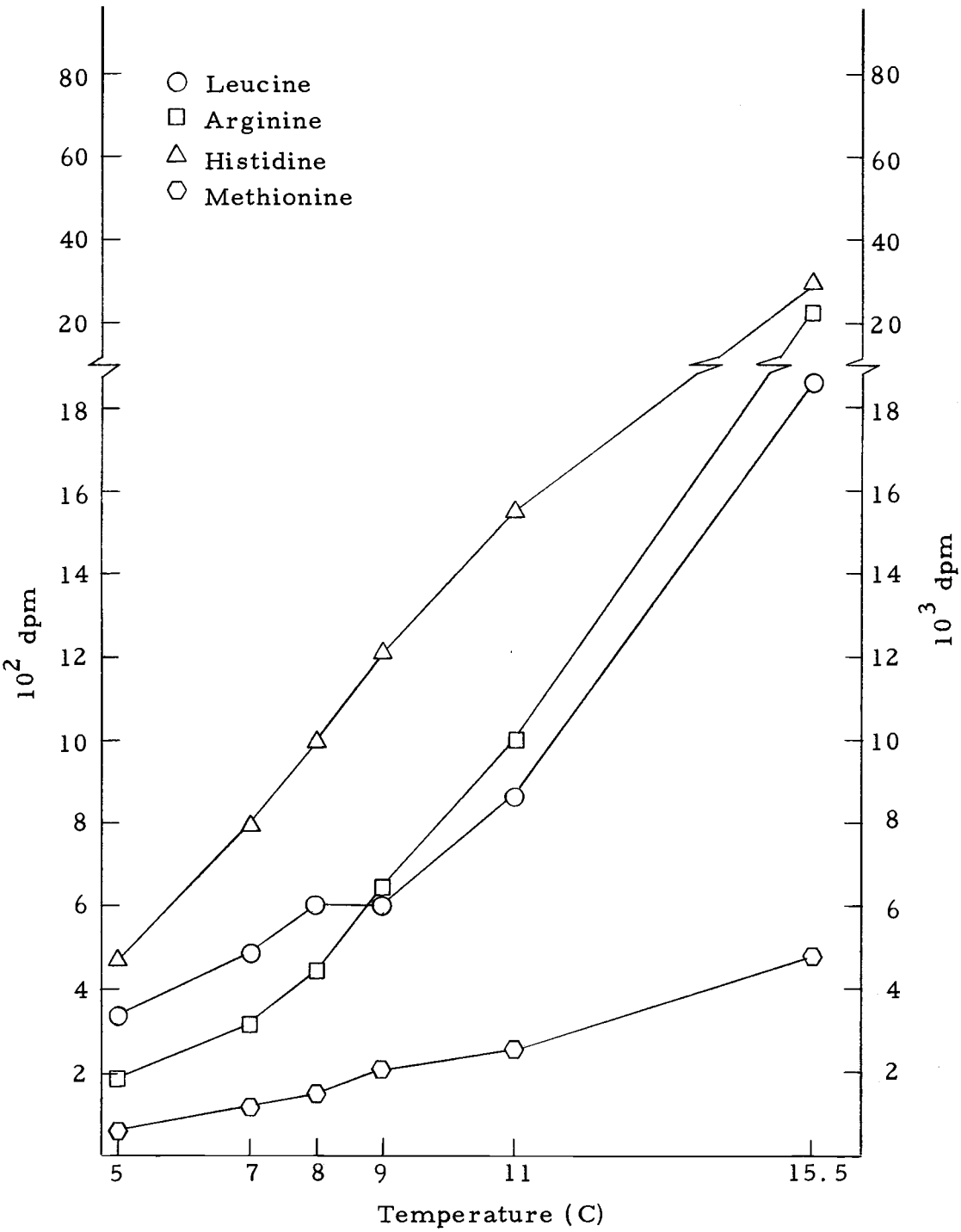

Figure 4. Uptake profile by UC41 previously grown at 15C. One-hour uptake of each amino acid was examined in MM+. Counts are given in 10^2 dpm for histidine methionine and in 10^3 dpm for leucine and arginine.

TABLE 5. RESPIRATION BY UC175 PREVIOUSLY GROWN AT 15 C.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	5.0	472	0	0.0
	7.0	912	0	0.0
	8.0	1,503	0	0.0
	9.0	2,348	0	0.0
	11.0	4,318	0	0.0
	15.5	9,477	0	0.0
arginine	5.0	87	35	40.2
	7.0	263	51	19.4
	8.0	498	61	12.2
	9.0	748	79	10.6
	11.0	1,788	182	10.2
	15.5	4,478	467	10.4
methionine	5.0	50	0	0.0
	7.0	50	0	0.0
	8.0	74	0	0.0
	9.0	112	0	0.0
	11.0	227	0	0.0
	15.5	343	0	0.0
histidine	5.0	68	30	44.1
	7.0	200	66	33.0
	8.0	326	79	24.2
	9.0	472	64	13.6
	11.0	695	83	11.9
	15.5	1,322	113	8.5

TABLE 6. RESPIRATION BY UC41 PREVIOUSLY GROWN AT 15 C.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	5.0	3,367	0	0.0
	7.0	4,922	0	0.0
	8.0	6,097	0	0.0
	9.0	6,006	0	0.0
	11.0	8,654	0	0.0
	15.5	18,652	0	0.0
arginine	5.0	1,878	159	8.5
	7.0	3,173	342	10.8
	8.0	4,436	483	10.9
	9.0	6,516	584	9.0
	11.0	10,043	896	8.9
	15.5	21,527	2,451	11.4
methionine	5.0	63	0	0.0
	7.0	116	0	0.0
	8.0	155	0	0.0
	9.0	211	27	11.3
	11.0	255	46	15.3
	15.5	483	147	23.3
histidine	5.0	473	52	11.0
	7.0	808	64	7.9
	8.0	1,003	48	4.8
	9.0	1,210	49	4.0
	11.0	1,546	69	4.5
	15.5	3,004	101	3.4

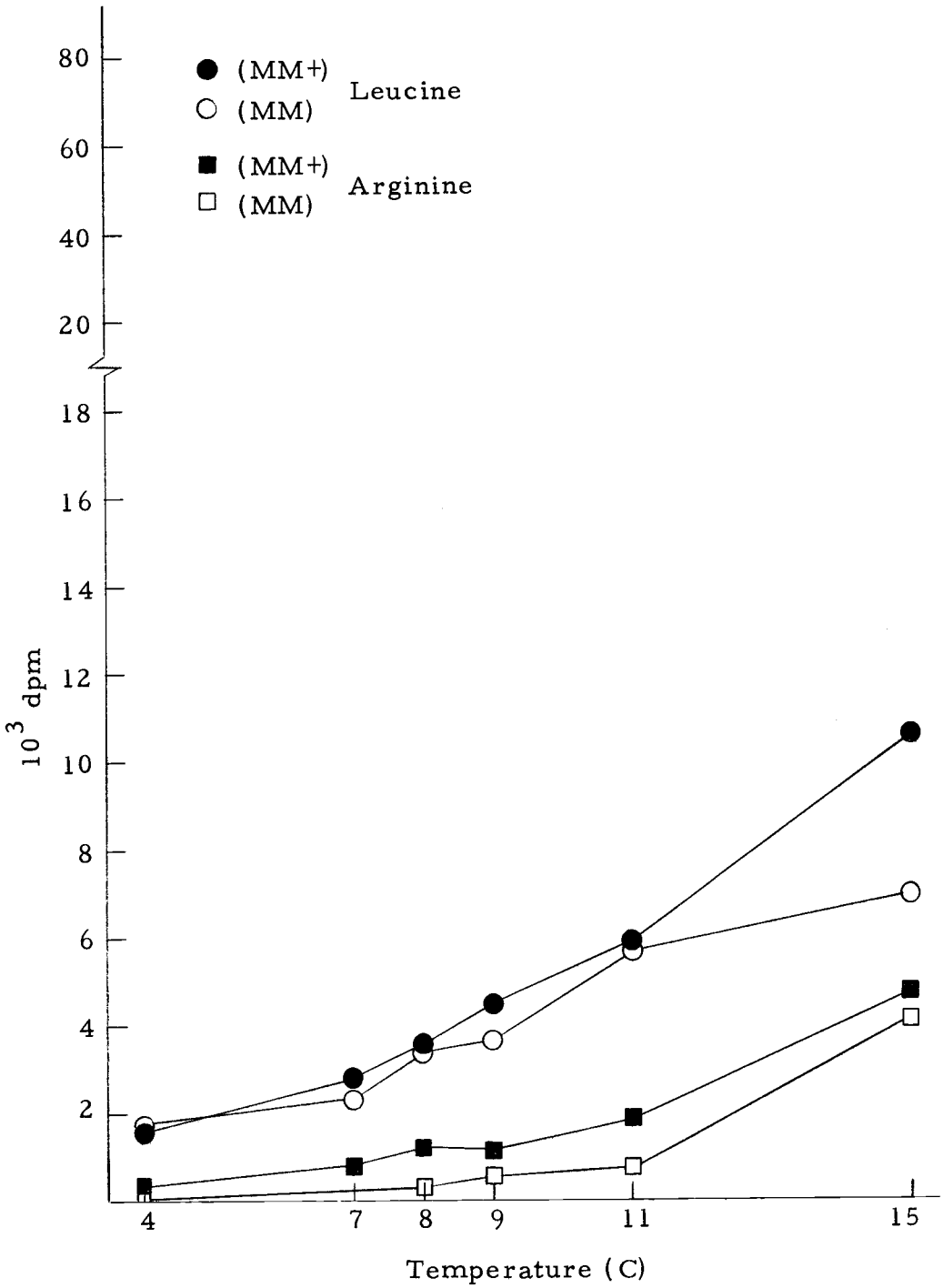

Figure 5. Uptake profile of leucine and arginine by UC41 in MM and MM+. One-hour uptake of each amino acid was examined for cells previously grown at 15 C.

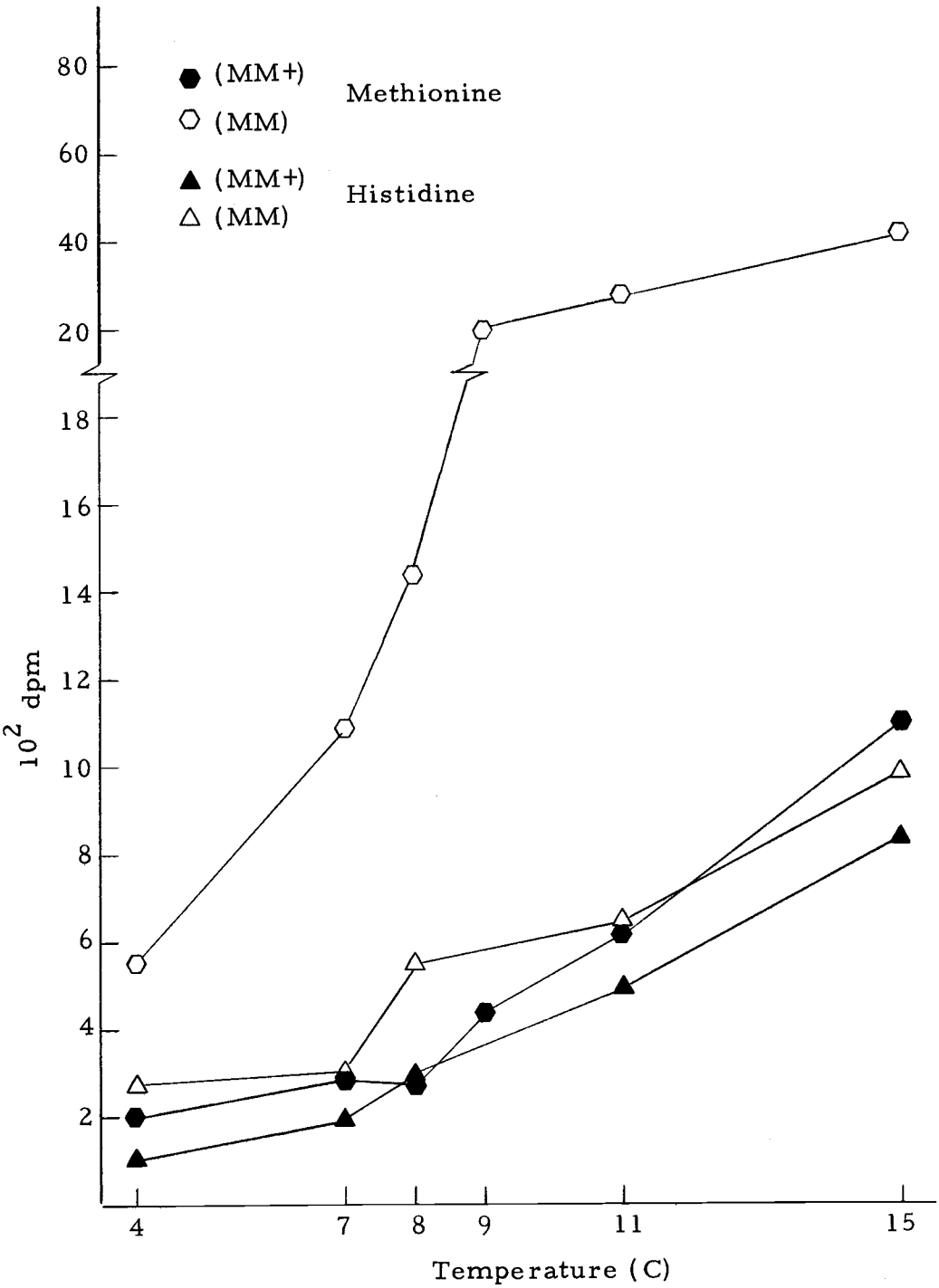

Figure 6. Uptake profile of histidine and methionine by UC41 in MM and MM+. One-hour uptake of each amino acid was examined for cells previously grown at 15 C.

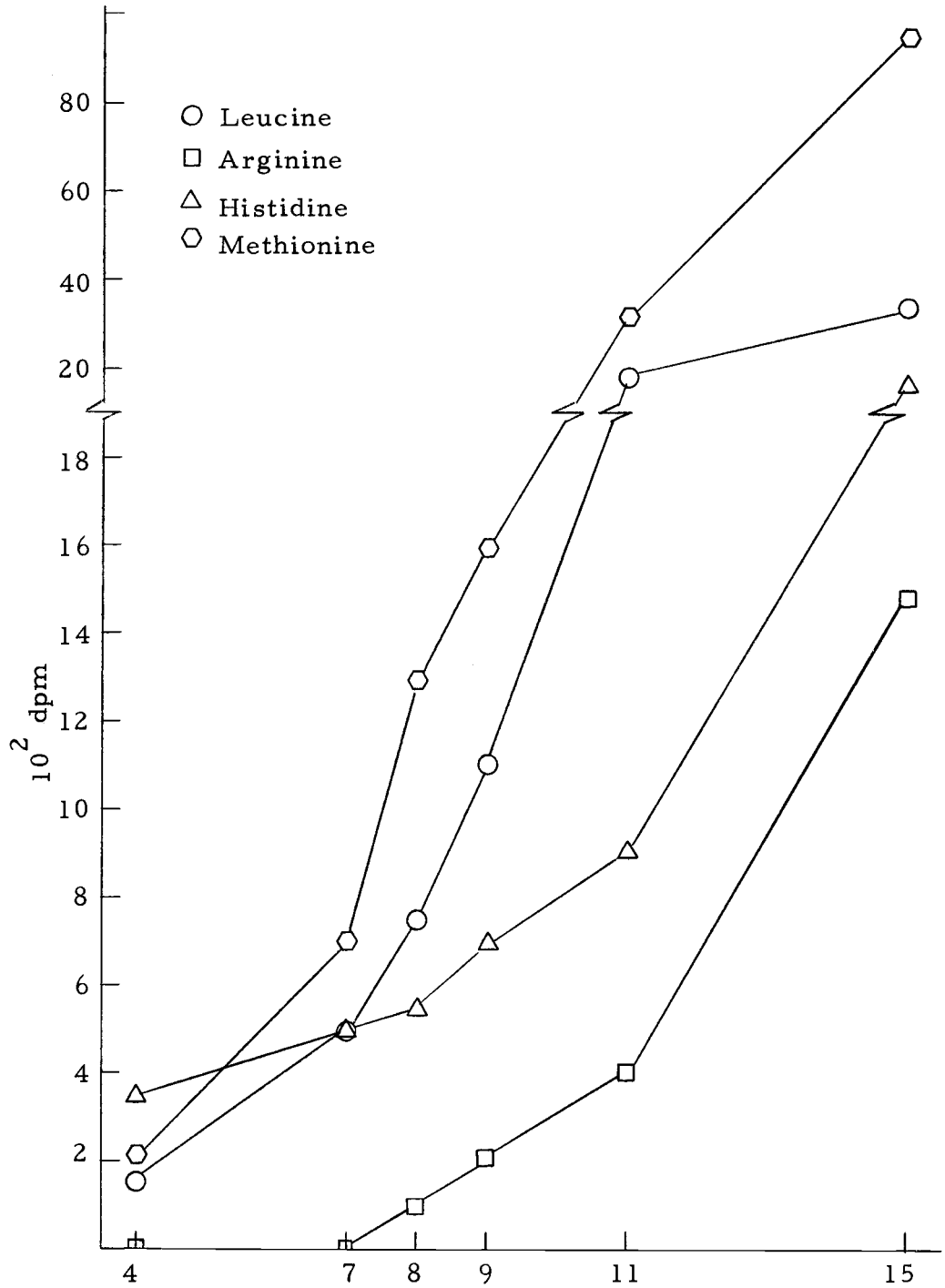

Figure 7. Uptake profile by UC41 in 0.5 g/l nutrient broth. One-hour uptake of each amino acid was examined for cells previously grown at 15 C.

examined (Tables 7, 8, 9). Labelled leucine was not respired by UC41 in any of the test media. No respiration of C-14-arginine was seen in MM until 11 C, at which temperature 12.4% of the total arginine taken up was respired. In MM+ arginine was respired at an average of 15% over the 4 C to 15 C range. UC41 did not respire arginine at 4 C or 7 C in 0.5 g/l nutrient broth; however, respiration was evident at and above 8 C (Table 9).

The auxotroph's respiration of C-14-methionine in MM was much less than it was in MM+. Methionine was respired only at 15 C in 0.5 g/l nutrient broth. Of the total labelled histidine taken up, approximately the same percentage was respired in both MM and MM+ while a higher percentage was respired in nutrient broth.

Uptake comparisons were studied for UC175 in each medium (Figures 8, 9, 10 and Tables 10, 11, 12). Similar patterns were seen to exist in total uptake and respiration by UC175 as existed in UC41 with the following exceptions. Levels of uptake in 0.5 g/l nutrient broth by UC175 were much less than by UC41. UC175 respired labelled methionine only at 15 C in all three media. Respiration of C-14-arginine did not occur until 11 C in 0.5 g/l nutrient broth.

Effects of various washes on retention of labelled amino acids.

Using 1% NaCl as the wash, UC41 uptake of labelled methionine was relatively low (below the minimum growth temperature) but quickly increased at and above 8 C. Distilled water-washed cells showed a similar pattern with a smaller increase above the medium growth temperature. The uptake pattern derived from the acid wash was like previous

TABLE 7. RESPIRATION BY UC41 IN MM+.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	4.0	1,768	0	0.0
	7.0	2,806	0	0.0
	8.0	3,589	0	0.0
	9.0	4,507	0	0.0
	11.0	5,839	0	0.0
	15.0	10,647	0	0.0
arginine	4.0	399	68	17.0
	7.0	791	98	12.4
	8.0	1,293	100	7.7
	9.0	1,247	163	13.1
	11.0	1,850	260	14.1
	15.0	4,756	818	17.2
methionine	4.0	229	53	23.1
	7.0	319	67	21.0
	8.0	298	57	19.1
	9.0	425	106	24.9
	11.0	626	135	21.6
	15.0	1,098	318	29.0
histidine	4.0	154	51	31.1
	7.0	216	58	25.2
	8.0	411	56	13.3
	9.0	388	45	11.3
	11.0	560	53	9.3
	15.0	810	76	9.2

Table 8. Respiration by UC41 in MM.

Substrate	Temperature of uptake °C	Total substrate available µg	Amount of substrate taken up µg	Amount of substrate taken up molecules	Percentage of total taken up %	Amount of substrate respired µg	Amount of substrate respired molecules	Percent respired %
C-14-leucine	4	0.0391	3.4×10^{-4}	62,100	0.87	0	0	0
	7		4.5	81,500	1.16	0	0	0
	8		6.8	122,800	1.73	0	0	0
	9		7.1	128,600	1.81	0	0	0
	11		10.9	198,300	2.79	0	0	0
	15		13.5	245,600	3.45	0	0	0
C-14-arginine	4	0.0444	0.3×10^{-4}	4,000	0.07	0	0	0
	7		*	-	-	-	-	-
	8		0.6×10^{-4}	8,200	0.14	0	0	0
	9		1.1	14,900	0.25	0	0	0
	11		2.0	26,900	0.44	2.4×10^{-5}	3,300	12.4
	15		10.9	148,200	2.45	7.7	6,400	4.3
C-14-methionine	4	0.539	1.6×10^{-4}	25,500	0.30	8.0×10^{-6}	1,300	5.0
	7		3.2	49,700	0.59	9.6×10^{-6}	1,500	3.0
	8		4.2	66,200	0.78	1.2×10^{-5}	1,800	2.8
	9		5.9	92,700	1.09	1.6	2,500	2.7
	11		7.9	124,800	1.47	1.9	2,900	2.4
	15		12.4	195,000	2.30	5.3	8,400	4.3
C-14-histidine	4	0.0404	0.8×10^{-4}	12,300	0.20	2.1×10^{-5}	3,200	25.7
	7		0.9	13,600	0.22	2.1	3,200	23.3
	8		1.7	25,000	0.41	2.6	3,800	15.1
	9		1.3	20,200	0.33	2.4	3,800	18.9
	11		1.8	27,600	0.45	2.2	3,400	12.4
	15		2.8	42,400	0.69	3.3	5,000	11.8

* lab accident.

TABLE 9. RESPIRATION BY UC41 IN 0.5 G/L NUTRIENT BROTH.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	4.0	114	0	0.0
	7.0	356	0	0.0
	8.0	477	0	0.0
	9.0	754	0	0.0
	11.0	1,279	0	0.0
	15.0	3,378	0	0.0
arginine	4.0	0	0	0.0
	7.0	0	0	0.0
	8.0	81	16	19.8
	9.0	220	39	17.7
	11.0	431	90	20.9
	15.0	1,531	314	20.5
methionine	4.0	258	0	0.0
	7.0	757	0	0.0
	8.0	1,350	0	0.0
	9.0	1,661	0	0.0
	11.0	3,556	0	0.0
	15.0	9,521	158	1.7
histidine	4.0	397	257	64.7
	7.0	502	268	53.4
	8.0	560	253	45.2
	9.0	721	266	36.9
	11.0	953	286	30.0
	15.0	1,899	316	16.6

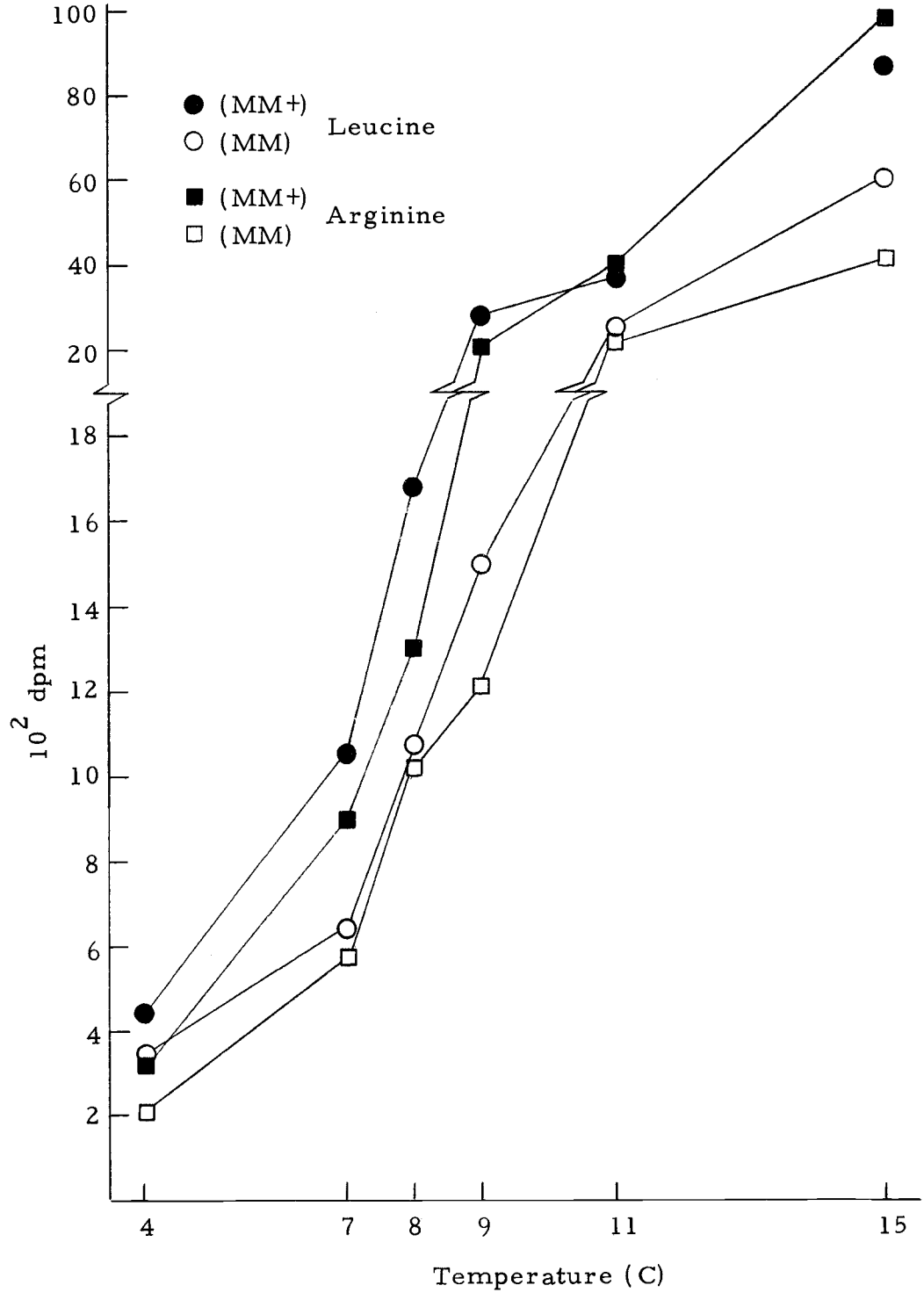

Figure 8. Uptake profile of leucine and arginine by UC175 in MM and MM+. One-hour uptake of each amino acid was examined for cells previously grown at 15 C.

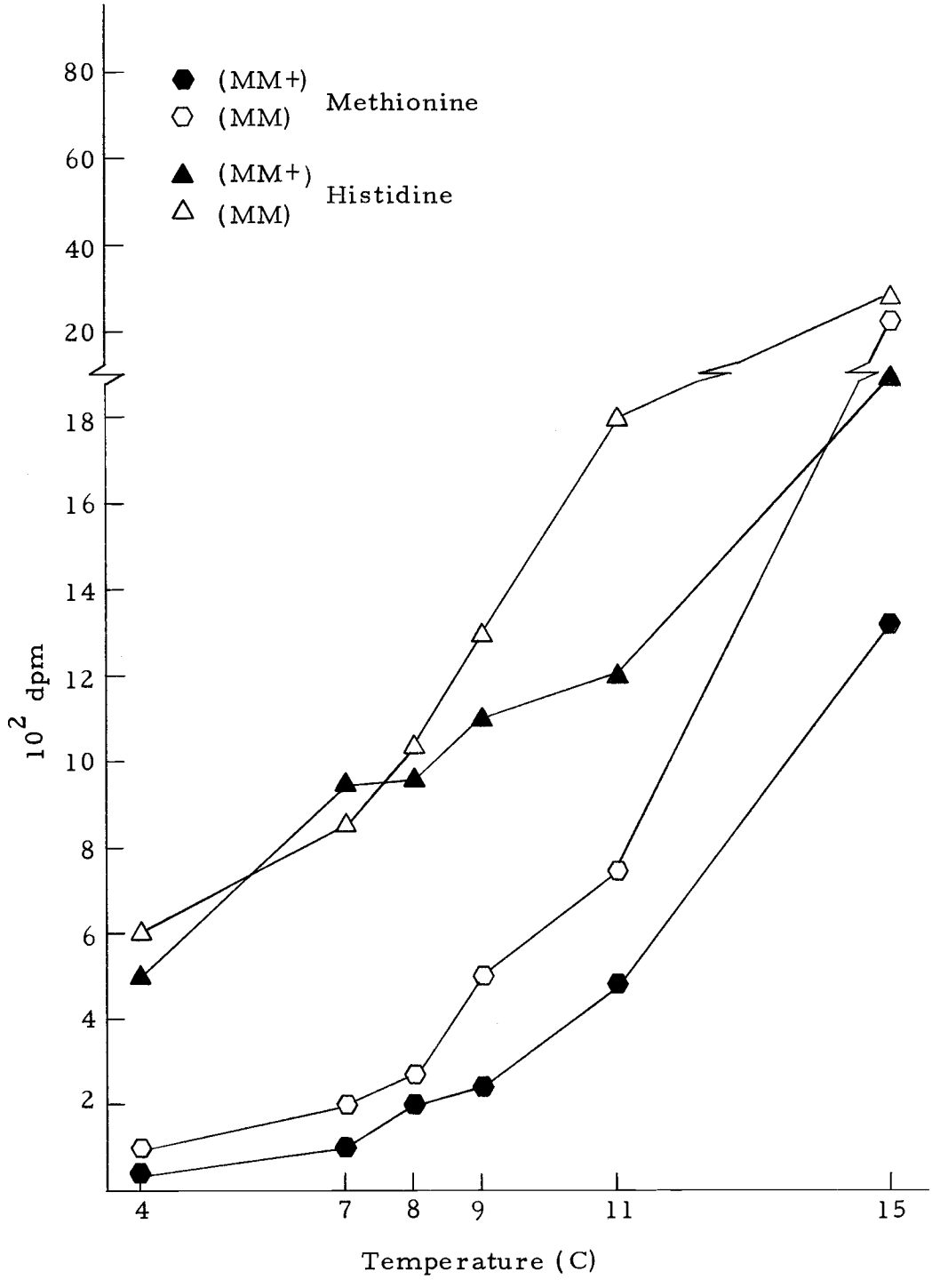

Figure 9. Uptake profile of histidine and methionine by UC175 in MM and MM+. One-hour uptake of each amino acid was examined for cells previously grown at 15 C.

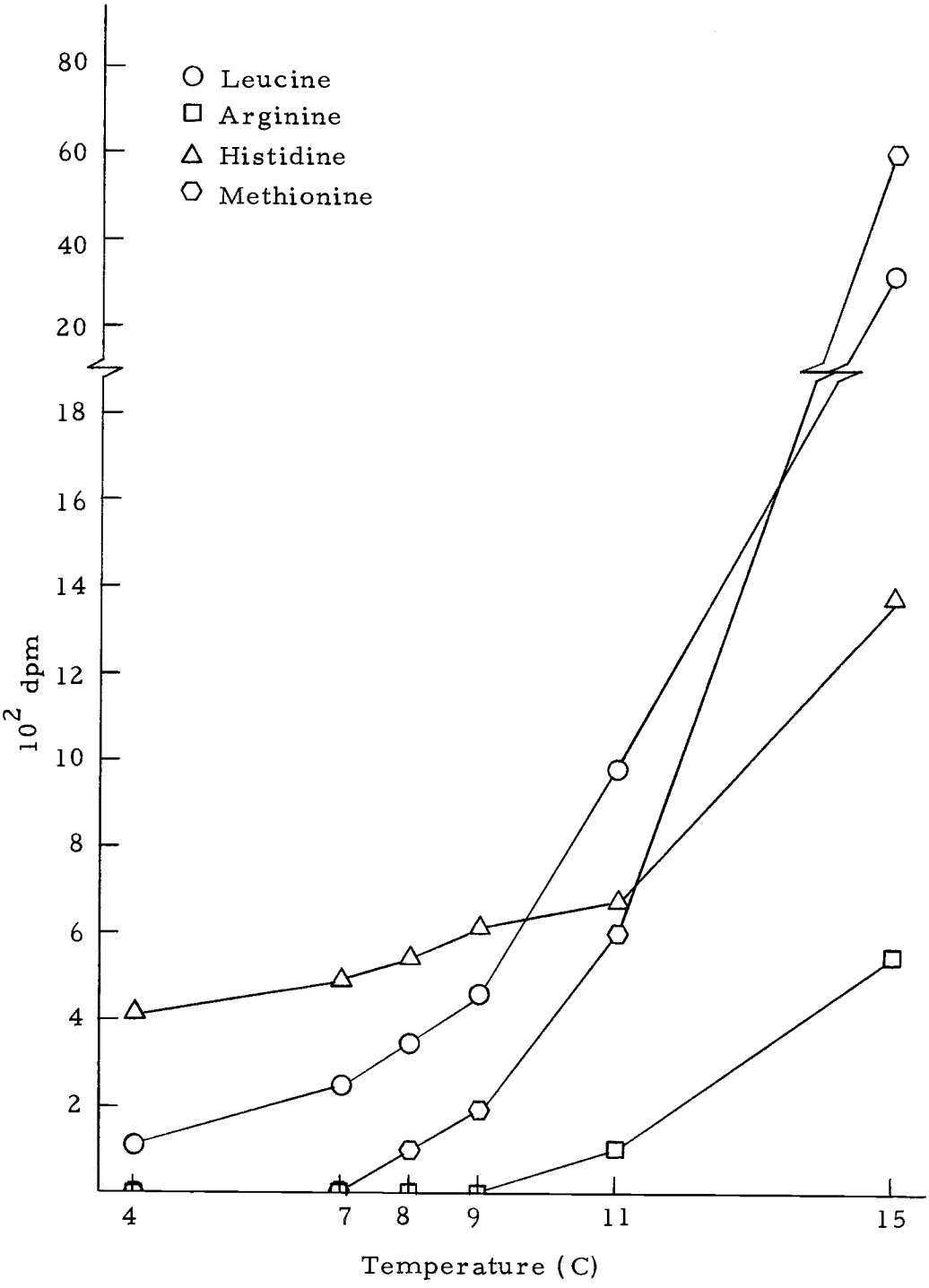

Figure 10. Uptake profile by UC175 in 0.5 g/l nutrient broth. One-hour uptake of each amino acid was examined for cells previously grown at 15 C.

TABLE 10. RESPIRATION BY UC175 IN MM+.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	4.0	421	0	0.0
	7.0	1,033	0	0.0
	8.0	1,688	0	0.0
	9.0	2,585	0	0.0
	11.0	3,515	0	0.0
	15.0	8,497	0	0.0
arginine	4.0	370	82	22.1
	7.0	876	88	10.0
	8.0	1,149	121	10.5
	9.0	2,038	139	6.8
	11.0	3,739	216	5.7
	15.0	9,810	376	3.8
methionine	4.0	58	0	0.0
	7.0	119	0	0.0
	8.0	227	0	0.0
	9.0	253	0	0.0
	11.0	481	0	0.0
	15.0	1,337	35	2.6
histidine	4.0	481	208	43.2
	7.0	941	208	22.1
	8.0	990	252	25.4
	9.0	1,101	262	23.8
	11.0	1,192	269	22.6
	15.0	1,899	325	17.1

Table 11. Respiration by UC-175 in MM.

Substrate	Temperature of uptake °C	Total substrate available µg	Amount of substrate taken up		Percentage of total taken up	Amount of substrate respired		Percent respired
			µg	molecules	%	µg	molecules	%
C-14-leucine	4	0.0360	0.6×10^{-4}	11,700	0.18	0	0	0
	7		1.3	22,800	0.35	0	0	0
	8		2.1	37,800	0.58	0	0	0
	9		2.8	50,800	0.78	0	0	0
	11		4.8	86,800	1.33	0	0	0
	15		11.8	213,900	3.27	0	0	0
C-14-arginine	4	0.0713	0.6×10^{-4}	8,700	0.09	1.5×10^{-5}	2,100	24.4
	7		1.5	20,700	0.21	2.1	2,900	14.1
	8		2.6	34,800	0.36	2.8	3,700	10.7
	9		3.2	43,900	0.45	2.9	4,100	9.3
	11		5.4	93,500	0.76	3.8	5,500	7.0
	15		10.2	139,200	1.43	4.7	6,500	4.6
C-14-methionine	4	0.1023	0.2×10^{-4}	3,600	0.02	0	0	0
	7		0.5	7,500	0.05	0	0	0
	8		0.8	12,800	0.08	0	0	0
	9		1.4	22,400	0.14	0	0	0
	11		2.1	33,800	0.21	0	0	0
	15		6.6	104,700	0.65	3.0×10^{-5}	4,800	4.6
C-14-histidine	4	0.0473	1.7×10^{-4}	26,000	0.36	8.2×10^{-5}	12,600	48.3
	7		2.4	36,800	0.51	8.4	12,800	34.8
	8		2.9	44,200	0.61	8.8	14,500	30.4
	9		3.6	55,100	0.77	9.5	14,500	26.4
	11		5.1	77,900	1.08	10.5	16,100	20.6
	15		8.0	122,200	1.70	12.3	18,800	15.4

TABLE 12. RESPIRATION BY UC175 IN 0.5 G/L NUTRIENT BROTH.

C-14-substrate	Temperature (C)	Total dpm taken up	dpm respired	Per cent respired (%)
leucine	4.0	129	0	0.0
	7.0	211	0	0.0
	8.0	310	0	0.0
	9.0	474	0	0.0
	11.0	978	0	0.0
	15.0	3,209	0	0.0
arginine	4.0	0	0	0.0
	7.0	0	0	0.0
	8.0	0	0	0.0
	9.0	0	0	0.0
	11.0	102	19	18.6
	15.0	562	83	14.8
methionine	4.0	0	0	0.0
	7.0	0	0	0.0
	8.0	101	0	0.0
	9.0	208	0	0.0
	11.0	631	0	0.0
	15.0	4,906	29	0.6
histidine	4.0	454	338	74.4
	7.0	475	314	66.1
	8.0	516	327	63.4
	9.0	586	345	58.9
	11.0	703	345	49.1
	15.0	1,407	368	25.0

uptake patterns observed for C-14-methionine (Figure 11).

UC175 exhibited similar uptake patterns. Below 8 C there was little labelled mthionine associated with cells when washed with 1% NaCl and with distilled water; however, when washed with acid, the same uptake pattern as in earlier experiments was obtained (Figure 12).

Lability of the uptake mechanism to cold osmotic shock.

The cold osmotic shock procedure of Neu and Heppel (24) was used to examine the nature of the amino acid-cell association. UC41 was subjected to osmotic shock and subsequently tested for its ability to take up labelled histidine. Following equilibration the cells amassed nearly identical quantities of C-14-histidine at all temperatures. At t=15 minutes the auxotroph had taken up nearly equal amounts of substrate from 4 C to 8 C while uptake at 15 C was increased. After one hour, relatively little uptake was noted below 8 C with the amount of uptake increasing from 8 C to 15 C (Figure 13).

Twenty-four hour uptake analysis.

The uptake of labelled leucine by UC41 in MM+ was studied at various temperatures after a 24-hour incubation period (Figure 14). After this period of incubation, cells incubated at or above 8 C were capable of removing all available leucine from the solution.

Kinetics of uptake and respiration.

The auxotroph exhibited a decreasing turnover time (T_t) of C-14-arginine with increasing temperature (Table 13). The uptake constant,

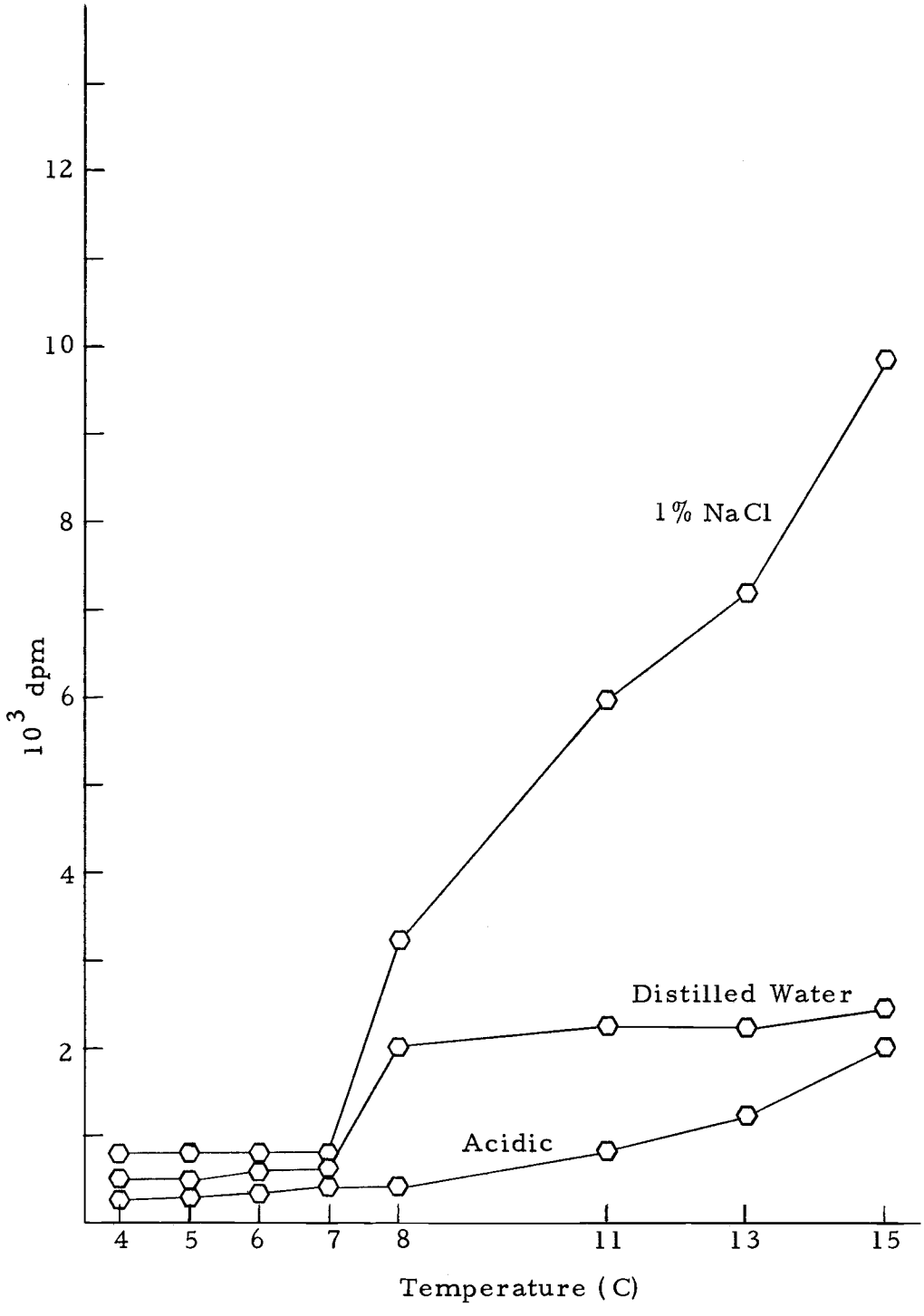

Figure 11. Effect of various washes on the association of methionine with UC41. One-hour uptake of the amino acid was examined for each wash. Cells were previously grown at 15 C.

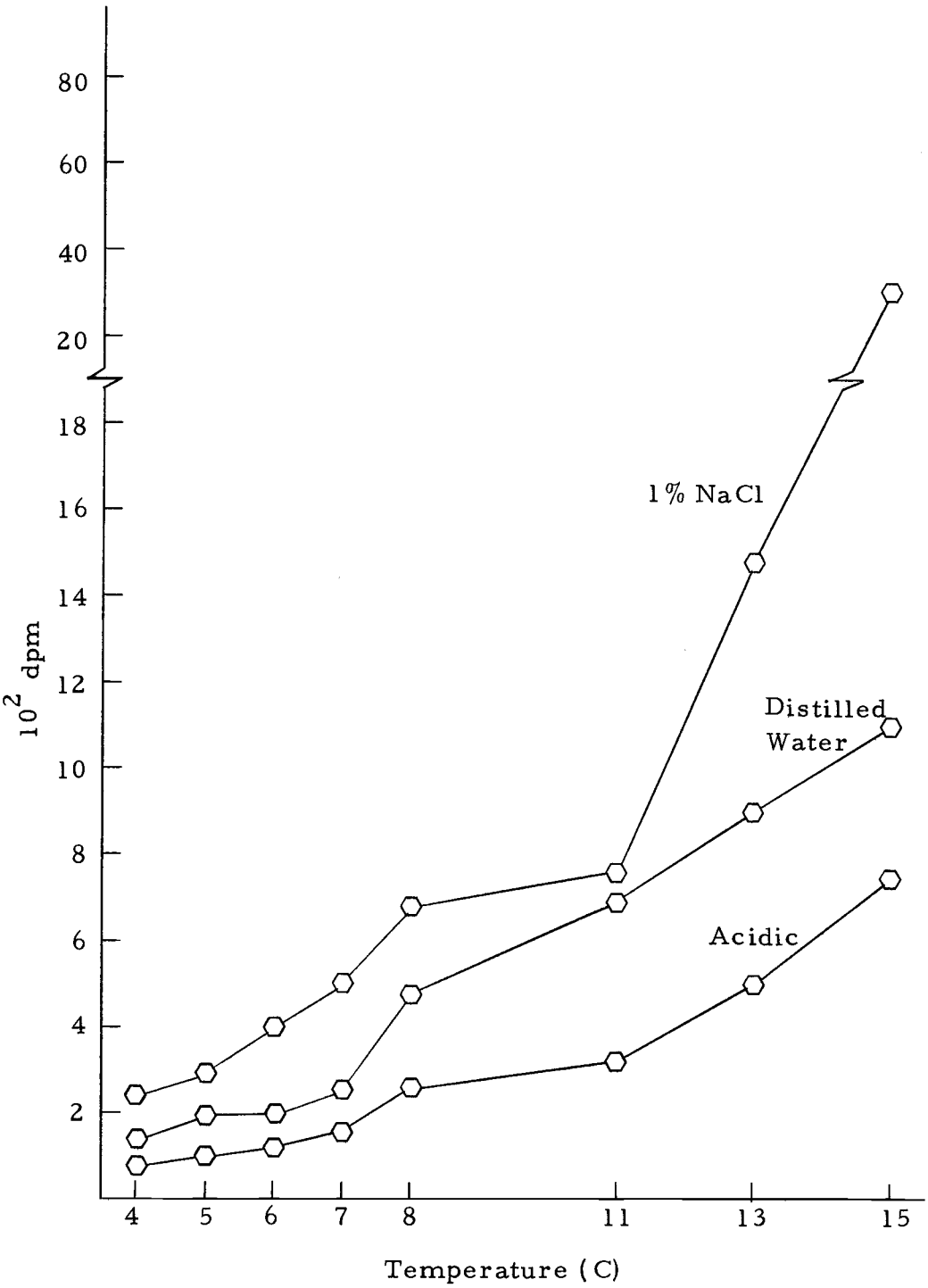

Figure 12. Effect of various washes on the association of methionine with UC175. One-hour uptake of the amino acid was examined for each wash. Cells were previously grown at 15 C.

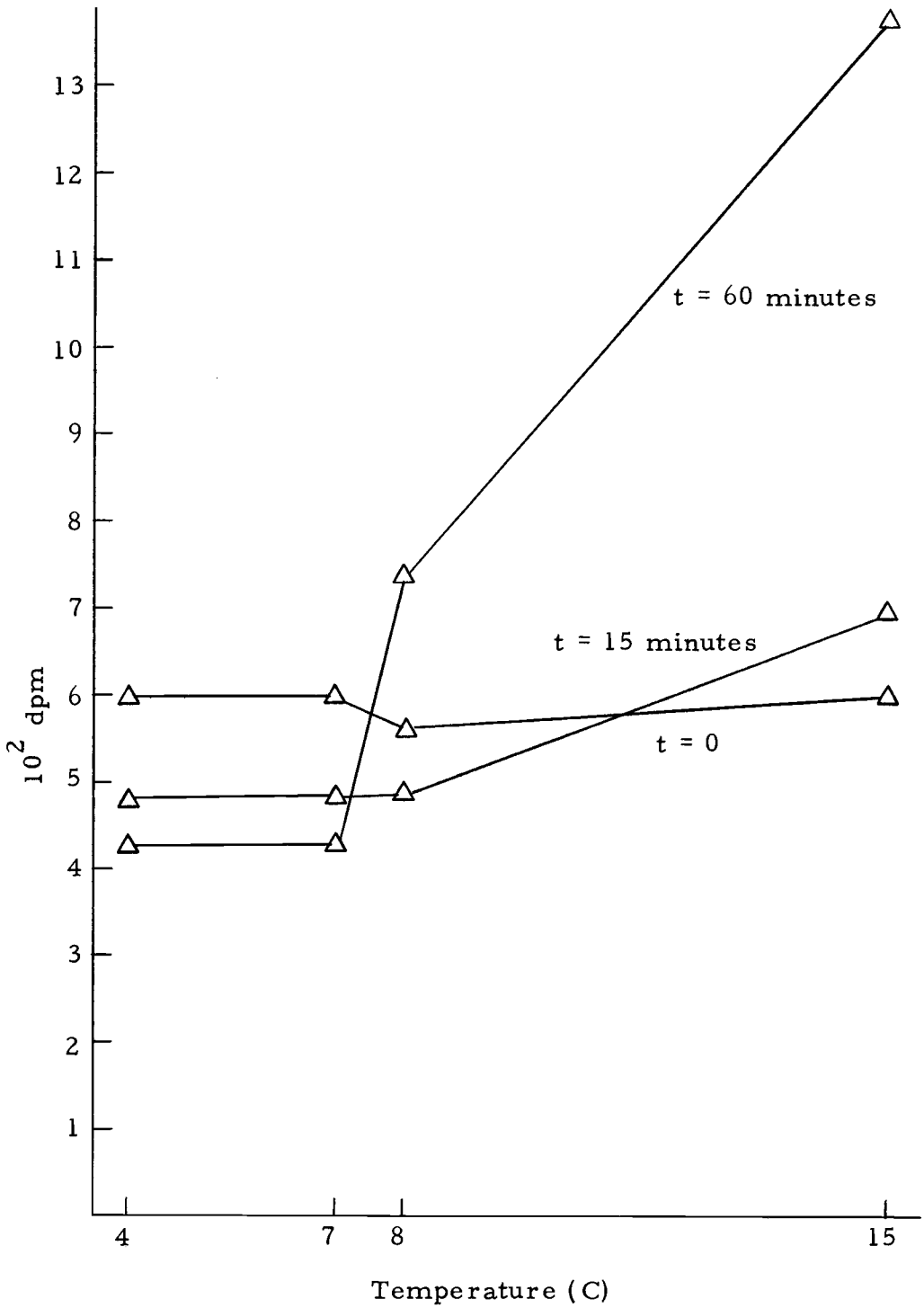

Figure 13. Effect of osmotic shock on the uptake of histidine by UC41. After equilibration (time indicated above) the labelled amino acid was added to the cells and immediately filtered. Cells were grown at 15 C prior to shock procedure.

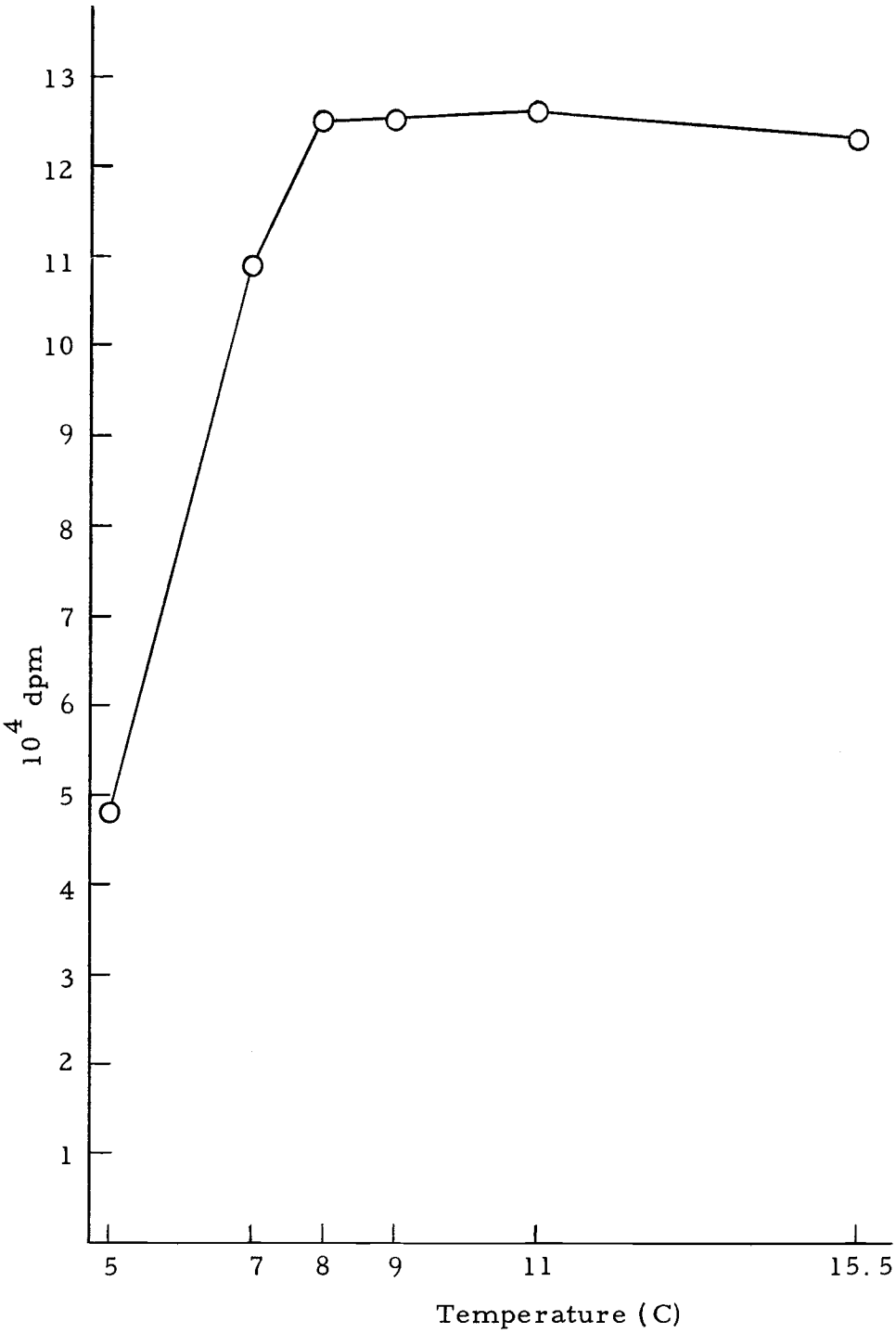

Figure 14. Twenty-four hour uptake of leucine by UC41. The cells were previously grown at 15 C and the uptake was examined in MM+.

TABLE 13. UPTAKE AND RESPIRATION KINETICS OF ARGININE BY UC41.

	Total Uptake			Respiration		
Temperature (C)	Vmax[a] (ug/l/hr)	T_t[b] (hr)	K_t[c] (ug/hr)	Vmax (ug/l/hr)	T_t (hr)	K_t (ug/hr)
4.0	0.212	15.1	3.2	0.128	29.5	3.8
7.0	0.285	11.0	3.1	0.138	29.1	4.0
8.0	0.245	7.0	1.7	0.110	26.9	3.0
9.0	0.282	4.7	1.3	0.109	24.6	2.7
15.0	0.435	2.5	1.1	0.150	19.2	2.9

[a] Vmax is the maximal velocity for mineralization of the substrate (which would apply when transport is at maximal velocity.

[b] T_t is the time necessary to turnover the concentration of substrate added.

[c] K_t is a constant related to uptake.

K_t, varied depending on whether the cells were incubated above or below the minimum temperature. Below 8 C the K_t was about 3.1 µg/l while above 8 C the K_t was only about 1.3 µg/l. From these same data, a separate analysis of the respiration of arginine also showed decreasing turnover time with increasing temperature. The K_t values were also different above and below the minimum growth temperature.

DISCUSSION

Minimum temperature of growth.

Shaw et al. (35), Hoffman (12), and Ingraham (14) have shown the minimum growth temperature for E. coli is between 7.5 C and 8.0 C in glucose minimal medium. Experiments with UC41 and UC175 using cells grown at 37 C gave different minimum growth temperatures depending on the strain. When comparing the minimum growth temperatures of UC41 and UC175 in MM+, 10.5 C and 12.5 C were obtained, respectively, when determined by both absorbance determinations and spread plate counts. The minimums are considerably higher than the 8.0 C expressed by Ingraham (14). Quite possibly the abrupt change from logarithmic phase at 37 C to 0 C centrifugation and washing did irreparable damage to the cells, particularly at the lower temperatures tested. This would be in keeping with the findings of several investigators (13, 23, 25, 26, 37). The differences of minimum growth temperatures obtained may be a result of a physiological property of the strains themselves--the 2 C lower minimum expressed by UC41 compared to that expressed by UC175.

When the two organisms were grown at 15 C prior to determination, a minimum growth temperature between 7.5 C and 8.0 C was attained by UC41 and UC175. This could indicate an adaptation through probably a physiological change, thus, less "damage" or shock to the cell occurred when the temperature is lowered for centrifugation and washing.

Amino acid uptake followed by acidic wash.

Only with regard to uptake of C-14-leucine did UC41 and UC175 show

any similarity of increasing uptake with increasing temperature for cells grown at 37 C. UC175 displayed a superiority of uptake ability. One could assume that the wild type is expressing the superior uptake ability that it had at 37 C. Little or varied uptake of the other three amino acids may reflect, in part, some of the damage caused by lowering the cells from 37 C to 0 C centrifugation and washings. The arginine uptake mechanism is inoperable for both strains over the temperature range employed while the mechanisms for methionine and histidine uptake show relatively little or no increasing uptake with increasing temperature.

Upon lowering the growth temperature from 37 C to 15 C, all subsequent uptake patterns showed increasing uptake with increasing temperature, as one would expect, assuming the rate of uptake is dependent upon the uptake temperature. Also with increasing temperature, molecular velocities are increased, thus, increasing the probability of molecules coming in contact with the uptake mechanism. In general, the uptake ability of UC41 was far greater than that of UC175 over the range 4 C to 15 C. This is indicated in the uptakes in MM, MM+, and 0.5 g/l nutrient broth (Figures 5, 6, 7, 8, 9, 10); however, as previously shown, they both have the same minimum growth temperature.

One might assume that UC41 is better able to adapt to the near minimum temperature and therefore out-compete its wild type component. It may be that the mutations induced in UC41 were of such a nature that, since the organism could not biosynthesize its own amino acids, more effort and energy was placed into the production of more uptake sites on the cell membrane thus increasing its uptake capacity. This could

parallel the fact that since the wild type can produce its own amino acids, normal or minimal complement of uptake mechanisms is maintained regardless of the environmental conditions within its environmental limitations.

Another possible explanation of the greater uptake ability of the auxotroph could lie in the arrangement or placement of the uptake sites on the cell membrane. It is possible that the change in temperature (37 C to 15 C) to near minimum might better expose the uptake mechanisms of UC41 thus increasing its uptake capacity, or, conversely, the change might partially hide the uptake sites of UC175, therefore, decreasing its apparent capabilities.

Konings and Freese (16), Lombardi and Kaback (18), Piperno and Oxender (29), and Short et al. (38) have shown that the specific uptake mechanisms of leucine, arginine, histidine, and methionine are all separate and independent of one another. This would seem logical since there is no common link shared among or between the amino acids with regard to their intracellular biosynthetic pathways. Thus when examining the effect that different media have on the apparent uptake ability, predictable if not totally explainable results are obtained. First of all in the MM and MM+ comparison, leucine uptake was nearly identical for both UC41 and UC175 up to 15 C in both media. Therefore, the other amino acids do not appear to have an enhancing or inhibiting effect on the leucine uptake mechanism. The increasing uptake at 15 C by both organisms in MM+ may be a result of growth or multiplication since UC41 cannot reproduce without all four amino acids being present. This could also be the case with UC175 since glucose and the other three

amino acids are available for energy and building blocks in MM+ while they are not contained in MM.

Arginine uptake was enhanced by the presence of glucose and the three amino acids. Again this may be a result of greater need for growth factors (i.e., arginine) in the presence of energy and building blocks. Methionine showed better uptake in MM without any additions by both UC41 and UC175. One could assume that the other three amino acids or glucose was partially inhibiting the uptake of methionine and this would seem to be the more logical conclusion. UC41 uptake of histidine was nearly the same in both media with a slight possible inhibition expressed by the presence of glucose and the three amino acids; UC175 uptake of histidine showed a definite inhibition in the presence of the additions, again probably competitive in nature.

Uptake studies in 0.5 g/l nutrient broth yielded both expected and unexpected results. Uptake of both leucine and arginine was effectively inhibited by the quantities of leucine and arginine indigenous to nutrient broth as one would expect. Arginine uptake inhibition was the more devastating particularly with respect to UC175. UC41 did not take up arginine or respire it below the minimum growth temperature. This could be the result of the lower uptake capabilities below 8 C in correlation with probable competition by arginine and other basic amino acids in the nutrient broth.

Histidine uptake was not changed by the presence of nutrient broth with regard to UC175 while a near two-fold increase of uptake in 0.5 g/l nutrient broth by UC41 was obtained. Uptake of methionine particularly above 8 C by both strains was greatly enhanced by the presence of

nutrient broth. These enhancements were unexpected in light of the inhibitions found in the experiments employing MM+ and MM. It seems unlikely that nutrient broth would physically act to enchance the uptake of either histidine or methionine. It would seem more likely that the intracellular metabolism or biosynthesis which is carried out during the hour incubation might require increased quantities of methionine and histidine. This may indeed be the case with methionine since DNA synthesis being carried out in the cell could require higher quantities of methionine for methylation purposes. The enhancement of histidine uptake in UC41 is less tangible particularly since histidine would probably in the presence of easier utilizable carbon energy sources be used solely as a protein building block. One cannot rule this out since one does not have a quantitative analysis of the amounts of histidine in 0.5 g/l nutrient broth. There may be little histidine (or methionine for that matter), yet excess amounts of arginine and leucine, in nutrient broth. Assuming a higher metabolic and biosynthetic rate in 0.5 g/l nutrient broth over the rate in MM or MM+ one might expect to see greater uptake of all four amino acids which in this case would be expressed by only histidine (and methionine) while competitive inhibition would shield this phenomenon in regard to leucine and arginine.

Effects of various washes on retention of labelled amino acids.

An effort was made to look at the type or types of association of the amino acid substrates with the cell, particularly the uptake mechanism association. To study this a 1% NaCl solution was used to

remove any unbound or unassociated substrate without removing any substrate which could be in any way associated with the cell. A distilled water wash was employed to remove all the substrate that was loosely associated with or bound to the outside of the cell without causing any internal loss. The acidic wash was employed to remove all that was bound to the outside plus any substrate which was neither involved in cellular respiration or in cellular metabolism or biosynthesis.

Acid-washed cells of UC175 and UC41 involved in methionine uptake gave uptake curves comparable to the curves obtained in the previous experiments all of which employed the acid-wash technique (Figures 3 and 4). For both strains the 1% NaCl wash showed very little association or binding of methionine below 8 C followed by accelerated association or binding at and above 8 C. The distilled water wash also showed little tightly bound or associated methionine below 8 C whereas above 8 C a higher degree of association was seen. The curves for the distilled water wash show, particularly with respect to UC175, an almost flat curve above 8 C, that is, not a rapid increase with increasing temperature, which nearly parallels the acidic wash curve. This would seem to indicate a specific number of sites or specific volume (or area) to which only a specific number of molecules of this particular substrate can be associated or bound. They do not appear to exist below 8 C. These sites or areas are subject to removal by acidic wash. It is significant, however, that the curves below 8 C are nearly the same with respect to all three washes with only a small difference in the amounts of substrate associated among the washes. This

may be an important factor in the mechanism of minimum temperature of growth, particularly since methionine in the form of formyl-methionine is important in initiation of protein synthesis.

Lability of the uptake mechanism to cold osmotic shock.

Many workers (1, 2, 3, 15, 17, 24, 26, 28, 39, 40) have reported the loss of specific binding proteins and enzymes as a result of cold osmotic shock. The cells remain viable and the proteins can be added back to the cells and are functional. An experiment was set up to examine the osmotic shock effect on uptake near the minimum growth temperature. Following the shock procedure and after a fifteen minute equilibration at the test temperature a comparative examination was made with cells given labelled histidine at various times. The initial curve (t=0) is flat indicating that the amount of histidine associated with the cells would not seem to be a function of temperature or of the uptake mechanism--a non-specific binding (Figure 13). After fifteen minutes at the test temperature (t=15) before addition of the labelled histidine, the curve is again flat from 4 C through 8 C with an increase in uptake becoming apparent at 15 C. At t=60 minutes, the curve is flat below the minimum temperature of growth, at 8 C there is an increase in uptake and at 15 C even greater increase. Therefore, fifteen minutes (or less) after equilibration at 15 C following cold osmotic shock, the binding protein for histidine is again actively taking up labelled histidine. It would seem likely that a new binding protein has been synthesized to replace that which had been lost through osmotic shock. After one hour following equilibration the 8 C cells

had significantly produced histidine-binding protein while at 15 C the cells appeared to have near normal levels of binding protein for histidine. Cells equilibrated at 7 C showed no apparent synthesis of new binding protein. One would not expect synthesis according to the findings of Das and Goldstein (7) and Friedman et al. (9).

Twenty-four hour uptake analysis (acidic wash).

Cells of UC41 were harvested aseptically and allowed to take up labelled leucine for 24 hours. At 8 C and above, the auxotroph removed all the available leucine from the medium as evidenced by the leveling of the curve above 8 C (Figure 14). Below 8 C lower levels of uptake were obtained indicating a temperature-dependent rate of uptake. This study would seem to indicate that the facility for taking up substrate is functional for at least leucine below the minimum growth temperature and the rate of uptake below the minimum may in some manner be important in determining the minimum growth temperature.

Possible relationships of energy of maintenance to uptake.

Several investigators (19, 20, 22, 30, 31) have reported an energy requirement for E. coli maintenance. The results for uptake in MM without any additives may lend themselves to estimations for energy of maintenance. Converting dpm/tube to ug/tube and finally to molecules/cell, one gets a number which may reflect the amount of substrate necessary to maintain a cell, expecially at and below the minimum temperature of growth of the cell. The results would indicate that the cells have different minimum requirements for each of the amino acids

tested. Examining the leucine uptake by UC175 (Table 11) about 23,000 molecules/cell were taken up at 7 C in one hour while 38,000 molecules/cell were taken up at 8 C. Both UC41 and UC175 were able to maintain their populations at 7 C for at least 168 hours. Assuming therefore, that UC175 is capable of extended maintenance at 7 C but not reproduction, this would suggest that 23,000 molecules/cell of leucine are necessary to maintain UC175 at 7 C for one hour in the absence of other carbon and energy sources. The remaining 15,000 molecules/cell taken up at 8 C could be that amount of energy which must be taken up over the maintenance amount to allow reproduction. Additional estimations could be made regarding energy derived from the other three amino acids in this manner.

SUMMARY

Both strains of E. coli K12, UC41 and UC175, had a minimum growth temperature of 8 C in glucose minimal medium containing the amino acids histidine, arginine, leucine, and methionine when grown at 15 C prior to testing. The auxotroph has a greater relative ability to bind and take up the four C-14-labelled amino acids in direct comparison experiments. The auxotroph having an apparent physiological adaptation enabled it to better compete particularly at near minimum temperatures. UC41 did not take up C-14-arginine below the minimum growth temperature in 0.5 g/l nutrient broth.

Cold osmotic shock removed the specific histidine-binding protein of UC41. When incubated at near minimum temperatures following the removal, the binding protein was re-synthesized and functional at and above 8 C within one hour but not below 8 C indicating a lack of re-synthesis of that particular binding protein.

Washes of 1% NaCl, distilled water, and sulfuric acid-NaCl following incubation and filtration of UC41, which was taking up C-14-methionine, did not affect the association of the labelled substrate with the cell below 8 C. This was not the case at and above 8 C where the 1% NaCl wash showed a large amount of methionine associated with the cells. The differences between the amounts of cell-associated methionine following the acidic wash and the distilled water wash yielded a near constant level of association over the temperature range 8 C to 15 C. This could be interpreted as being a specific number of binding sites or a specific volume (pool size). The finding may be particularly important in determining a minimum temperature of growth.

Methionine was important in the synthesis of protein--formyl-methionine being an essential part of the initiation complex--if it was absent the initiation complex of ribosomes to mRNA will not form. Since UC41 could not synthesize its own methionine it must rely on the uptake of it from the environment. It has been shown that the organism was unable to bind methionine below the minimum growth temperature in a manner similar to that at and above the minimum. This temperature-dependent binding was probably responsible for the minimum growth temperature of the auxotroph. It may be possible that the wild type was governed by a similar mechanism. It may be possible that the wild type was governed by a similar mechanism. It may be that the enzyme necessary for the formylation of the methionine was non-functional below 8 C.

An estimation of the number of molecules of a substrate which was necessary for the maintenance of a cell at 7 C was made. With this as a reference point, the amount of additional substrate taken up at 8 C was estimated as the amount of substrate necessary for reproduction of a cell.

A kinetic analysis of C-14-arginine uptake and respiration revealed a distinct pattern below the minimum growth temperature from that at and above the minimum, with regard to the uptake constants. With respect to both processes the lower constants at and above the minimum growth temperature indicated a faster rate of reaction.

The data obtained from this study indicated a direct relationship between minimum growth temperature and binding, uptake, and respiration. The inability of E. coli incubated below 8 C to bind methionine

at levels similar to levels above 8 C, the lower levels of uptake and respiration below the minimum growth temperature, and the higher uptake constants below 8 C all lent evidence for the direct relationship.

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