ENERGETICS OF AMINO ACID UPTAKE IN THE PHOTOSYNTHETIC HALOPHILE *ECTOTHIORHODOSPIRA HALOPHILA*

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ENERGETICS OF AMINO ACID UPTAKE

IN THE PHOTOSYNTHETIC HALOPHILE

ECTOTHIORHODOSPIRA HALOPHILA

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CHAPTER I

INTRODUCTION

Bacteria are capable of accumulating substrates and ions against concentration gradients utilizing active transport systems which require energy to function. Gram-negative organisms have been shown to possess two distinct classes of active transport systems which do not chemically modify the substrate.[2,3] Shock sensitive systems contain a dissociable binding protein in addition to the permease. These shockable systems are coupled to the phosphate bond energy of adenosine triphosphate (ATP) or a similar high energy compound by a mechanism not using the membrane adenosine triphosphatase (ATPase).[3,31] The second class are termed membrane-bound or respiratory-linked systems. These shock-resistant systems contain permeases but do not contain dissociable binding proteins. The driving force for the second type is not necessarily derived from ATP. Systems of the latter class have been extensively studied in membrane vesicles.[12] (for review) Some controversy remains as to the mechanism of energy coupling to these membrane bound systems. Initially, Kaback and coworkers[16] proposed a mechanism of oxidation-reduction coupling at discrete portions of the respiratory chain. Others[1,7,15] have interpreted their results as supporting Mitchell's chemiosmotic theory of membrane transport.[21,22] This theory states

that the energy-transducing reactions of electron transport, ATP synthesis, and active transport are linked energetically and mechanistically by the proton motive force. This force is derived from the efflux of protons during electron transport and/or by hydrolysis of ATP by the membrane ATPase.[1,3] The proton motive force is composed of transmembrane gradients of electrical and chemical potentials:

$$\Delta p = \Delta \Psi - z\ \Delta pH$$

where $\Delta \Psi$ is the membrane potential (interior negative), ΔpH is the pH gradient, and z is a factor to convert pH units into electrical units equalling about 59 mV/pH unit at 25°C. These separate components of the proton motive force have been studied individually by the use of specific ionophores and inhibitors.[6,30] From these studies Hamilton and Niven have formulated a model to explain the driving force for the uptake of various amino acids by Staphylococcus aureus. Basic amino acid uptake was driven by $\Delta \Psi$ in a uniport mechanism. Neutral amino acid uptake was driven by Δp in a H^+ symport mechanism, and acidic amino acid uptake was driven by $-z\Delta pH$ in a H^+ symport mechanism. A unique opportunity to test the underlying assumptions of the chemiosmotic theory was provided by the discovery of bacteriorhodopsin, a pigment on the cytoplasmic membrane of the extreme halophile, Halobacterium halobium. When this pigment is irradiated with visible light, a cyclic photochemical reaction occurs with extrusion and

uptake of protons. Thus, a chemiosmotic gradient is established by this novel reaction. Energy coupling mechanisms for transport of amino acids have been studied in this bacterium, and the results were interpreted as being consistent with the chemiosmotic theory of membrane transport.[10,20]

Only preliminary studies have been made on active transport systems of photosynthetic bacteria. In studies with Chromatium sp. strain D, it was concluded that this organism lacks systems of sufficiently high affinity for efficient amino acid transport.[29] The uptake of dicarboxylic acids, pyruvate and alanine have been studied in Rhodopseudomonas spheroides.[5,8] Ectothiorhodospira halophila differs from R. spheroides, the most studied photosynthetic bacteria, in that the latter, a member of the Athiorhodaceae, is able to grow both anaerobically in the light and aerobically in the dark. In addition to this metabolic difference, R. spheroides possesses its photosynthetic apparatus in membrane vesicles dispersed throughout the cytoplasm[4,8] while E. halophila contains a lamellar system.[26]

The paucity of data from this important class of organisms lends interest to this study. The biochemistry of E. halophila is also of interest to exobiologists because the possible Martian organisms have been postulated to possess its rare combination of extreme halophily, photosynthetic capabilities, and anaerobic growth.[9]

Ectothiorhodospira halophila was isolated from a sample

of salt encrusted mud from the shore of Summer Lake in Southern Oregon by Raymond and Sistrom in 1967.[26] The organism was not named at this time and was designated S.L.-1. Trüper in 1968 redescribed the genus Ectothiorhodospira which had been originally described by Pelsh in 1937.[28] Its species are gram-negative purple sulfur bacteria, spiral to vibroid in shape, motile by means of polar flagella, and dividing by binary fission. The bacteria are strictly anaerobic, photoautotrophic, and obligately photosynthetic. They are photosynthetic in the presence of oxidizable substrates such as reduced sulfur compounds. Globules of elementary sulfur are deposited extracellularly during growth. The pigments are bacteriochlorophyll a and carotenoids of the spirilloxanthin series. Molecular oxygen is not produced during photosynthesis.

Raymond and Sistrom[27] later characterized S.L.-1 as a member of the genus Ectothiorhodospira, naming it E. halophila because of its high salt requirement for growth. This organism differs from E. mobilis, the type species, in that E. halophila has single polar flagella rather than tufted polar flagella; its temperature optimum for growth is 47°C versus 25°C for E. mobilis. E. halophila requires at least 9% NaCl for growth as opposed to 2% for E. mobilis. E. mobilis requires vitamin B_{12} for growth and E. halophila does not. E. halophila also differs from the type species in being unable to utilize sulfate as its sole source of organic sulfur.

The optimum pH for growth of E. halophila is 7.4 - 7.9.

Cells lyse in less than 1-3% NaCl, grow well in 11-22% NaCl, and will grow slowly in 30% NaCl. Photosynthetic hydrogen donors are sulfide, sulfur and thiosulfate. Poly-β-hydroxybutyric acid is the storage material. Deoxyribonucleic acid base composition is 68.4 moles percent guanosine plus cytosine.

The Thiorhodaceae exhibit a broad range of morphological variation in their photosynthetic apparatus. Three main types are evident, all of which appear to be outgrowths or invaginations of the cell membranes.[4,23] These types include membranous vesicles, tubular membranes, and a lamellar system. E. halophila typically possess two bundles of lamella composed of 8-12 closely adpressed membrane pairs.[26] This structure consisting of stacks of lamella are analogous to the grana stacks of higher plants, and may be the most complex configuration yet seen in photosynthetic bacteria.

CHAPTER II

MATERIALS AND METHODS

Growth

Cells were grown in the medium described in Table 1 in 168 ml milk dilution bottles under incandescent light at 41.5°C. Each milk dilution bottle was inoculated with about 20 ml of a stock culture stored at 4°C.

Cell Suspensions

Two-day cultures were harvested by centrifugation, washed two times in a buffer of 1.0 molar NaCl and 0.02 molar tris(hydroxymethyl) aminomethanehydrochloride(Tris), pH 8.0, and resuspended in the same buffer to a turbidity of 150 Klett units in a Klett-Summerson colorimeter equipped with a No. 66 filter. This turbidity corresponds to 0.41 mg of cell protein/ml. All harvesting and washing operations were performed at 4-5°C. The cells were kept on ice under ordinary lab light prior to assay. The cells retained approximately 50% of their activity for the uptake of proline and glutamate 24 hours after harvesting. No cells over 24 hours old were used for transport assays. Protein was determined on aliquots of cells lysed in 0.02 molar Tris buffer using the method of Lowry *et al.*[19] with bovine serum albumin as the standard.

Table 1. Growth Medium for E. halophila
(per liter)

NaCl	-- 200 gm	NH_4Cl 10% Soln.	--	4 ml
Tris buffer pH 8.0	-- 40 ml	NaOH 2 M	--	3 ml
Phosphate buffer- 1 M pH 7.0	2 ml	Solution C(1)	--	5 ml
$(NH_4)_2SO_4$ 10% Soln.	-- 1 ml			

The following quantities are sterilized separately and added to the individual cultures as they are started.

Ascorbate, 5%	-- 10 ml*	Vitamin Mix(2)	0.021 ml*
$NaHCO_3$, 10%	-- 20 ml	Na Acetate, 20%	10 ml
$Na_2S_2O_3 \cdot 5H_2O$, 10%	-- 10 ml		

(1) Solution C (quantities/liter)

Nitrilotriacetic Acid	10.0 gm
$MgCl_2 \cdot 6H_2O$	24.0 gm
$CaCl_2 \cdot 2H_2O$	3.335 gm
$FeCl_3 \cdot 4H_2O$	1.071 gm
$NH_4Mo_7O_{24} \cdot 4H_2O$	0.090 gm
Trace element solution	50 ml

(2) Vitamin Mix (mg/ml)

Nicotinic Acid	2.0
Thiamine	1.0
p-amino benzoic acid	0.2
Biotin	0.02
Vitamin B_{12}	0.001

*Sterilized by Millipore filtration.

Amino Acid Uptake

Cell suspensions were incubated in 16 x 100 mm glass tubes at 41.5°C in a Braun Warburg apparatus equipped with incandescent lamps to the exterior of the plexiglass bath. The light intensity from the source was about $2mW \cdot cm^{-2}$ as measured with a Kettering-YSI Model 65 Radiometer. Dark incubations were conducted in aluminum foil covered tubes under laboratory lights reduced as far as practical. All assays were conducted with N_2 bubbling through the medium at about 80 cc/min. After specified preincubation of 5 ml cell suspension, usually 5 min., ^{14}C-amino acid was added, 0.5 ml was removed at intervals, the cells rapidly collected on membrane filters (Gelman GA-6, 0.47 micron, 25 mm dia.) and washed with 1 ml of chilled Tris-M NaCl buffer. Unless otherwise indicated the ^{14}C-amino acids were diluted in 10^{-4} M ^{12}C-amino acid to give a final concentration of 2×10^{-6} M in the incubation mixture. The filter disks were then placed in scintillation vials and dissolved in a solution containing 1 ml of water, 2 ml methyl cellosolve and 10 ml of a cocktail made of dioxane, 10% naphthalene (w/v) and 0.5% diphenyloxazole (w/v). Samples were counted on a Beckmann LS-100C liquid scintillation counter. All data points represent at least duplicate experiments. Many experiments were done in triplicate. Initial rates were determined over 4 min intervals immediately following ^{14}C-amino acid addition. All measurements were corrected for retention of ^{14}C-amino acid by the filters in zero time controls which

were 0.09 nmoles for ^{14}C-glutamate and 0.05 nmoles for ^{14}C-proline. Uptake of the lipid soluble cation ^{3}H-triphenylmethylphosphonium bromide (^{3}H-TPMP^{+}) was assayed in a similar manner as described for the amino acids. The membrane filters retained 0.8 nmoles of TPMP^{+} in zero time controls.

ATP Assays

Intracellular ATP was extracted by injecting 0.1 ml of cell suspension into 0.9 ml of boiling, 0.02 M Tris, pH 7.4 and boiling in a water bath for an additional two min. The ATP content of the extract was quantified using firefly assay as previously reported.[10]

Membrane Vesicles

Membrane vesicle preparations were made by ultrasonic disruption of spheroplasts. Cells suspended in 0.02 M Tris buffer containing 20% sucrose, and 0.01 M EDTA were treated with muramidase (0.5 mg/ml) for 20 min at room temperature. The spheroplasts were then centrifuged and resuspended in the Tris-M NaCl buffer to which was added 10 mM sodium ascorbate and 20 μM dichloroindophenol to maintain the proper redox potential.[8] Membrane vesicles were prepared by sonication of the spheroplasts for four 30-sec intervals using a Heat Systems Co. Sonifier Cell Disrupter equipped with a micro tip. The preparation was incubated with 0.1 mg/ml deoxyribonuclease (DNAase) for 15 min to reduce the viscosity and then centrifuged for 20 min at 5,000 x g to remove cell debris and any

remaining whole cells. The vesicles were then harvested by centrifugation for 20 min at 45,000 x g and resuspended using a syringe equipped with a 21 gauge needle. The preparation was examined microscopically to ascertain that no whole cells remained. All preparative operations were at 4° to 5°C.

Thin Layer Chromatography and Autoradiography

Thin layer chromatography was done on silica gel plates (Silica gel 60F-254) obtained from EM Laboratories, Inc. The solvent systems were sec-butyl alcohol: formic acid: water (7:1:2), and chloroform: 40% methanol: 17% NH_4OH soln. (4:4:2).

Autoradiography was accomplished by exposing duPont Cronex medical x-ray film to the thin layer plates. Developing and fixing reagents were Kodak liquid x-ray developer and replenisher (cat. 146 5335) and Kodak rapid fixer (cat. 146 4114).

Chemicals

U ^{14}C-L-proline (sp. act. 232 ci/mole) and U ^{14}C-L-glutamic acid (sp. act. 290 ci/mole) were obtained from New England Nuclear. 1-^{14}C-glutamic acid (sp. act. 4.84 ci/mole) was from Cal Biochem. Tritiated triphenylmethylphosphonium ion (sp. act. 114 ci/mole) was a gift from H. R. Kaback. Unlabeled triphenylmethylphosphonium bromide (K and K Laboratoreis) was used in a final concentration of 1 mM as an inhibitor and at 0.02 mM as a carrier for the labeled ion. N,N'-dicyclohexylcarbodiimide (DCCD) was from Eastman and was used in 0.1 mM final concentration. Carbonylcyanide m-chlorophenyl

hydrazone (CCCP), used at a 10 nM final concentration; 2-heptyl-4-hydroxy-quinoline-N-oxide (HQNO), used at a 0.4 mM final concentration; and valinomycin, used at 0.002 mg/ml final concentration were all from Sigma. The purified and stabilized luciferin-luciferase mixture was from du Pont. All other chemicals were reageant grade and were obtained from the standard sources. (See Appendix for a description of inhibitor actions.)

CHAPTER III

RESULTS

When compared at varying NaCl levels the initial rate for ^{14}C-glutamate uptake was maximum around 1.0 M NaCl (Figure 1). The initial rate of ^{14}C-proline uptake was highest between 1.0 and 2.0 M NaCl (Figure 1). Thus, 1.0 M NaCl was chosen as the salt concentration for both glutamate and proline assays so as to achieve highest activities and to simplify preparative procedures.

Several experiments were performed to ascertain that the amino acids were actually transported against a concentration gradient and not chemically altered by the cells. Analyses were made on ^{14}C-labeled amino acids which had been taken up by the cells and then recovered by lysing the cells with distilled water. In the case of ^{14}C-proline the lysate was evaporated and then extracted with absolute ethanol. Over 95% of the total ^{14}C was recovered in this ethanol soluble fraction. Thin layer chromatographic and autoradiographic analysis showed that over 90% of the ethanol soluble ^{14}C was contained in one spot which exhibited chromatographic behavior identical to authentic proline. A different analytical approach was used in the case of ^{14}C-glutamic acid. For these experiments the cells were allowed to

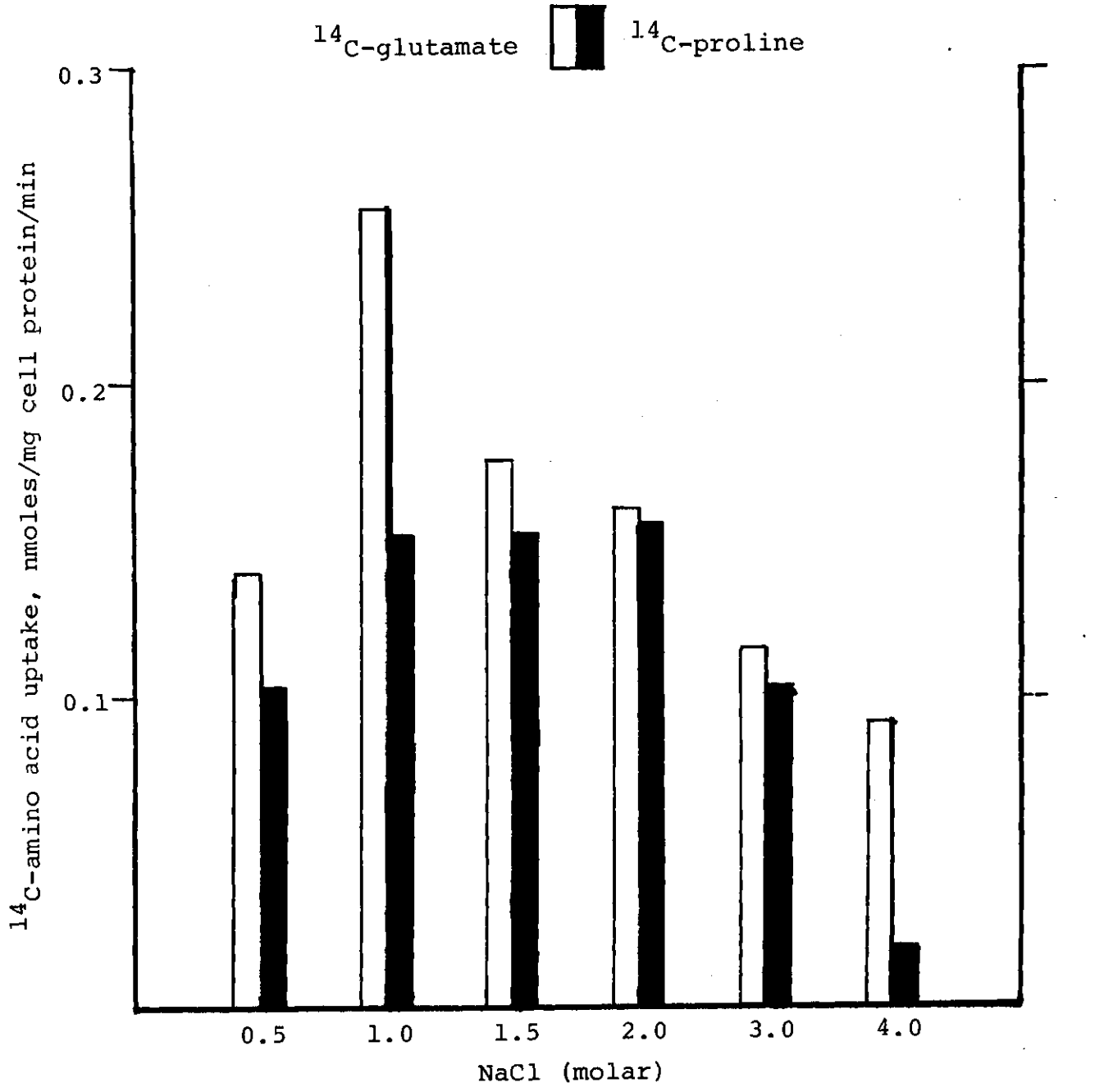


Figure 1. Initial Rates of Amino Acid Uptake at Varying Salt Concentrations in the Assay Medium.

accumulate 1-^{14}C-glutamate and then were lysed as above. Essentially all of the ^{14}C was recovered in the water soluble fraction. This ^{14}C extract was mixed with ^{12}C-L-glutamic acid and then treated with glutamate decarboxylase, an enzyme which specifically cleaves the number one carbon of L-glutamate. The CO_2 which evolved was trapped in hyamine hydroxide and its radioactivity determined. The finding that the ^{14}C-fraction recovered from the cells was decarboxylated to the same extent as authentic 1-^{14}C-glutamic acid was evidence that the accumulated amino acid had not been chemically altered.

An efflux of ^{14}C-proline occurs when an excess of ^{12}C-proline is added to the incubation after the cells have accumulated ^{14}C-proline; a loss of ^{14}C-proline from the cells also occurs when the light is turned off (Figure 2). These results suggest that the transport system is bilateral, and that exchange between intracellular and extracellular amino acids occurs. The addition of an excess of ^{12}C-glutamate caused a slow efflux of previously accumulated ^{14}C-glutamate (Figure 3). The difference in the efflux rates between proline and glutamate may well be attributable to the difference in the Km values for these two systems (see below). An alternative explanation is considered in the conclusions section.

Proline uptake in the light continues rapidly for about 30 min until a maximum intracellular concentration is accumulated (Figure 4). Double reciprocal plots[17] showed a Km of 3.0×10^{-7} M and a Vmax of 0.16 nmoles/mg cell protein/min.

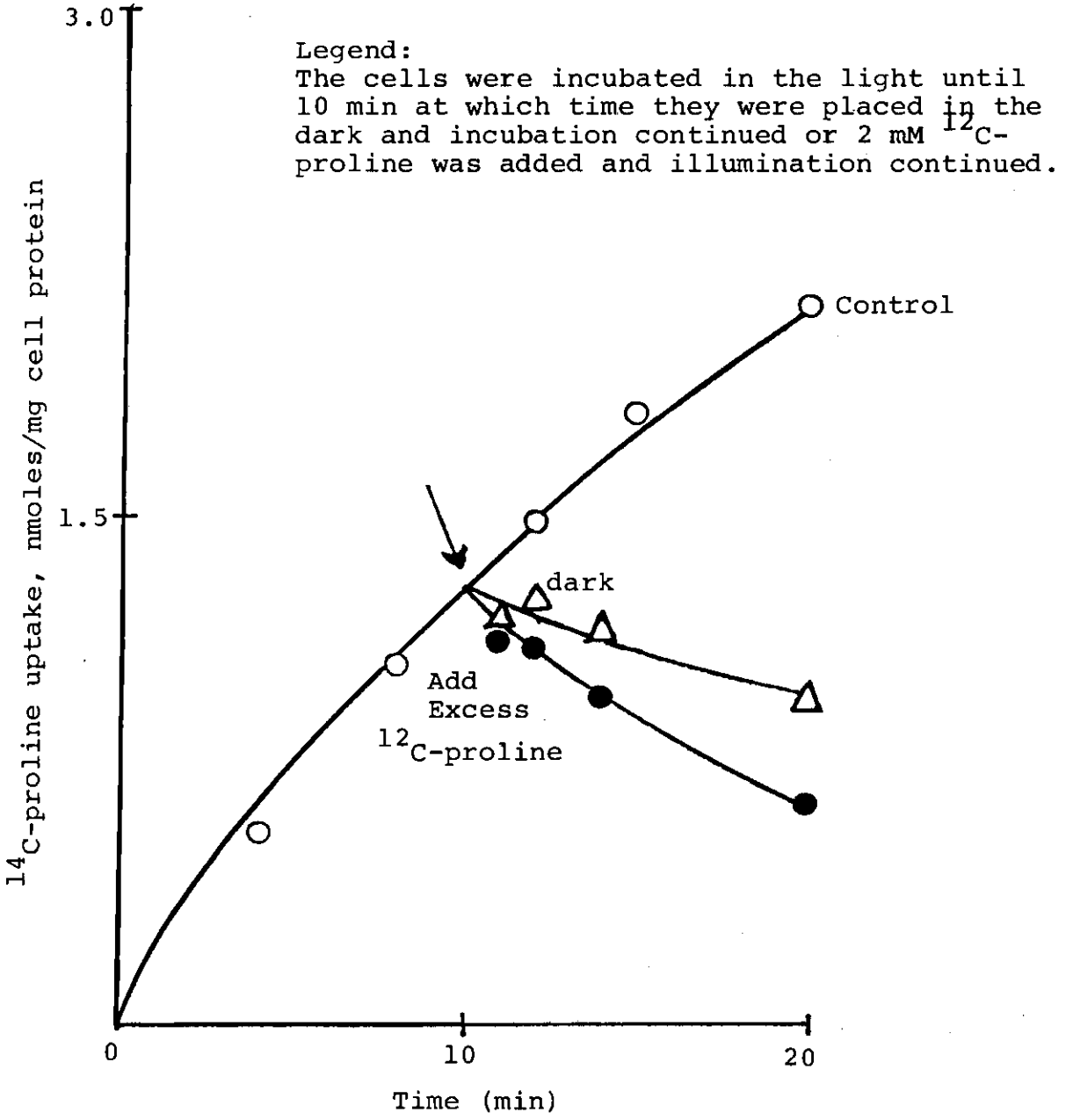


Figure 2. ^{14}C-Proline Efflux in the Dark or by the Addition of Excess ^{12}C-Proline.

Legend:
Assay conditions were as usual in the light. At 6 min, 2mM ^{12}C-glutamate was added and the illumination continued.

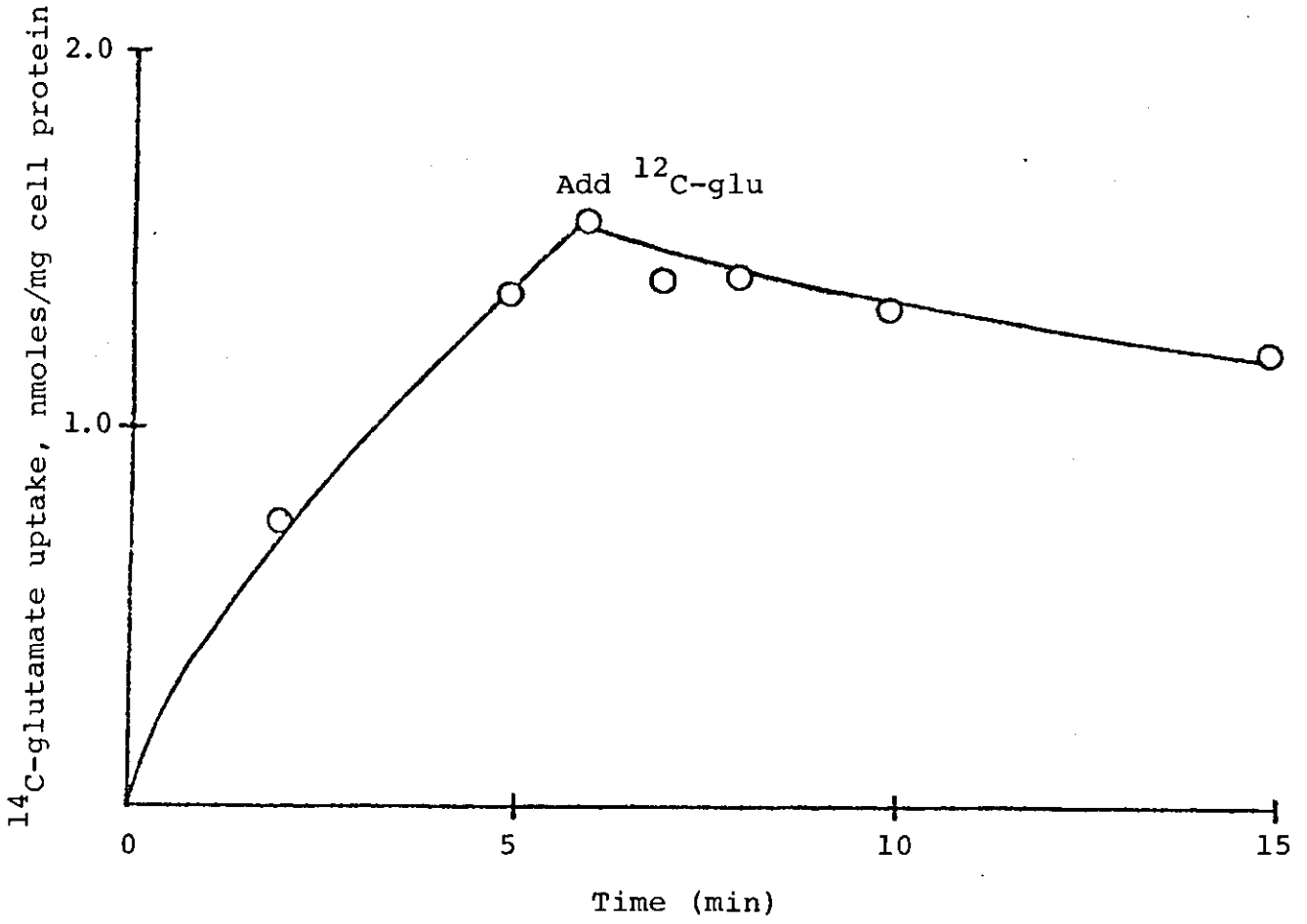


Figure 3. ^{14}C-Glutamate Efflux Caused by Excess ^{12}C-Glutamate.

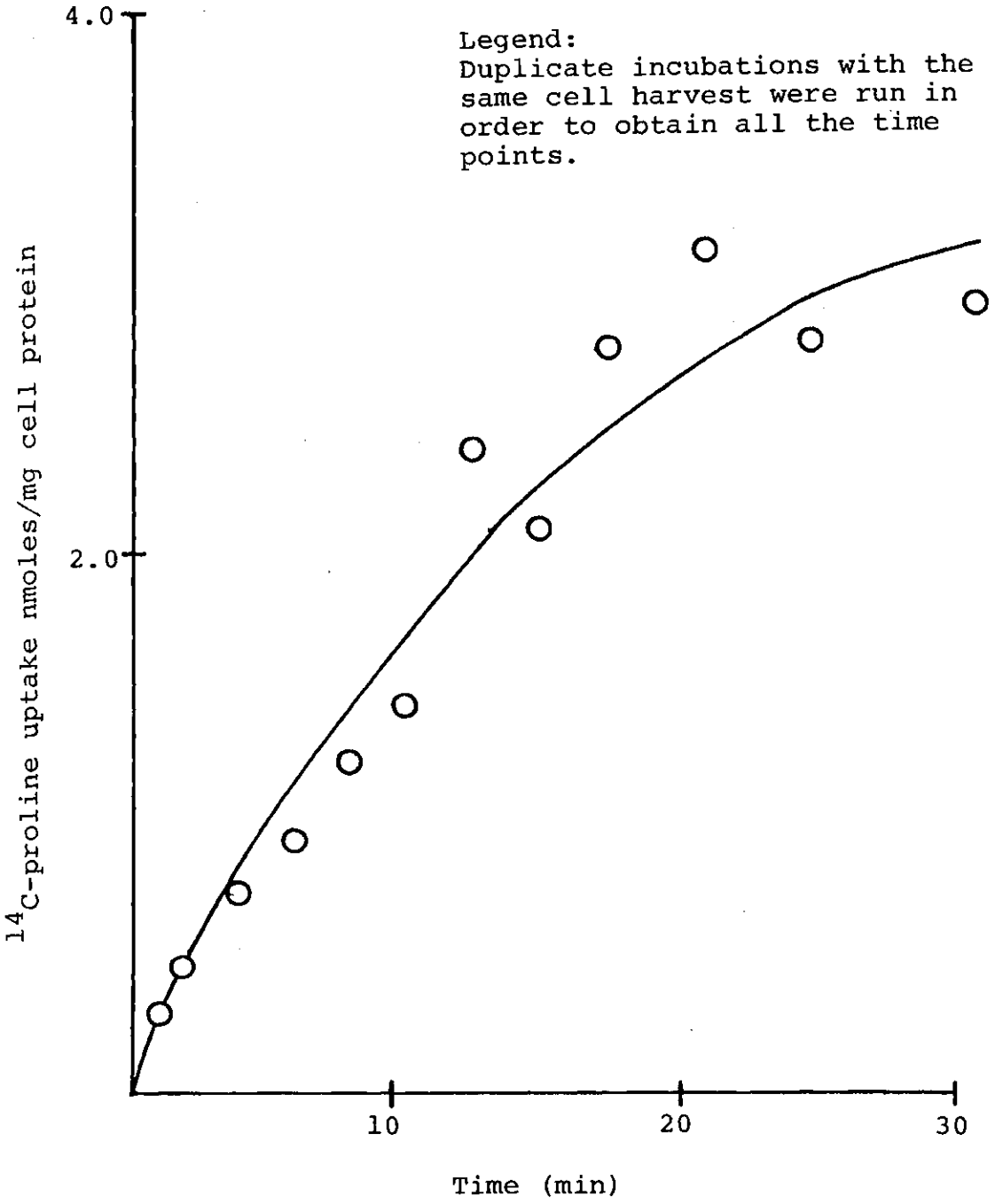
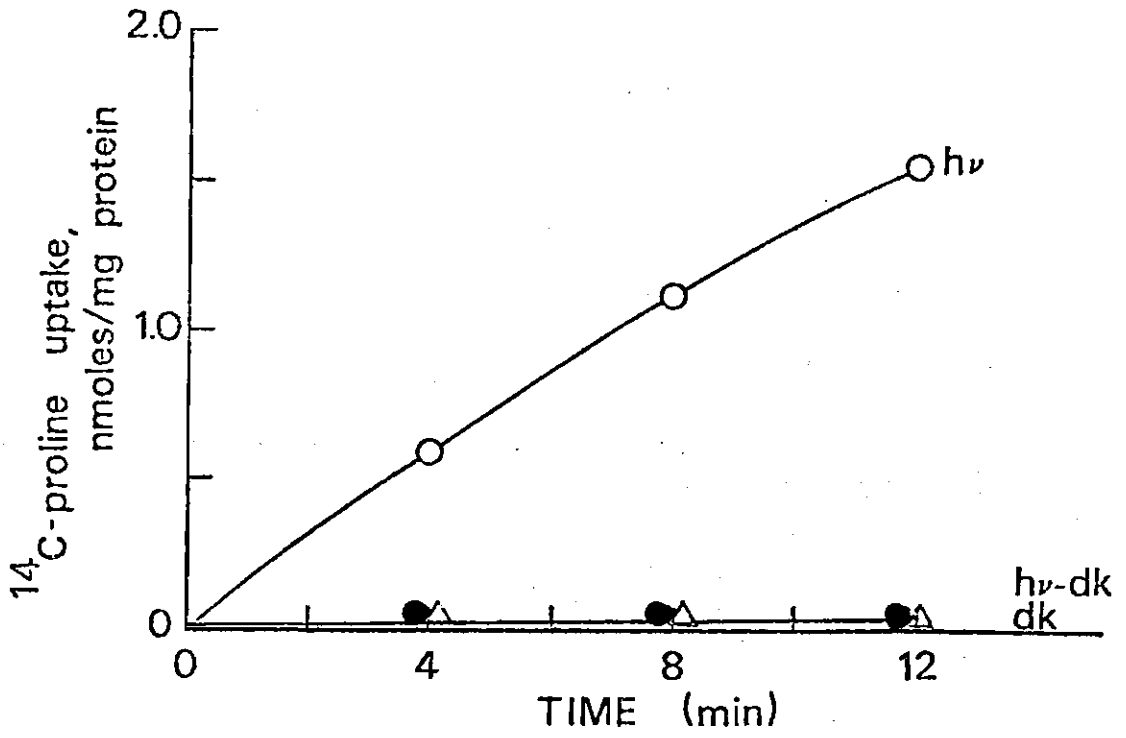


Figure 4. Time Course of ^{14}C-Proline Uptake by Illuminated Cells.

Proline uptake is inhibited by bubbling O_2 through the assay medium or by increasing the light levels to about 30 $mW.cm^{-2}$ (data not shown). Proline uptake does not occur in the dark, nor is proline uptake stimulated by preincubating the cells in the light, placing them in the dark, and immediately adding ^{14}C-proline (Figure 5).

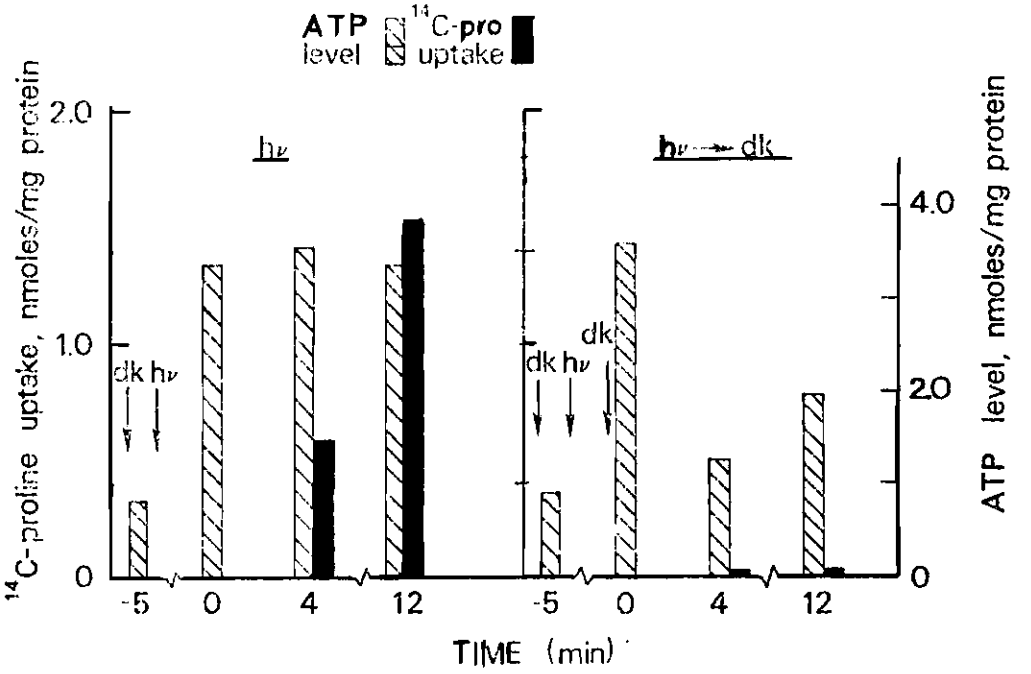
Photophosphorylation proceeds rapidly in the light in E. halophila under the standard assay conditions. Intracellular ATP levels reach a maximum of about 3.5 nmoles/mg of cell protein after 5 min in the light (Figure 6). This level is maintained during the course of amino acid uptake in the light. The intracellular ATP levels drop when illumination is discontinued. The ATP level maintained after preillumination is higher than the initial level, however. The high ATP levels generated by light preincubation do not stimulate uptake of ^{14}C-proline in the dark.

Carbonylcyanide m-chlorophenylhydrazone (CCCP), a proton permeant which reduces the proton motive force and the accompanying phosphorylation, almost completely inhibits proline uptake (Figure 7). 2-Heptyl-4-hydroxy-quinoline-N-oxide (HQNO), an inhibitor of photosynthetic electron transport, is also a strong inhibitor of proline uptake (Figure 7). The ATPase inhibitor, N,N'-dicyclohexylcarbodiimide (DCCD) stimulates uptake. DCCD has been shown to decrease cell permeability to the back flux of protons in E. coli.[25] If it affects E. halophila in a similar fashion, the stimulation of proline



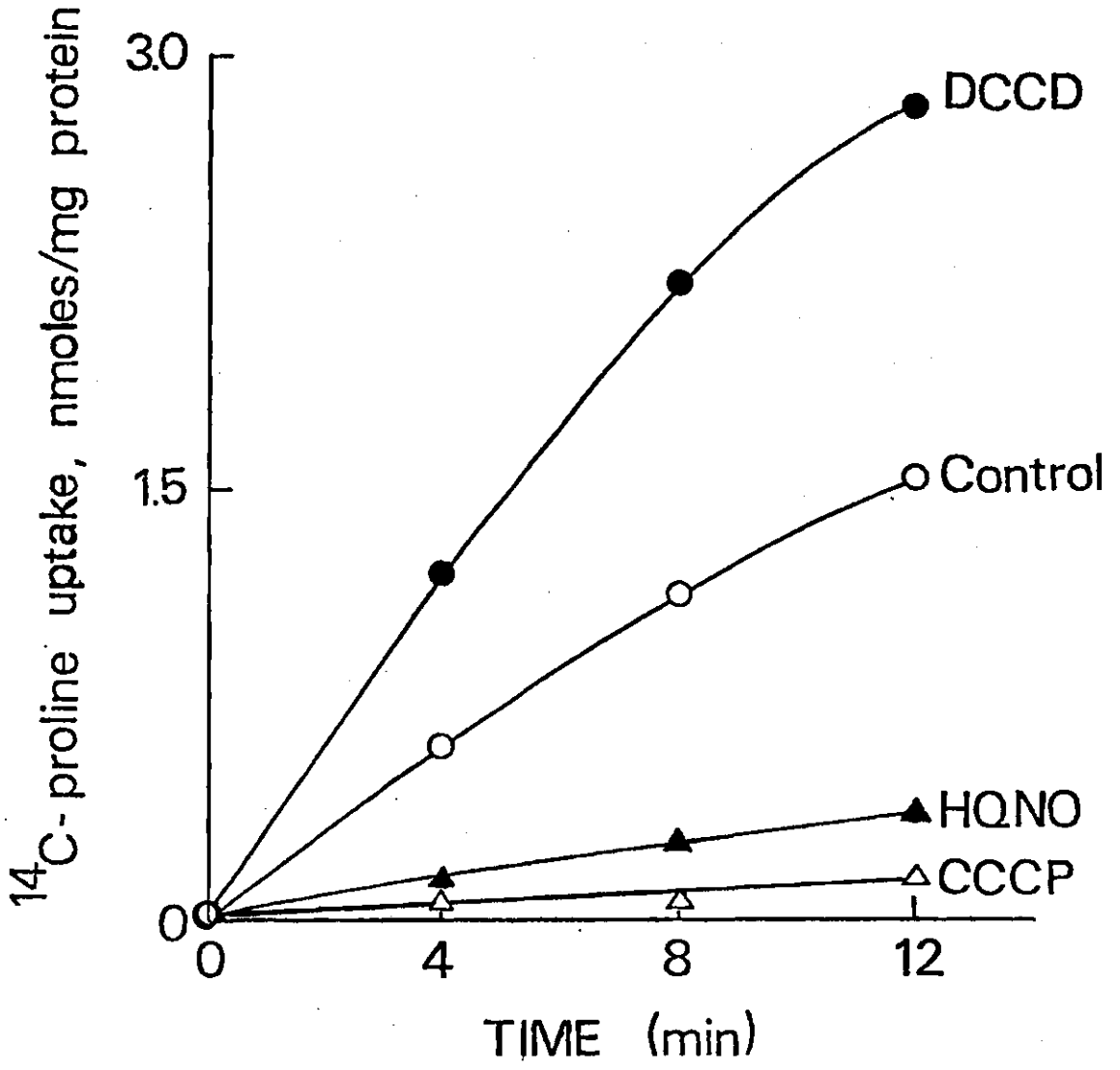
Legend:
Cells illuminated throughout the entire experiment (hυ). Cells maintained in the dark throughout the experiment (dk). Cells preilluminated for 5 min and then placed in the dark immediately prior to ^{14}C-proline addition (hυ-dk).

Figure 5. Light Requirement for ^{14}C-Proline Uptake.



Legend:
hυ= cells were illuminated throughout the experiment.
hυ→dk = cells illuminated for 5 min, and then placed in the dark.
^{14}C-proline was added at 0 time in both experiments.

Figure 6. Time Course of ^{14}C-Proline Uptake and Intracellular ATP Levels.



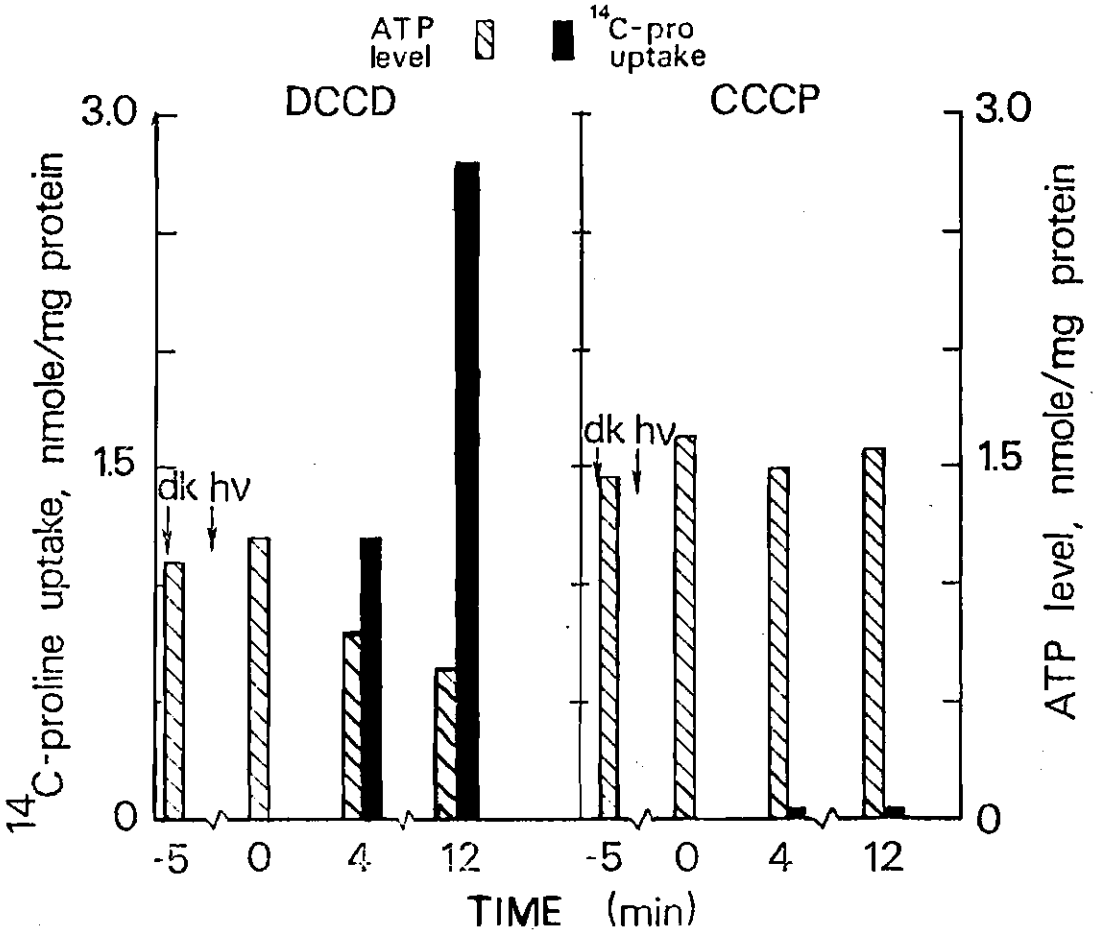
Legend:
Cells were illuminated for the entire experiment. Inhibitors were added 5 min before the addition of ^{14}C-proline at 0 time. All inhibitors were dissolved in ethanol. The control contained a corresponding level of ethanol (0.2%).

Figure 7. Effects of Inhibitors on Proline Uptake.

uptake may be due to an increase in the proton motive force. In Figure 8 the time course of proline uptake and ATP levels are compared in the presence of DCCD and CCCP. The addition of DCCD causes a drop in the intracellular ATP levels, while the uptake of ^{14}C-proline continues to proceed rapidly. In contrast, CCCP causes a slight diminution of ATP levels and almost completely abolishes ^{14}C-proline uptake. The slight rise in ATP levels in cells exposed to CCCP suggests that some photophosphorylation is occurring. If so, it would follow that the proline uptake system requires a higher level of the proton motive force than does the phosphorylation system. From Figures 6 and 8 it is also apparent that proline transport is not proportional to ATP levels.

Initial rates of ^{14}C-glutamate uptake in the light are higher than initial rates observed with ^{14}C-proline. Also, the maximum accumulation of glutamate is attained in a shorter period (Figure 9). Double reciprocal plots[17] showed the Km for this system to be 1.0×10^{-4} M and the Vmax is 1.0 nmole/mg cell protein/min. Glutamate is taken up in the dark by a system exhibiting a Km of 4.5×10^{-5} M and a Vmax of 1.6 nmole/mg cell protein/min. It is not clear whether this slight difference in Km values is indicative of two distinct systems.

Some clues as to the nature of the glutamate transport system(s) were revealed from inhibitor studies. CCCP was the most potent inhibitor of glutamate uptake by illuminated cells. In contrast, DCCD severely inhibits the uptake of ^{14}C-glutamate



Legend:
Inhibitors were added at -5 min. ^{14}C-proline was added at 0 min. Illumination was as indicated.

Figure 8. Effects of DCCD and CCCP on Photophosphorylation and Proline Uptake.

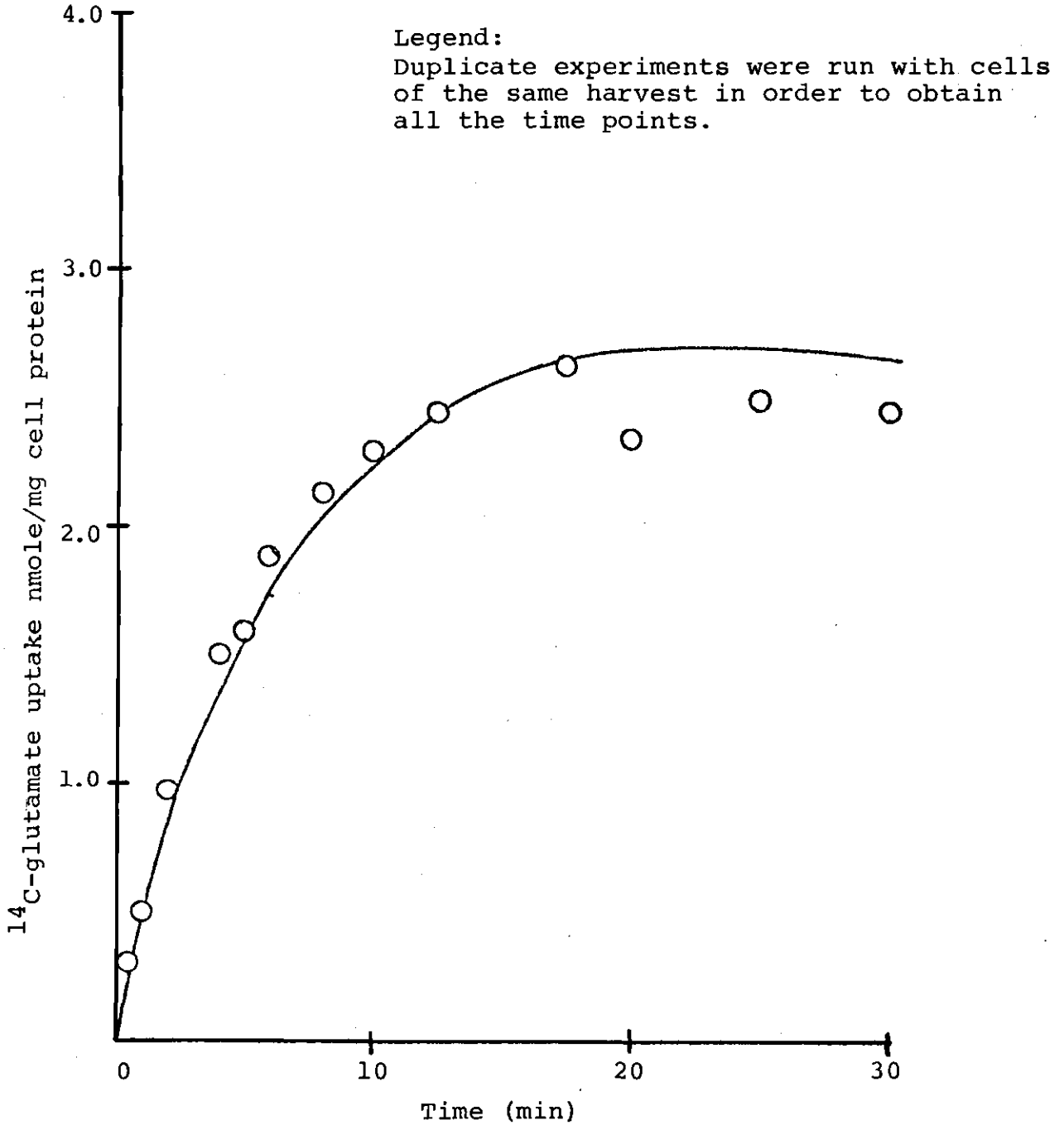
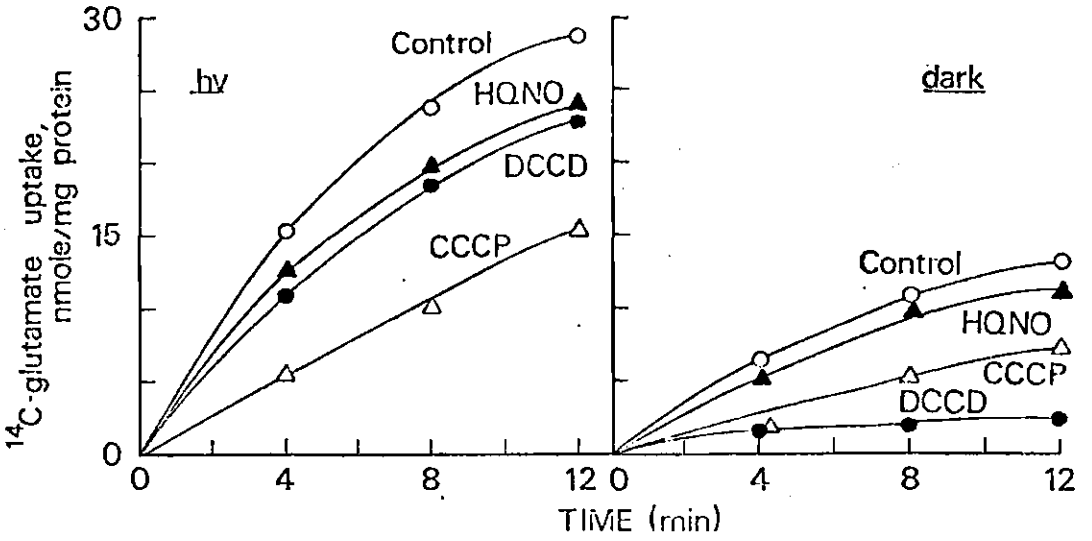


Figure 9. Time Course of ^{14}C-Glutamate Uptake by Illuminated Cells.

by dark exposed cells (Figure 10). This result indicates an indispensible role for ATP in the uptake of ^{14}C-glutamate in the dark.

Triphenylmethylphosphonium ion ($TPMP^+$) is a lipid soluble cation which crosses the membrane in response to a membrane potential (interior negative). This movement of the cation across the membrane reduces the electrical potential. $TPMP^+$ is an effective inhibitor of ^{14}C-proline and ^{14}C-glutamate uptake in the light (Table 1). In contrast, $TPMP^+$ inhibition of glutamate uptake in the dark is only 56%. This disparity may be explained if a large amount of membrane damage is caused by the larger influx of $TPMP^+$ in the light. This also indicates that $\Delta\psi$ is an essential component of the driving force for glutamate. This finding is in contrast to the model of Hamilton and Niven[6] who proposed that glutamate uptake in Staphylococcus aureus is driven by $-z\Delta pH$ by a $^+$H-symport mechanism. Table 2 also contains data on the inhibition of proline or glutamate transport caused by a 1000-fold excess of the other amino acid. Proline transport is inhibited by glutamate, but glutamate transport is not inhibited by excess proline.

The intracellular accumulation of tritium labeled $TPMP^+$ (^{3}H-$TPMP^+$) may be quantitated as illustrated in Figure 11. This accumulation is not inhibited by ^{12}C-proline nor by glutamate (Table 2). As anticipated an increased level of



Legend:
hυ = cells preilluminated 5 min before the addition of ^{14}C-glutamate at 0 time and illumination was continued. dark = cells kept in the dark 5 min before the addition of ^{14}C-glutamate at 0 time and then maintained in the dark. Inhibitors were dissolved in ethanol and added at -5 min. Controls contain a corresponding level of ethanol (0.2%).

Figure 10. Effects of Inhibitors on Glutamate Uptake in the Light and Dark.

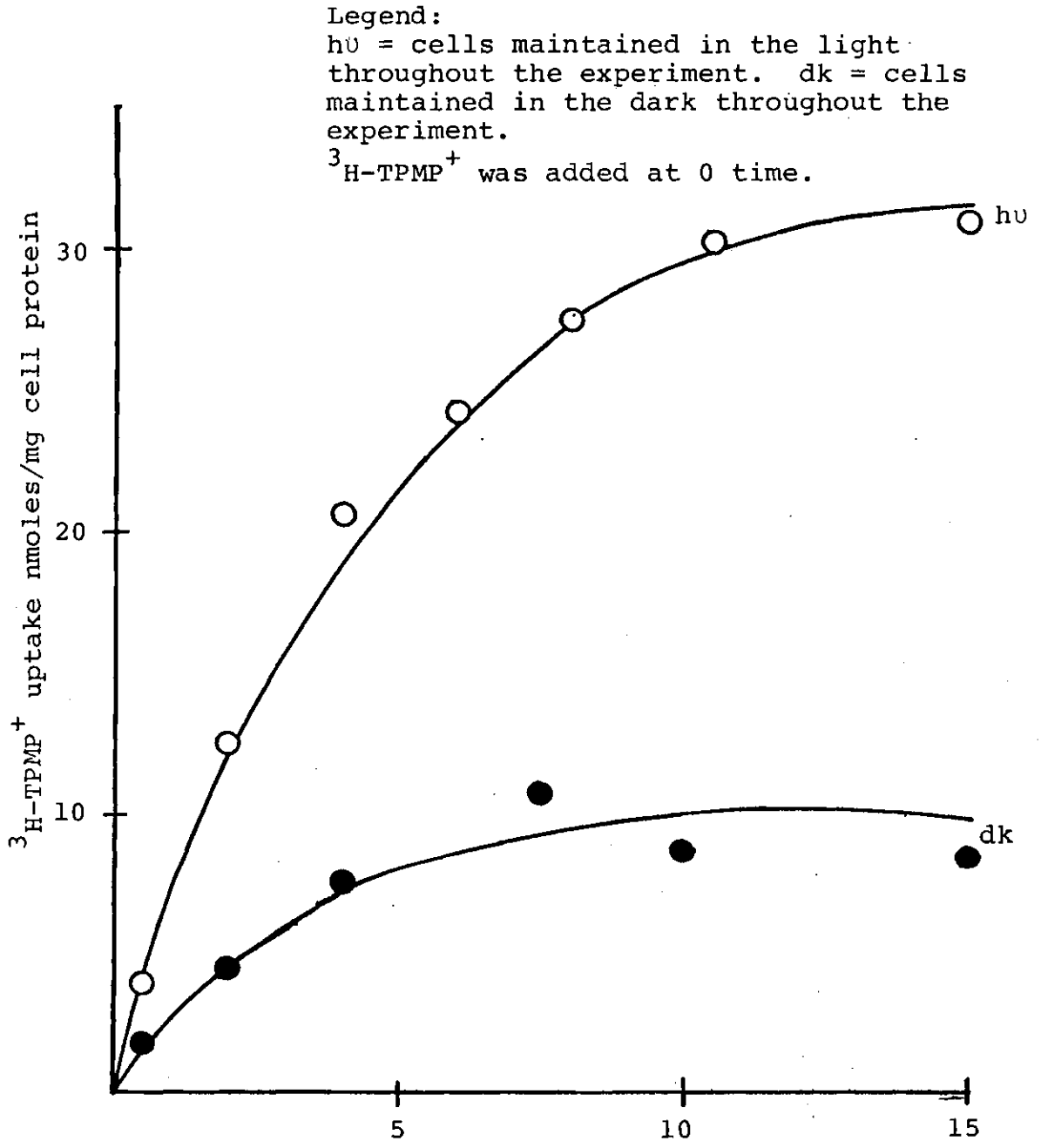


Figure 11. Uptake of ^{3}H-TPMP^{+} in the Light and in the Dark.

Table 2. Competition between the Different Transportable Species

Transported Species	Inhibitor	Uptake nmole/ mg cell protein @ 10 min.	Inhibition
^{14}C-proline (in light)	none	2.1	-
	^{12}C-glutamate	1.2	43%
	$TPMP^+$	0.09	96%
^{14}C-glutamate (in light)	none	3.2	-
	^{12}C-proline	3.2	none
	$TPMP^+$	0.21	93%
^{14}C-glutamate (in dark)	none	2.4	-
	$TPMP^+$	1.05	56%
^{3}H-$TPMP^+$ (in light)	none	30.2	-
	proline	30.2	none
	^{12}C-glutamate	30.2	none

Conducted under standard assay conditions with inhibitors and the ^{14}C or H^3 labeled substrate added at 0 time. When used, the final concentrations of ^{12}C-glutamate, ^{12}C-proline and unlabeled $TPMP^+$ were 2 mM, 2 mM and 1 mM, respectively.

^{3}H-TPMP^{+} accumulation by light exposed cells indicates that the membrane potential is greater in the light than in the dark. From this we can conclude that in E. halophila photosynthetic electron transport is capable of generating a larger proton motive force than the hydrolysis of ATP by the membrane ATPase. It is also possible to conclude that much more ^{3}H-TPMP^{+} is accumulated than are ^{14}C-amino acids. This level of accumulation is about 10-fold higher than that of the amino acids. ^{3}H-TPMP^{+} accumulation is inhibited strongly by CCCP in the light. The initial rate of TPMP^{+} uptake was stimulated by DCCD, but the accumulation was completed in 10 min. As proposed above, saturation may be due to membrane damage caused by increased fluxes of TPMP^{+}. DCCD severely inhibits uptake of ^{3}H-TPMP^{+} by dark exposed cells, again indicating a role for the membrane ATPase. CCCP shows a strong inhibition of uptake of TPMP^{+} in the dark, but this inhibition is slightly less pronounced than that of DCCD (Table 3).

Table 4 shows the effects of varying relative amounts of KCl and NaCl while maintaining a constant salt concentration of 1.0 M. Proline uptake is stimulated with increasing K^{+} concentration. This effect is consistent with the observation of Lombardi and Kaback in membrane vesicles of E. coli.[18] Glutamate uptake is inhibited as Na^{+} concentration decreases. This result is consistent with previous finding of a Na^{+} dependent glutamate transport system in membrane vesicles of E. coli.[13] Valinomycin causes a moderate inhibition of

Table 3. DCCD and CCCP Inhibition of Uptake of ^{3}H-TPMP^{+} by Light- or Dark-exposed Cells.

Conditions	Inhibitor	H^{3}-TPMP^{+} uptake, nmole/mg protein		
		2 min	4 min	10 min
Light	None	12.7	19.5	27.7
Light	CCCP	2.22	2.22	2.22
Light	DCCD	14.5	23.3	17.0
Dark	None	3.2	7.51	8.74
Dark	CCCP	0.74	0.99	1.35
Dark	DCCD	0.86	0.62	0.86

Conducted under standard assay conditions with H^{3}-TPMP^{+} added at zero time, and inhibitors added at -5 min. The no inhibitor control contained 0.2% ethanol.

Table 4. The Influence of Varying Na^+ and K^+ Ratios on Proline and Glutamate Uptake in the Presence and Absence of Valinomycin

Assay Conditions[a]	Relative Activity[b]		
	^{14}C-Proline Uptake (light)	^{14}C-Glutamate Uptake (light)	^{14}C-Glutamate Uptake (dark)
1 M NaCl	1.0	1.0	1.0
1 M NaCl plus VAL	1.0	0.97	0.9
0.75 M NaCl and 0.25 M KCl	1.31	0.94	0.9
0.75 M NaCl and 0.25 M KCl plus VAL	0.79	0.05	0.14
0.25 M NaCl and 0.75M KCl	1.49	0.75	0.7
0.25 M NaCl and 0.75M KCl plus VAL	0.15	0.11	0.23

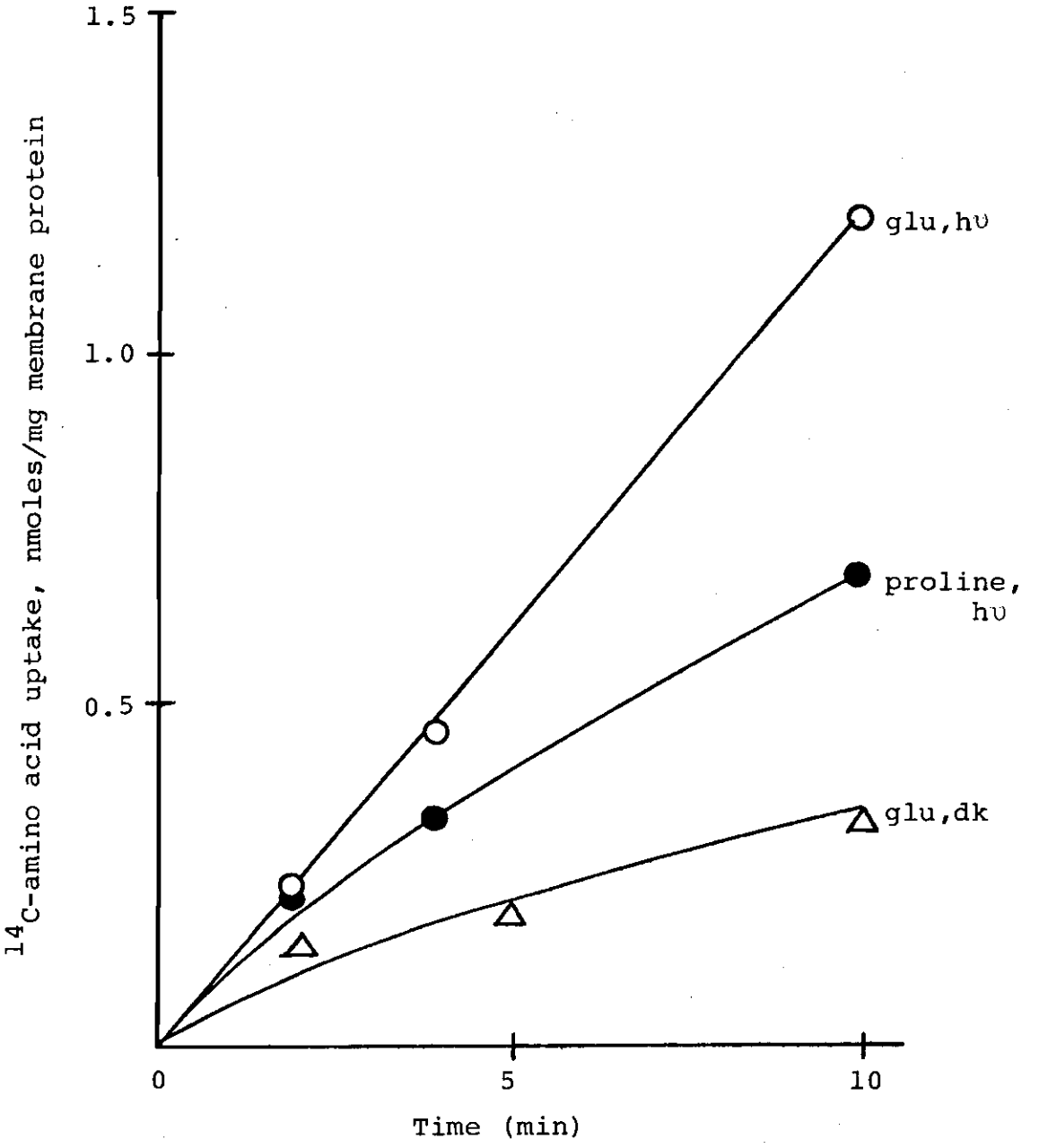
[a]Cells were harvested and resuspended in Tris buffer containing the indicated NaCl-KCl mixtures. The conditions of assay were otherwise standard except that the membrane filter disks were washed with the same NaCl-KCl mixture as was used in the incubation. Valinomycin (VAL) was added at -5 min.

[b]Calculated from initial rates; the three activities observed with cells in 1 M NaCl were each set equal to 1.0.

proline uptake by cells suspended in 0.25 M KCl and causes an almost complete inhibition by cells in 0.75 M KCl. Glutamate uptake in the light and dark are almost completely inhibited in media containing 0.25 M or more K^+ ion. ATP formation was strongly inhibited in cells in the media containing 0.75 M KCl (data not shown).

Membrane vesicles were prepared according to the procedure described in the methods section. It was difficult to obtain active preparations consistently. We were able with repeated attempts to obtain several active preparations which provided reproducible results. These vesicles took up ^{14}C-amino acids as illustrated in Figure 12. The same patterns of proline transport stimulation and glutamate transport inhibition with increasing proportions of K^+ ion in the assay buffer were observed with membrane vesicles (data not shown).

Preparations of membrane vesicles had low but detectable ATP levels which did not appear to rise with illumination, indicating that the membrane vesicles were not catalyzing photophosphorylation (data not shown).



Conducted as described for whole cell experiments except that the vesicles were not bubbled with N_2.

Figure 12. Amino Acid Uptake by Membrane Vesicles.

CHAPTER IV

CONCLUSIONS

Our findings with proline transport by E. halophila in the light are consistent with the chemiosmotic theory of membrane transport. Glutamate transport in the light is more difficult to analyze because of the light-independent uptake which occurs with this amino acid. The glutamate transport system which functions in the dark is inhibited by DCCD and thus appears to be driven by a membrane ATPase. This raises the question as to why proline uptake could not be driven by a comparable mechanism in the dark. The results of the $TPMP^+$ experiments clearly showed that a membrane potential was being generated in the dark. This potential appears to be derived from proton extrusions catalyzed by the membrane ATPase since uptake of $^3H\text{-}TPMP^+$ was strongly inhibited by DCCD and CCCP in the dark.

The difference between proline and glutamate uptake systems might be explained by the differences in the amounts of energy needed to drive the two systems. There are several results consistent with the hypothesis that glutamate transport requires less energy than does proline transport. The most obvious is the lack of proline transport in the dark. The results of the $^3H\text{-}TPMP^+$ experiments show that the membrane

potential is greater in the light than in the dark. Glutamate transport also proceeds faster in the light than does proline transport. The fact that CCCP, which lowers the proton motive force, inhibits proline transport more than glutamate transport supports the hypothesis. HQNO, which reduces that portion of the proton motive force derived from photosynthetic electron transport, inhibits proline uptake more strongly than glutamate uptake. More support comes from the fact that glutamate transport is unaffected by excess proline, while proline transport is inhibited by excess glutamate. This result differs from the findings of Lombardi and Kaback[18] who found that proline transport was not inhibited by any other amino acid in E. coli, another gram-negative organism. In their work the inhibiting amino acid was present in a 100-fold excess at a concentration of 1 mM. We used a 1000-fold excess at 4 mM in our experiments. The competition at these elevated levels may be for an energy source rather than for a transport system.

The experiments using valinomycin as an inhibitor of glutamate uptake are partially consistent with Mitchell's theory. With valinomycin present proline uptake decreased as $\Delta\psi$ was lowered by increasing the amounts of K^+ ion in the external medium. This decrease of neutral amino acid uptake follows the model proposed by Hamilton and Niven.[6] This result is also consistent with the findings of Asghar et al. with other neutral amino acids.[1] Glutamate transport dropped off precipitously when 0.25 M K^+ was introduced into the medium. The fact

that ATP synthesis and proline uptake were still occurring at a 0.25 M K^+ level indicates that substantial $\Delta\rho$ exists. This inhibition of glutamate transport by valinomycin is unexplained at this time. We do know that valinomycin has some anomalous effects in E. halophila. We were not able to demonstrate amino acid or ^{3}H-TPMP$^+$ uptake in the dark driven by a valinomycin induced K^+ efflux from cells suspended in low K^+ medium (data not presented). This was true of whole cells and membrane vesicles although ^{3}H-TPMP$^+$ was not used with vesicles. This valinomycin induced uptake was predicted and demonstrated with other gram negative bacteria.[6]

Most of our experiments are consistent with the chemiosmotic theory of membrane transport. The phenomena which need further explanation are the possibility of an energy threshold for proline transport and the odd effects of valinomycin. As explained in the introduction, E. halophila has a complex membrane structure associated with its photosynthetic apparatus. Since the proton extrusion which accompanies photosynthetic electron transport is initiated in the lamellar structure, valinomycin may well be causing K^+ flux between the cytoplasm and the photosynthetic apparatus rather than between the cytoplasm and the external medium.

APPENDIX 1

MODES OF ACTION AND PROPOSED EFFECTS OF INHIBITORS USED

Inhibitor	Mode of Action	Effect on $\Delta\psi$	Effect on $-z\Delta pH$
DCCD	ATPase inhibitor[25]	none	increase
CCCP	increases membrane permeability to protons[6]	decrease	decrease
HQNO	inhibits electron transport[11]	decrease	decrease
$TPMP^+$	lipid soluble cation[25]	decrease	none
Valinomycin	specific K^+ carrier, lipid soluble[6]	decrease (when extracellular K^+ present)	none

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