AN ABSTRACT OF THE THESIS OF

John Frank Carpenter for the degree of Master of Science in

Zoology presented on Jan. 7,1981

Title: The Effects of External Salinity on Carbon Dioxide Incorporation into Free Amino Acids in Artemia Nauplii.

Redacted for Privacy

Abstract Approved:

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In Artemia salina nauplii acclimated in 0.25 M, 0.5 M, 1.0 M, 1.5 M, 2.0 M, and 2.5 M NaCl incorporation of $^{14}CO_2$ into free amino acids was greatly stimulated in the higher salinities. The ^{14}C label was found in aspartic acid, serine, glutamic acid, proline and alanine. Hypersalinity also caused an increase in concentrations of unlabeled free amino acids in the naupliar cytosol, an increase in the amount of ammonia excreted into the medium and an increase in aerobic glycolysis.

The Effects of External Salinity on Carbon Dioxide Incorporation into free Amino Acids in *Artemia* Nauplii

by

John Frank Carpenter

A THESIS

submitted to

Oregon State University

in partial fulfillment of
the requirements for the
degree of

Master of Science

Completed January 7, 1981

Commencement June 1981

APPROVED:

Redacted for Privacy

Professor of Zoology in charge of major

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Date thesis is presented Jan. 7,1981

Typed by Suzi Sargent for John Frank Carpenter

ACKNOWLEDGEMENTS

I would like to thank Dr. Frank Conte for his guidance and support during the course of this study. I am also very grateful to Steve Hand and Joel Lowy for their assistance in lab and critical evaluations of proposed experiments. I am greatly in debt to Dr. Robert Becker for his valuble advice and the generous amount of time he allotted me on his amino acid analyser, and I appreciate the efforts of Thom Baily and Steve Shaw to make the most efficient use of that time.

Finally, I would like to express my deepest appreciation to my dad, Bob Worrick, and my dear friends, Bill and Virginia Jones, for their encouragement and support throughout my college career.

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Introduction

Croghan (1958a) found that it is possible for the crustacean, *Artemia salina*, to adapt to seawater ranging from 0.26% NaCl to crystallizing brine. Survival in these media is possible since in both adults (Croghan, 1958a-e; Thuet, et al., 1968; Smith, 1969a, b; Geddes, 1975a-c) and nauplii (Conte et al., 1972; Conte, 1977; Russler and Mangos, 1978) the solutes in the hemolymph can be maintained at much lower concentrations and different compositions than those of the external environment. The hypoosmotic state is maintained by active transepithelial ion transport and dependent upon the cationic enzyme, Na+K- activated ATPase (Croghan, 1958b,c; Smith, 1969 a,b; Conte, et al., 1972; Conte, et al., 1980). Ewing, et. al., (1972) found an increase sensitivity of nauplii to ouabain (a specific inhibitor of Na+K- activated ATPase) as the salinity of the external medium increased, indicating a dependency on Na+K- activated ATPase activity for survival. The finding by Augenfeld (1969) that Na+K- activated ATPase activity increases in the adult in response to increased external salinity is consistent with this concept.

When external salinities increase there is a greater demand being placed upon the osmoregulatory mechanism, and there must be a concomitant increase in demand for chemical energy. This is supported by the observation that a decrease in cellular [ATP] occurs when

external salinity increases (Ewing, et. al., 1980). Two lines of experimental evidence suggest that most of the increase in chemical energy needed for enhanced levels of ion transport at higher salinities does not come from aerobic metabolism. First, oxygen consumption by nauplii appears to be independent of the NaCl gradient (Conte, 1980; Conte, et al., 1980; Ewing, et. al., 1980). Second, oxygen consumption accompanying naupliar transitions from 0.5 M NaCl to 2.5 M NaCl does not change significantly in the presence of ouabain which can be interpreted that oxygen utilization is not directly influenced by levels of Na+K-activated ATPase activity (Edwards, 1975).

As Conte (1980) points out, when additional energy is needed to cope with this type of environmental stress and is not derived from aerobic metabolism, one must assume that some other kind of facultative metabolic pathway needs to be activated to compensate for declining levels of cellular ATP. Standard anaerobic glycolysis is used in most animals and yields large quantities of lactic acid. However, in brine shrimp nauplii, glycolysis does not occur in this fashion. If comparison is made of animals incubated at 0.5 M NaCl in contrast to those in 2.5 M NaCl a significantly greater increase in glycolysis occurs at the higher salinity but little production of lactate is made. Furthermore, if ouabain (10^{-4} M) is added to either incubation medium it will cause a reduction in glycogen utilization but will cause little change in lactate production (Conte, 1980; Conte, et. al., 1980).

Conte (1977, 1980) has proposed that a C-4 dicarboxylic acid pathway serves as a facultative anaerobic shunt in nauplii to help meet this increased energy requirement. The pathway involves the fixation of

carbon dioxide with phosphoenolpyruvate to form oxaloacetate. The resulting oxaloacetate can be transferred into aspartate by transamination or malate by reduction.

Previous studies on carbon dioxide fixation in the encysted embryo have demonstrated incorporation of ^{14}C-label into compounds found in cold and hot TCA-soluble and TCA-insoluble fractions of homogenates. (Clegg, 1967; Clegg, 1976). Further characterization of these compounds has shown them to be purine and pyrimidine bases of nucleic acids found in both adults, nauplii, and embryos (Warner and McClean, 1968) and as proteins, enzymes such as the Na+K+ activated ATPase in nauplii (Peterson, et. al., 1978) and cytoplasmic malate dehydrogenase in nauplii (Hand and Conte, 1980). Of particular interest to the present study are results presented by Clegg (1976) on embryos still encased in the embryonic shell where he demonstrated that ^{14}C-label appeared into organic acids. Aspartic acid and malic acid were the most highly labeled compounds and appeared derived from labeled oxaloacetate, consistent with Conte's proposed anaerobic shunt for the swimming nauplii.

Also, earlier work by Emerson (1967) measuring the levels of free amino acids in nauplii incubated in distilled water, 0.5 M NaCl, and 1.0 M NaCl showed that the levels of all free amino acids were lowest in nauplii incubated in distilled water. There was a greater concentration of all amino acids, except glutamic acid and histidine, in nauplii incubated in 1.0 M NaCl than in those incubated in 0.5 M NaCl. Similarly, Boulton and Huggins (1976) found in nauplii an increase in total ninhydrin positive substances with increased salinity.

The present study was undertaken to determine if the incorporation of carbon dioxide into free amino acids in nauplii is influenced by environmental salinity. Both the specific activity of the amino acids and the total concentration of free amino acids were measured in nauplii incubated at 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 M sterilized NaCl. In addition the concentrations of free amino acids excreted into the acclimation media were measured.

Methods and Materials

1. Source and Hatching of Nauplii

Dried brine shrimp cysts were purchased from San Francisco Bay Brand, Newark, Calif. 94560 and stored at -20°C. Cysts were sterilized by antiformin treatment as described by Clegg (1976) and Abdul-Baki (1974). Bacteria-free nauplii were obtained by incubating 15 g. of sterile cysts in 500 ml. of autoclaved diluted Instant Ocean (I.O./dist H_2O in 1:1) containing 60 µg/ml penicillin at 30°C for 21 hrs. After incubation, free-swimming nauplii were harvested aseptically from unhatched cysts and broken shells by the method of Finamore and Clegg (1968).

2. Salt Acclimation Media and Incubation with ^{14}C-Bicarbonate

Bacteria-free nauplii harvested from two hatching flasks were divided volumetrically into two batches of nearly equal quantities of nauplii. Each batch was filtered in a Miracloth filter cone to remove the excess seawater and transferred into a 2 liter Fernbach flask containing 500 ml of artificial acclimation (Millipore filtered with membrane 0.45 µ pore size) medium that was on an elliptical shaker table to continuously swirl and oxygenate the medium. The acclimation media were adjusted to be at pH = 7.8 and contained 3 mM $NaHCO_3$, 6 mM $CaCl_2$, and 10 mM KCl. Each medium was brought to the desired salinity by additional amounts of solid NaCl. Nauplii were acclimated for 30 min. prior to adding of the labeled bicarbonate. To one flask 100 µCi of $NaH^{14}CO3$ was added. The other flask was used to monitor animal density,

survival, pH and dissolved O_2 throughout the experimental period. Samples for these parameters, and an aliquot to monitor ^{14}C-bicarbonate radioactivity were taken at 0.1 hr, at 1.5 hr, and at 3.0 hr. during acclimation.

3. Harvesting of ^{14}C-labeled Nauplii

After the 3 hr. acclimation period, the nauplii in the ^{14}C-bicarbonate flask were immediately separated from the medium by gravity filtration through a Miracloth filter. The collected conditioned medium was adjusted to pH 2.2 with 0.5 M sulfuric acid and rotary evaporated to dryness at 37°C. The labeled nauplii were washed off the filter cone with 20 ml of cold 0.5 M perchloric acid and homogenized in a glass homogenizer. The homogenate was centrifuged in a Sorvall SC-5B Refrigerated Centrifuge at 4°C for 20 min. at 27,000 xG. The pellet was analyzed for protein by modification of the Lowry method (Peterson, 1977). The protein-free supernatant was adjusted to pH 9 with 5 M potassium hydroxide to precipitate perchlorate salts and centrifuged at 5100 xG for 15 min. The volume of the resulting supernatant was measured and the supernatant was reacidified to pH 2.0 with 0.5 M hydrocholoric acid. A volume (2X) of 95% ethanol was added to precipitate glycogen. The precipitate was centrifuged at 12,000 xG for 20 min. to pellet the glycogen. Purification and analysis of glycogen is described later. The glycogen-free and protein-free supernatant (referred to as naupliar cytosol) containing the soluble organic acids including the amino acids, and was brought to dryness on a Buchler rotary evaporator at 37°C.

4. Isolation and Analysis of Glycogen

The crude glycogen pellet was brought into solution by the addition of 1.75 g of NaCl into 60 mls. glass distilled water (0.5 M). One-hundred and twenty mls of absolute ethanol was added to the solution, mixed well and stored in a freezer overnight at -10°C. The resulting precipitate was centrifuged at 4°C for 30 min. at 12,000 xG. The pellet was redissolved in 12 mls 30% potassium hydroxide, heated in a boiling water bath for 10 min. and cooled in an ice bath. Twenty mls 95% ethanol was added and the resulting solution was mixed well and stored in a freezer overnight at -10°C. The resulting precipitate was centrifuged at 4°C for 10 min. at 12,000 xG. The pellet was resuspended in 10 mls. of 95% ethanol, allowed to stand for 10 min at 0°C in an ice bath and centrifuged at 4°C for 10 min. at 12,000 xG. The pellet was air dried and redissolved in 5 mls glass distilled water. The final aqueous solution was analyzed for glucose equivalents by the Anthrone method (Lowy, 1978).

5. Isolation and Analysis of Free Amino Acids from Conditioned Acclimation Media and Naupliar Cytosol

A. Extraction of amino acids from conditioned acclimation media.

Before extraction, a tritiated tyrosine aliquot was added to the dried organic salt mixture to monitor efficiency of extraction. Free amino acids were extracted from the dried salt mixture by liquid partitioning using 95% ethanol. The first extraction consisted of 45

mls of 95% ethanol which was removed by pipetting and was repeated 3 times. All washes were combined and rotary-evaporated to dryness at 37°C. The amino acid salt mixture was extracted a second time in a similar manner. The dried amino acid salt mixture from this second extraction was extracted a third time with two washes consisting of 3 ml 95% ethanol. The final ethanol extract was rotary-evaporated to dryness and redissolved in 2 mls glass distilled water. To this solution 4 mls of a saturated potassium oxalate solution was added to precipitate excess calcium present in acclimation media (Ca^{++} ion was found to greatly interfere with the performance of the amino acid analyzer). The calcium oxalate suspension was centrifuged for 10 min at 4300 xG to pellet the insoluble salt. The "calcium-free" (<5 micrograms/ml) supernatant was removed by a Pasteur pipette, placed in a 50 ml round bottom flask and rotary-evaporated to dryness. The amino acid salt residue had an additional 95% ethanol extraction consisting of 3 washes of 3 ml to separate the free amino acids from the excess potassium oxalate salt. The potassium oxalate free washes were combined and rotary-evaporated to dryness. The dried amino acids were redissolved in 1 ml glass distilled water and 0.1 ml was removed for analysis of ninhydrin positive substances referred to as NPS (Moore and Stein, 1948).

B. Extraction of amino acids from naupliar cytosol

Free amino acids were extracted from the dried organic acid mixture by liquid partitioning using 95% ethanol. First extraction consisted of three 5 ml 95% ethanol washes of the residue. The ethanolic washes were

combined, total 15 ml, decanted into a 50 ml round bottom flask and rotary evaporated to dryness at 37°C. The second extraction of this residue consisted of three 3 ml washes of 95% ethanol which were removed by Pasture pipette abd transferred into a 50 ml round bottom flask and rotary evaporated to dryness at 37°C. The amino acid residue was redissolved in 2 mls of glass distilled water, and 0.1 ml removed for analysis of NPS (Moore and Stein 1945).

C. Analysis of Free Amino Acids by Automated Amino Acid Analyzer

Identification and quantification of amino acids were performed with a Beckman/Spinco 120B modified automated analyzer using a single column system (Spackman, et. al. 1958). For each amino acid sample, in addition to a standard chemical analysis, an analysis of radioactivity was performed. Prior to chromatography a tritiated tyrosine spike was added. Volumetric fractions (25 drops/tube) of the column eluate were collected before it entered the chemical (ninhydrin) reaction coil. A signal from the fraction collector occurred at the change of tubes and was used to activate an event recorder to mark the chart. Since the same chromatograph chart and chart speed were used for recording both chemical and radioactivity events, all tubes became synchronized to the chemical chart analysis. Each sample collected for analysis of ^{14}C content was given an aliquot of Handifluour Scintillation Cocktail (sample/cocktail was a 1:10 ratio). Radioactivity was measured by a Packard Model 3310 Liquid Scintillation Spectrophotometer. The radioactivity (cpm) for each fraction was plotted against the time of fraction collection. Since the positions of the tubes were synchronized

to the chemical chart, the radioactive peaks obtained were superimposed to the events occurring on chemical chart. To identify C^{14} labeled free amino acids, the position of the tritiated tyrosine peak served as a reference point.

Results

1. Free Amino Acids Excreted in the Acclimation Media

The chemical concentrations of ninhydin-positive substances (NPS) and free amino acids found in the conditioned acclimation media from two different experiments are shown in Tables I and II. The results from both experiments indicate that a 2-3 fold increase of total NPS occurs when the external salinity rises from 0.25 M to 2.5 M. A large portion of the elevatd NPS is due to the incremental rise in ammonia, by far the major constituent of the total NPS. As the ammonia excretion is stimulated so is the excretion of free amino acids with the total amino acid concentration showing a doubling over the same salinity range. Two amino acids, alanine and serine, appear to be the major amino acids excreted accounting for 30-35% of the total amino acids. Alanine shows the most dramatic salt dependency in which a 2-fold increase occurs as the concentration of salts rise from 0.25 M to 2.5 M whereas serine shows a slight decrease(18-15%). Most of the other free amino acids evidenced a slight increase or a variable change with the rise in salinity. Since the amino acids are found in very low concentrations, it was important to ascertain whether the changes observed were due to variations in the extraction procedure. The efficiency of the ethanol extraction was determined by the recovery of tritiated tyrosine marker in the final extracts. The percentage of recovery between the various salinities was 80-85% as shown in Table III, and therefore cannot be responsible for the observed changes.

TABLE I: Free Amino Acids in the Conditioned Acclimation Media as a Function of Salinity

	0.25 M NaCl			1.0 M NaCl			2.0 M NaCl		
	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)
Aspartate	35.3	0.9	2.4	55.2	1.0	2.5	64.7	0.7	3.0
Threonine	75.6	1.8	5.1	97.1	1.8	4.4	104.1	1.2	4.8
Serine	261.1	6.3	17.6	298.1	5.5	13.6	306.5	3.5	14.3
Glutamate	40.8	1.0	2.8	55.4	1.0	2.5	86.5	1.0	4.0
Proline	81.1	1.9	5.5	86.7	1.6	4.0	85.5	1.0	4.0
Glycine	150.8	3.6	10.2	187.8	3.4	8.6	202.9	2.3	9.4
Alanine	165.2	4.0	11.1	377.8	6.9	17.2	447.8	5.1	20.8
Cysteine	11.7	0.3	0.8	19.8	0.4	0.9	--	--	--
Valine	63.8	1.5	4.3	96.7	1.8	4.4	103.3	1.2	4.8
Methionine	30.1	0.7	2.0	53.1	1.0	2.4	39.6	0.5	1.8
Isoleucine	66.4	1.6	4.5	81.5	1.5	3.7	80.8	0.9	3.8
Leucine	99.6	2.4	6.7	187.5	3.4	8.6	143.7	1.7	6.7
Tyrosine	117.9	2.8	8.0	149.2	2.7	6.8	65.3	0.8	3.0
Phenylalanine	85.5	2.1	5.8	90.3	1.7	4.1	50.9	0.6	2.4
Histidine	13.6	0.3	0.9	40.7	0.8	1.9	67.6	0.8	3.1
Lysine	97.6	2.3	6.6	154.5	2.8	7.0	138.1	1.6	6.4
Arginine	87.4	2.1	5.9	162.2	3.0	7.4	163.6	1.9	7.6
Ammonia	2688.4	64.5	--	3268.7	59.8	--	6588.8	75.3	--
total NPS	4171.9	100.0	--	5462.3	100.0		8709.7	100.0	--
total A.A.'s	1483.5	--	100.0	2193.6	--	100.0	2150.9	--	100.0

TABLE II: Free Amino Acids in the Conditioned Acclimation Media as a Function of Salinity

	0.5 M NaCl			1.5 M NaCl			2.5 M NaCl	
	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)
Aspartate	150.6	2.9	5.8	100.8	1.2	3.0	105.7	1.4	3.1
Threonine	128.8	2.5	4.9	150.7	1.8	4.5	174.0	2.3	5.1
Serine	414.9	8.0	15.9	426.6	5.2	12.8	487.6	6.3	14.4
Glutamate	125.1	2.4	4.8	115.2	1.4	3.5	175.3	2.3	5.2
Proline	104.2	2.0	4.0	166.4	2.0	4.8	196.4	1.5	5.8
Glycine	259.0	5.0	9.9	276.0	3.4	8.3	322.2	4.2	9.5
Alanine	317.4	6.1	12.2	587.6	7.2	17.7	662.7	8.6	19.6
Cysteine	24.1	0.5	0.9	--	--	--	--	--	--
Valine	102.3	2.0	3.9	136.2	1.7	4.1	154.9	2.0	4.6
Methionine	28.5	0.6	1.1	36.6	0.5	1.1	50.9	0.7	1.5
Isoloeicine	96.2	1.9	3.7	114.2	1.4	3.4	112.9	1.5	3.3
Leucine	157.9	3.1	6.1	227.5	2.8	6.8	184.6	2.4	5.5
Tyrusineanine	181.4	3.5	7.0	200.7	2.7	6.6	115.7	1.5	3.4
Phenylanine	106.1	2.1	4.1	163.9	2.0	4.9	96.5	1.3	2.9
Histidine	73.5	1.4	2.8	72.6	0.9	2.2	86.9	1.1	2.6
Lysine	174.4	3.4	6.7	257.1	3.1	8.3	262.3	3.4	7.7
Arginine	163.2	3.2	6.3	275.4	3.4	8.3	262.3	3.4	7.7
Ammonia	2560.7	49.6	--	4871.0	59.5	--	4355.1	56.3	--
Total NPS	5168.3	100.0	--	8193.1	100.0	--	7740.8	100.0	--
Total A.A.'s	2607.6	--	100.0	3322.1	--	100.0	3385.7	--	100.0

TABLE III

Extraction Efficiency of Free Amino Acids from the Conditioned Acclimation Media

Salinity (M)	Recovery (%)
0.25	81.3
0.5	81.8
1.0	80.6
1.5	81.3
2.0	82.4
2.5	84.9

Interestingly, none of the ^{14}C-labeled amino acids that were formed in the naupliar cytosol appeared in the conditioned acclimation media. Therefore, it is possible that the free amino acids found in the extracellular media are derived from a different metabolic source, such as the yolk platelets. If this is the case, the metabolic pools containing the ^{14}C-labeled amino acids must be compartmentalized in different manner than the unlabeled but identical amino acids.

2. Free Amino Acids Located in the Naupliar Cytosol

The chemical concentrations of free amino acids and NPS found in the intracellular compartment are shown in Tables IV and V. The results from two different experiments show that unlike the conditioned acclimation media, the total free amino acids constitute the largest component (>90%) of the NPS. Ammonia is less than 10% of the total NPS and the intracellular ammonia concentration appears to be at a saturated level because despite stimulated ammonia excretion caused by raising the external salinity, the internal concentration remains nearly constant between 0.25 M to 2.5 M. The majority of the individual free amino acids increase in concentration as the salinity is elevated. Alanine exhibits a 3-fold increase, whereas proline shows a 2-fold increase. Other amino acids (serine, threonine, glycine, tyrosine) show a less dramatic rise but appear to be elevated at higher salinities. The two acidic amino acids, aspartic and glutamic, remain nearly at the same concentration levels or show a slight decline at the highest external salt concentration (>2.0 M). It should be noted that if the data from both experiments are viewed together, there is a stepwise increase in total free amino acids across the salinity profile.

TABLE IV: Free Amino Acids in the Nauplius Cytosol as a Function of Salinity

	0.25 M NaCl			1.0 M NaCl			2.0 M NaCl		
	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)
Aspartate	1141	4.7	5.1	1443	5.1	5.5	1258	2.9	3.1
Threonine	722	3.0	3.2	848	3.0	3.2	1297	3.0	3.2
Serine	3257	13.4	14.5	3470	12.0	12.9	4485	10.2	10.9
Glutamate	2662	10.9	11.8	2711	9.6	10.4	2438	5.6	5.9
Proline	3393	13.9	15.1	3199	11.3	12.2	5831	13.3	14.2
Glycine	1765	7.2	7.9	1950	6.9	7.5	2545	5.8	6.2
Alanine	5704	23.4	25.4	8122	28.7	31.1	18,096	41.2	44.0
Valine	254	1.0	1.1	302	1.1	1.2	322	0.7	0.8
Isoleucine	117	0.5	0.5	146	0.5	0.6	98	0.2	0.2
Leucine	166	0.7	0.7	234	0.8	0.9	234	0.5	0.6
Tyrosine	673	2.8	3.0	780	2.8	3.0	1151	2.6	2.8
Histidine	517	2.1	2.3	322	1.1	1.2	478	1.1	1.2
Lysine	536	2.2	2.4	731	2.6	2.8	848	1.9	2.1
Arginine	1560	6.4	6.9	1960	6.9	7.5	2096	4.8	5.1
Ammonia	1911	7.8	--	2165	7.6	--	2720	6.2	--
Total NPS	24,375	100.0	--	28,295	100.0	--	43,875	100.0	--
Total A.A.'s	22,464	--	100.0	26,130	--	100.0	41,155	--	100.0

TABLE V: Free Amino Acids in the Naupliar Cytosol as a Function of Salinity

	0.5 M NaCl			1.5 M NaCl			2.5 M NaCl	
	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)	nmoles a.a./ 100 mg protein	Total NPS (%)	Total Amino Acids (%)
Aspartate	2518	9.1	9.8	2510	7.4	8.2	2720	5.3	5.9
Threonine	247	0.9	1.0	1300	3.8	4.2	1560	3.0	3.4
Serine	2660	9.6	10.3	3430	10.1	11.2	4640	9.0	10.1
Glutamate	3450	12.4	13.4	3340	9.8	10.9	2520	4.9	5.5
Proline	2380	8.5	9.2	3070	9.0	10.0	4780	9.2	10.4
Glycine	2270	7.4	8.8	2120	6.2	6.9	3110	6.0	6.8
Alanine	6150	22.1	24.2	9140	26.8	29.9	18,570	35.9	40.4
Valine	330	1.2	1.3	370	1.1	1.2	490	1.0	1.1
Isoleucine	210	0.8.	0.8	210	0.6	0.7	330	0.6	0.7
Leucine	230	0.8	0.9	--	--	--	400	0.8	0.9
Tyrosine	500	1.8	1.9	780	2.3	2.5	890	1.7	1.9
Histidine	500	1.8	1.9	990	2.9	3.2	880	1.7	1.9
Lysine	590	2.1	2.3	420	1.2	1.4	660	1.3	1.4
Arginine	3660	13.2	14.2	2920	8.6	9.5	5780	11.2	12.6
Ammonia	2000	7.3	--	3420	10.1	--	3950	7.6	--
to_tal NPS	27,780	100.0	--	34,040	100.0	--	51,710	100.0	--
total A.A.'s	25,780	--	100.0	30,590	--	100.0	45,930	--	100.0

3. Newly synthesized amino acids via the C-4 carbon dioxide pathway

In earlier experiments, Clegg (1976) clearly established that the non-enzymatic CO_2-adduct reactions were not responsible for the incorporation of $^{14}CO_2$ into the organic metabolite obtained from brine shrimp cells. Fig. 1 illustrates the chromatographic profile of amino acids obtained from an ethanolic extract obtained from the cytosol of nauplii which had been acclimated for 3 hrs in 2.0 M NaCl. The identity of the radioactivity peaks could be found through the alignment of radioactivity peaks over the amino acids after the tritiated tyrosine marker was positioned upon the tyrosine peak (Fig. 2). Five labeled amino acids were identified as being aspartic acid, glutamic acid, alanine, proline and serine. Two peaks of radioactivity (listed as unknown 1 and 2 in Fig. 2) have remained unidentifiable because we lacked suitable chemical standards that would co-chromatograph with these peaks.

Interpretation of the radioactive peak that co-chromatographed with serine could be questioned due to the breadth (encompassing the threonine peak) of the peak and the early report of Clegg (1976) that threonine was form from $^{14}CO_2$ fixation. We investigated this matter further, having obtained purified standards of ^{14}C-serine and ^{14}C threonine, by co-chromatographing the naupliar cytosolic ethanolic extracts in the following manner: ^{14}C-serine was added to the mixture of labeled amino acids contained in the ethanolic extracts and chromatographed. It can be seen in Fig. 3 that the only change in the radiochromatogram was an increase in the peak height over serine.

Figure 1: A typical chromatographic profile of amino acids isolated from the naupliar cytosol and/or conditioned acclimation media.

NOTE: Abbreviations for identification of amino acids:
D - aspartic acid; T - threonine; S - serine; E - glutamic acid; P - proline; G - glycine; A - alanine; C^2 - cystine; V - valine; M - Methionine, I - isoleucine; L - leucine; Y - tyrosine; F - phenylalanine; K- lysine.

Figure 1.

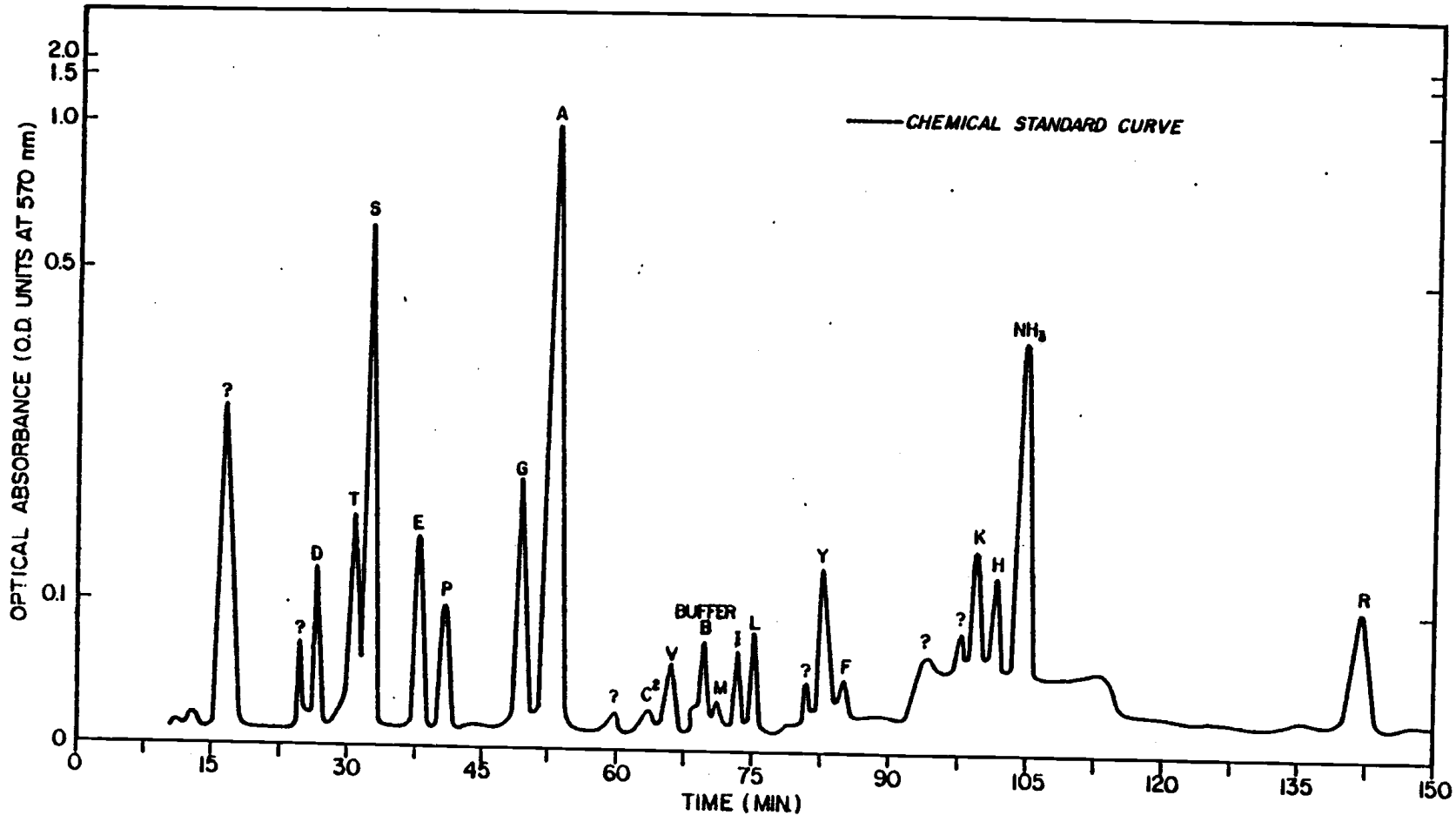

OPTICAL ABSORBANCE (O.D UNITS AT 570 nm)
2.0
1.5
1.0
0.5
0.1
0
CHEMICAL STANDARD CURVE
?
D
?
T
S
E
P
G
A
?
C
V
BUFFER
B
M
I
L
?
Y
F
?
?
K
H
NH3
R
0
15
30
45
60
75
90
105
120
135
150
TIME (MIN)

Figure 2: A typical chromatographic profile of the chemical concentration and radioactivity of unknown C-14 labeled amino acids isolated from the naupliar cytosol.

Figure 2.

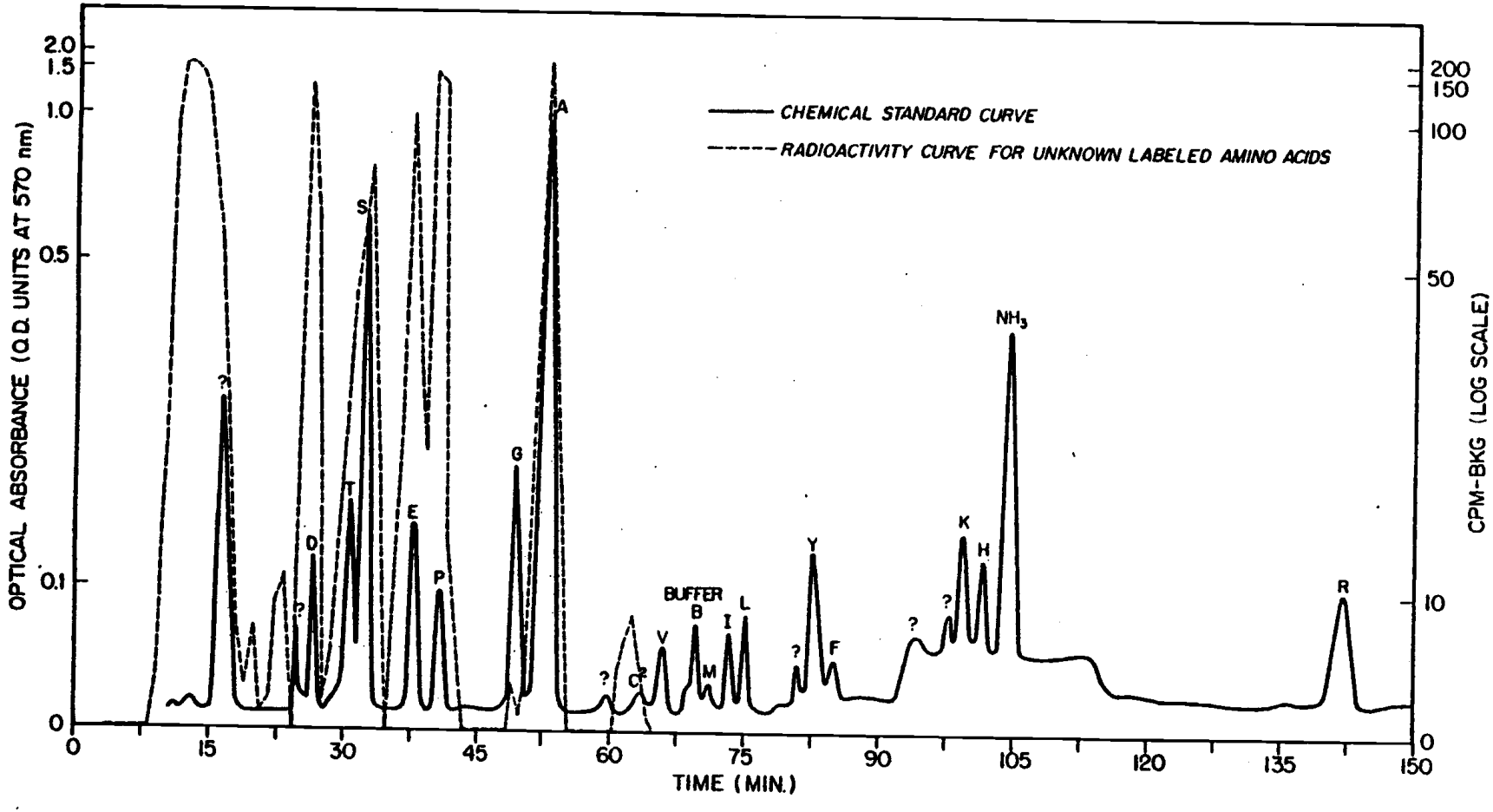

CHEMICAL STANDARD CURVE
RADIOACTIVITY CURVE FOR UNKNOWN LABELED AMINO ACIDS
OPTICAL ABSORBANCE (O.D. UNITS AT 570 nm)
CPM-BKG (LOG SCALE)
TIME (MIN.)
BUFFER
NH3
2.0
1.5
1.0
0.5
0.1
0
200
150
100
50
10
0
15
30
45
60
75
90
105
120
135
150

Figure 3: A typical chromatographic profile of the chemical concentration and radioactivity of unknown C-14 labeled amino acids isolated from the naupliar cytosol with an additional quantity of purified C-14 serine (500 cpm).

Figure 3.

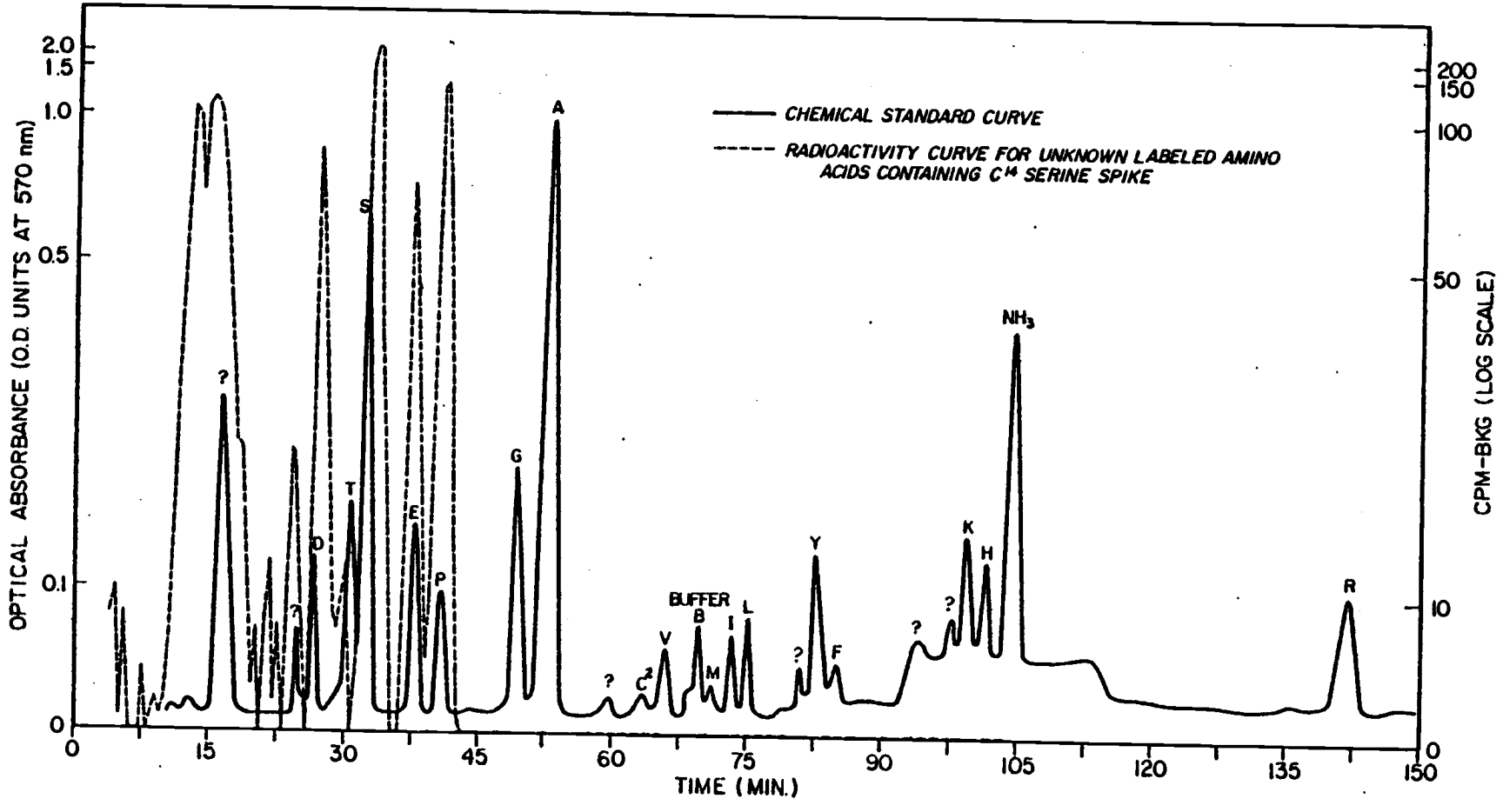
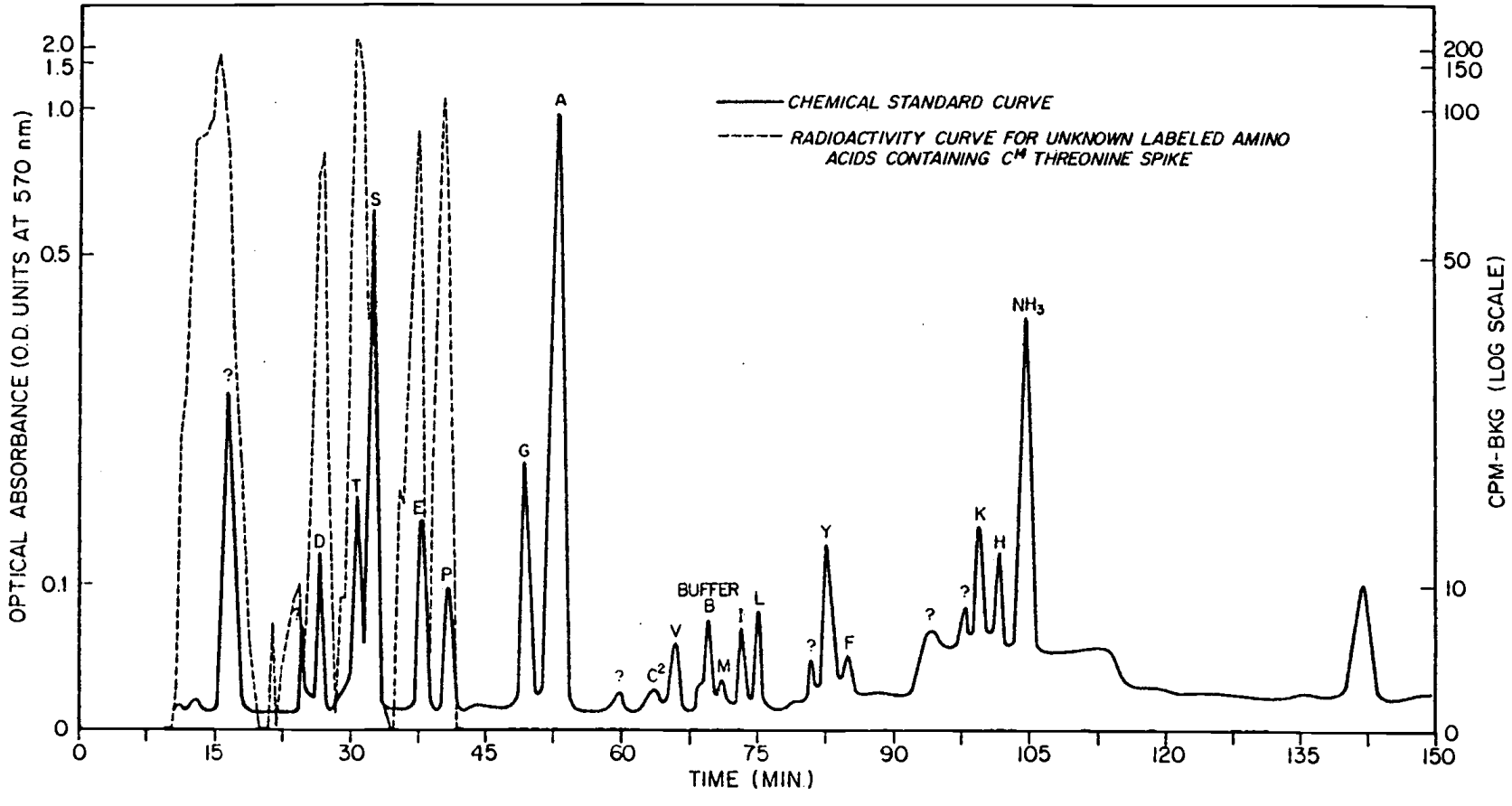

CHEMICAL STANDARD CURVE
RADIOACTIVITY CURVE FOR UNKNOWN LABELED AMINO ACIDS CONTAINING C14 SERINE SPIKE
OPTICAL ABSORBANCE (O.D. UNITS AT 570 nm)
CPM-BKG (LOG SCALE)
TIME (MIN.)
BUFFER
NH3

Figure 4: A typical chromatographic profile of the chemical concentration and radioactivity of unknown C-14 labeled amino acids isolated from the naupliar cytosol with an additional quantity of purified C-14 threonine (500 cpm).

Figure 4.


CHEMICAL STANDARD CURVE
RADIOACTIVITY CURVE FOR UNKNOWN LABELED AMINO ACIDS CONTAINING C^M THREONINE SPIKE
OPTICAL ABSORBANCE (O.D. UNITS AT 570 nm)
CPM-BKG (LOG SCALE)
TIME (MIN.)
2.0
1.5
1.0
0.5
0.1
0
200
150
100
50
10
0
15
30
45
60
75
90
105
120
135
150
?
D
T
S
E
P
G
A
?
C²
V
BUFFER
B
M
I
L
?
Y
F
?
?
K
H
NH₃

Whereas, when ^{14}C-threonine was co-chromatographed with the identical mixture, two peaks occurred as shown in Fig. 4. The ^{14}C-threonine peak occurs just prior to the serine peak and a second peak occurs as a small shoulder corresponding precisely with the serine chemical peak. Based upon this evidence, it is interpreted that the labeled amino acid is serine and not threonine.

4. Quantification of newly formed ^{14}C-labeled amino acids as a function of salinity

It was shown earlier that elevation of the external salinity caused an increase in intracellular amino acids (Tables IV and V). Could the increase seen in the intracellular amino acids be derived from metabolic pathways enhanced by the CO_2-fixation reaction? Results in Tables VI and VII were obtained from two separate experiments wherein nauplii were placed in different concentration of NaCl but had identical concentration of K+, Mg++ and Ca++ ions and specific activities of the added ^{14}C-bicarbonate. The concentration and specific activity of the labeled free amino acids were analyzed by chromatography. It can be seen that the same five amino acids were labeled in all of the salinities but at the higher salinities (>1.5 M) no additional amino acids were being formed despite an increase in synthesis via carbon dioxide fixation. The highest specific activity was exhibited by aspartic acid followed by glutamic, proline, serine, and alanine. A shift in salinity caused in increase in specific activity in the majority of labeled amino acids but alanine nearly doubled despite a 3-fold increase in chemical concentration. Clearly, alanine biosynthesis

TABLE VI. ^{14}C Labeled Amino Acids in the Naupliar Cytosol as a Function of Salinity

Labeled Peaks	0.25 M NaCl CO_2 Incorporation CPM/100 mg Protein	0.25 M NaCl CO_2 Incorporation (%)	0.25 M NaCl Specific Activity CPM/umole	1.0 M NaCl CO_2 Incorporation CPM/100 mg Protein	1.0 M NaCl CO_2 Incorporation (%)	1.0 M NaCl Specific Activity CPM/umole	2.0 M NaCl CO_2 Incorporation CPM/100 mg Protein	2.0 M NaCl CO_2 Incorporation (%)	2.0 M NaCl Specific Activity CPM/umole
Void Volume	20,493	--	--	21,080	--	--	23,789	--	--
Unknown 1	1,207	4.9	--	867	3.2	--	706	1.9	--
Aspartate	7,395	30.2	6,642	7,872	29.4	5,566	8,665	23.1	7,064
Serine	2,115	8.6	666	2,356	8.8	712	3,522	9.4	811
Glutamate	6,531	26.7	2,508	6,946	25.9	2,623	8,742	23.3	3,688
Proline	3,529	14.4	1,065	4,812	17.9	1,538	7,380	19.6	952
Alanine	2,197	9.0	395	2,879	10.7	363	7,845	20.9	445
Unknown 2	1,529	6.2	--	1,069	4.0	--	706	1.9	--
Final Tot. CPM	44,996	100.0	--	47,881	100.0	--	61,350	100.0	--
Init. Tot. CPM	40,772	--	--	45,920	--	--	39,720	--	--
% Recovery	--	110.0	--	--	104.0	--	--	154.0	--
Total A.A.'s	24,503	--	--	26,801	--	--	37,561	--	--

TABLE VII. ^{14}C Labeled Amino Acids in the Naupliar Cytosol as a Function of Salinity

Labeled Peaks	0.5 M NaCl CO_2 Incorporation CPM/100 mg Protein	0.5 M NaCl CO_2 Incorporation (%)	0.5 M NaCl Specific Activity CPM/umole	1.5 M NaCl CO_2 Incorporation CPM/100 mg Protein	1.5 M NaCl CO_2 Incorporation (%)	1.5 M NaCl Specific Activity CPM/umole	2.5 M NaCl CO_2 Incorporation CPM/100 mg Protein	2.5 M NaCl CO_2 Incorporation (%)	2.5 M NaCl Specific Activity CPM/umole
Void Volume	19,005	--	--	16,097	--	--	19,509	--	--
Unknown 1	612	2.9	--	879	3.6	--	672	2.0	--
Aspartate	6,406	30.7	2,677	6,378	26.2	2,679	7,206	21.5	2,792
Serine	1,515	7.3	393	1,669	6.8	514	2,432	7.3	552
Glutamate	6,981	33.4	2,120	6,836	28.0	2,148	6,140	18.3	2,566
Proline	3,080	14.8	1,359	5,061	20.8	1,738	7,021	20.9	1,338
Alanine	1,701	8.2	291	2,583	10.6	297	9,453	28.1	536
Unknown 2	559	2.7	--	968	4.0	--	610	1.8	--
Final Tot. CPM	39,859	100.0	--	40,471	100.0	--	54,300	100.0	--
Init. Tot. CPM	30,140	--	--	38,826	--	--	52,371	--	--
% Recovery	--	132.0	--	--	110.0	--	--	104.0	--
Total A.A.'s	20,854	--	--	24,374	--	--	33,534	--	--

by this metabolic pathway appears to very much salt-dependent and stimulated under these experimental conditions. The difference in the specific activity of aspartic acid between the two experiments is due to a decrease of aspartic acid content in one group of nauplii. It cannot be easily explained since the other amino acids have similar specific activities and chemical concentration per unit weight of nauplii (Table IV and V).

The peak which appears in the void volume contains a large amount of radioactivity due to the Kreb's cycle intermediates. Preliminary measurements indicate that malate is the major component but amino acids are absent. The total radioactivity found in this peak was not included in the calculation of the percentages of CO_2-incorporation into amino acids (Tables VI and VII).

5. Mortality of Nauplii, pH of Media and Dissolved Oxygen

To insure that the nauplii being labeled by ^{14}C-bicarbonate were not being influenced by other factors, monitoring of dissolved O_2 and pH of the media were made. pH remained on the alkaline side at 7.8 and was nearly constant between salinities, thus insuring that the specific activity of the external bicarbonate pool remained unchanged. Oxygen in the media fluctuated between salinities but at no time did the media become anoxic. Hence, anaerobic conditions of external environment were avoided. Lastly, naupliar mortality in both experiments was minimal and is only slightly higher at the elevated salinities. In all cases, 90% of the population was quite viable and therefore, one was not investigating dead or dying animals.

TABLE VIII. Dissolve O_2, pH of External Media and Mortality of Nauplii During Acclimation as a Function of Salinity

Time (hr)	0.25 M NaCl			1.0 M NaCl			2.0 M NaCl		
	ml O_2/l	pH	mortality (%)	ml O_2/l	pH	mortality (%)	ml O_2/l	pH	mortality (%)
0	4.1	6.850	0	3.4	7.025	2	4.3	7.150	3
1.5	4.8	7.210	1	4.8	7.487	3	4.2	7.478	2
3.0	1.3	6.612	1	2.6	7.085	2	N.A.	7.495	8

Time (hr)	0.5 M NaCl			1.5 M NaCl			2.5 M NaCl		
	ml O_2/l	pH	mortality (%)	ml O_2/l	pH	mortality (%)	ml O_2/l	pH	mortality (%)
0	2.4	7.326	0	2.6	7.592	0	1.5	7.400	1
1.5	0.9	7.494	4	0.9	7.350	5	2.0	7.412	10
3.0	1.8	6.945	1	1.8	7.499	3	1.8	7.420	N.A.

TABLE IX.

Glycogen Content of Nauplii as a Function of Salinity

Media Salinity (M)	Glycogen (ug glucose/100 mg. protein)	Radioactivity (CPM/ug glucose)
0.25	12,550	<1
1.0	11,810	<1
2.0	8,730	<1

6. Glycolysis and Glycogen Content of Nauplii as function of salinity

Since glycogen is the major substrate for anaerobic metabolism, the glycogen content of the acclimated nauplii for the three different salinities was measured. Table XI shows the values of glycogen found and it clearly shows that glycogen content decreased as salinity increased. These results support the earlier findings that glycolysis is coupled in some unknown fashion with electrolyte and water balance in these animals (Conte et. al., 1980).

Discussion

1. High Aerobic Glycolysis under Hypersaline Conditions

Since the environmental parameter being studied is the effect of external salts, it was necessary to provide assurances that other environmental factors did not alter the course of the investigation. One major environmental factor given considerable attention was the level of oxygen, because dissolved oxygen decreases substantially as salt concentrations approach saturation levels. Table VIII indicates that measured values for dissolved oxygen, although slightly variable, do not indicate major differences existing between salinities and that the media were definitely in an aerobic state. Therefore, our experimental conditions were neither anoxic or hypoxic. Furthermore low densities of nauplii were used, and partially filled acclimation flasks to maximize surface area exposure were used to aid gas exchange together with constant shaking of the flasks. It can be concluded from these data, that nauplii were being acclimated to hypersaline conditions under aerobic circumstances.

Despite the availability of oxygen and reasonable rates of oxygen consumption (Conte, 1980), a high rate of glycolysis was observed as shown in Table IX. These findings substantiate earlier work which showed high aerobic glycolysis occurring but being accompanied by negligible lactate production (Conte, 1980) which would not happen under typical anaerobic conditions (Ewing and Clegg, 1964). Racker (1976) reports finding high aerobic glycolysis occurring in tumor cells but it occurs with a concommitant increase in lactate production. Both of

these metabolic activities were found to be linked with ATPase activity, especially Na+K- activated ATPase. Similarly, it has been shown that the rate of aerobic glycolysis can be reduced substantially by the addition of ouabain (10^{-4}M) in the external salinities surrounding nauplii undergoing acclimation to hypersaline conditions (Conte, 1980, Conte et. al., 1980). When taken together, these findings suggest that Na+K-ATPase activity or its end products may control glycolysis under hypersaline conditions.

2. Relationships between Na+K-ATPase and CO_2-Fixation Reaction

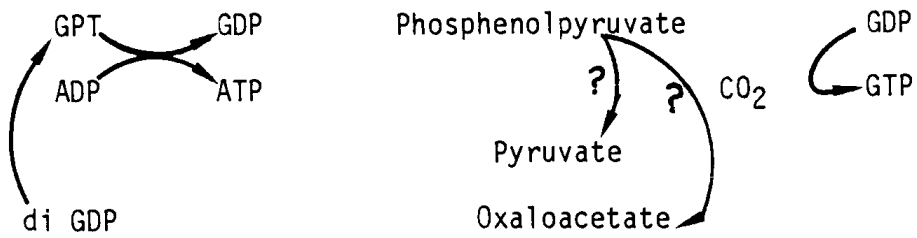
One must now ask "What is the common factor between Na+K-ATPase activity, the C-3 fragment derived from glucose metabolism and the carbon dioxide fixation reaction"? It has been generalized that the Na+K-ATPase reaction occurs as follows:

$$3Na^+ + ATP \xrightarrow[ATPase]{Na+K} 2K + ADP + Pi$$

where a net transport of Na^+ occurs during ATP hydrolysis. Since large quantities of Na+K-ATPase have been found in nauplii (Peterson et. al., 1978) and it appears responsible for development of naupliar osmoregulation (Conte et. al., 1978) it looks like that either the substrate (ATP) or the end-products (ADP or Pi) or the enzyme moiety itself may be the critical elements. Measurements of adenine nucleotide levels under hypersaline conditions have shown that [ATP] decreases and [ADP] remain nearly constant (Ewing et. al., 1980). Levels of Pi or its transport into or out of the embryo have not been determined.

Perhaps, other nucleotides can serve as equivalents to ATP and are recruited to replenish the low substrate levels of ATP needed by Na+K-

ATPase. Warner and co-workers (1972) have investigated the breakdown of yolk platelets in regard to nucleotide metabolism and found that of all the acid-soluble nucleotides, only ATP shows an increase in proportion to the decrease of the high energy storage compound di GDP (or Gp_4G). Unfortunately it is not known at this time if hypersaline conditions enhances the breakdown of yolk platelets. If this assumption is correct, then the CO_2 fixation reaction could be driven by the need of its end-products to replenish the ATP substrate required by the Na+K-ATPase reaction. This can be viewed diagramatically:



3. Salt-dependent CO_2-fixation reactions

Results from this study show that hypersalinity conditions surrounding nauplii causes CO_2 fixation to be stimulated as evidenced by:

(a) An increase in total $^{14}CO_2$ labeled metabolites (Table IV and V).

(b) An increase in specific activities of five ^{14}C-labeled amino acids

(c) A two-three fold increase in specific activity of alanine and intracellular concentration of alanine.

Clegg (1976) showed that in the encysted embryo the CO_2-fixation reaction is not the result of a simple CO_2 adduction, but requires a metabolic catalyst, Lowenstein (1967) reviews the known metabolic pathways for carboxylation and decarboxylation reactions involving C-4 dicarboxylic acids. They are as follows:

1) A reductive carboxylation of pyruvate that is catalysed by malic enzyme:

Pyruvate + CO_2 + NADPH + H^+ ⟶ malate + $NADP^+$

2) A reversible reaction catalyzed by phosphoenolpyruvate carboxykinase:

Oxaloacetate + GTP (or ITP) ⟶ phosphoenolpyruvate + CO_2 + GDP (or IDP)

3) A carboxylation of pyruvate that is catalysed by pyruvate carboxylase:

Pyruvate + CO_2 + ATP ⟶ oxaloacetate + ADP + Pi

4) An enzyme bringing about the carboxylation of propionate:

Propionate + CO_2 ⟶ succinate

In vivo studies by Clegg (1976) following the incorporation of ^{14}C-carbon dioxide, given in the gaseous form since the shell is impermeable to ions, found the major organic acids to be ^{14}C-labeled amino acids and the dicarboxylic acids, malate and oxaloacetate. Since ^{14}C-succinate was not found, it would appear that pathway (4) is eliminated. *In vitro* studies (Conte, 1977) on the carboxylation requirements of a crude ammonium sulfate fraction of naupliar post-mitochondrial supernatant preparation indicated that there is need of a nucleotide (specifically GDP or IDP) cofactor and a specific substrate phosphoenolpyruvate (PEP). Pyruvate did not serve as a substrate in this preparation. Thus, pathway (2) appears to be the most promising mechanism responsible for carbon dioxide fixation in the nauplius.

Since large amounts of ^{14}C-malate have been reported by Clegg (1976) to form from ^{14}C-oxaloacetate, the observed increase in glycolysis under hypersalinity conditions would benefit from the additional formation of NAD^+ resulting from this oxidation reduction reaction. The lack of appearance of lactate could be resolved by this alternate pathway. Interestingly, the enzyme responsible for pathway (2) is PEPCK which has been found in other animal tissues to be a gluconeogenic enzyme (Holten and Nordlie, 1965; Moce and Utter, 1975; Colombo, et al., 1978) because $^{14}CO_2$ fixation results in the formation of labeled glucose or glycogen in these tissues. In contrast, ^{14}C-label was not found in either glucose or glycogen isolated from the naupliar cytosol. (Table IX).

In conclusion, the effects of hypersalinity on *Artemia* nauplii can be summarized as follows: 1) increased aerobic glycolysis, 2) increased incorporation of $^{14}CO_2$ five free amino acids, 3) increased levels of free amino acids in the naupliar cytosol and 4) increased excretion of

NH_3 and free amino acids into the media. These findings are consistent with and supportive of Conte's (1980) proposed C-4 dicarboxylic acid facultative anaerobic shunt.

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