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CELLULAR RESPONSES OF ARTEMIA SALINA
TO ANAEROBIC CONDITIONS

BY

SOVERIN KARMIOŁ

A Thesis
Submitted to the Faculty of Graduate Studies and Research
Through the Department of Biology in
Partial Fulfillment of the Requirements
for the Degree of Master of Science
at the University of Windsor

Windsor, Ontario, Canada

1981

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DEDICATION

I would like to dedicate this thesis to my parents, Kuba and Fela Karmiol, who through much uncertainty supported by efforts. Also to my wife, Erika, whose patience made the completion of this thesis possible and to my young son, Benjamin, who makes all things possible.

ABSTRACT

The stability of the encysted gastrulae of Artemia salina under anaerobic conditions has been studied in this thesis. A comparison with encysted gastrulae not subjected to anaerobic conditions has revealed various differences: a greater resistance of the yolk platelets from anaerobic cysts to solubilization by NaCl, to disruption by incubation at 37°C and to extraction of carotenoids by organic solvents; a change in the cytochrome c oxidase and GTP:GTP guanylyltransferase enzyme activities; and an increased carotenoid content in the cysts subjected to anaerobic conditions. An argument is presented to show that encysted gastrulae of Artemia salina are capable of stabilizing enzyme and membrane systems under anaerobic conditions by complexing these systems with carotenoids. The limnology of the natural habitat of Artemia salina is discussed in an attempt to rationalize the response of encysted gastrulae of Artemia salina to anaerobic conditions.

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To the members of my committee, Dr. David Cotter, Department of Biology and Dr. Keith Taylor, Department of Biochemistry, thank you for the helpful criticism given and for agreeing to be members of my committee after the work was well in progress. And to my advisor, Dr. Alden H. Warner, for his infinite patience and excellent scientific training, which I still draw upon with confidence, a very special thank you.

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I. INTRODUCTION

In 1966, Dutrieu and Chrestia-Blanchine reported that the fully hydrated encysted gastrulae of Artemia salina could tolerate extended periods of anoxia (at least 5 months) without utilizing their carbohydrate and lipid reserves to any appreciable extent. Subsequently, these observations were confirmed and expanded by Ewing (1968) and Ewing and Clegg (1969) demonstrating that anaerobiosis also arrests morphogenesis and during anaerobic conditions there was no increase in the lactic acid concentration nor an increase in the lactate dehydrogenase activity. Also, the ability of gastrulae stored under anoxia to produce viable nauplii when placed in an aerobic environment at the same percentage (Ewing and Clegg, 1969) but not necessarily at the same rate (Stocco et al., 1972) as compared with gastrulae kept under aerobic conditions indicated that no major structural deterioration occurred under anaerobic conditions.

The existence of some form of energy for the maintenance of the structural integrity that must be occurring during anaerobic conditions in the hydrated embryo of Artemia seems to be a reasonable assumption. A candidate for this energy source may be the diguanosine nucleotides found so abundantly in the Artemia embryo (Finamore and Warner, 1963). In 1972, Stocco et al. demonstrated that although carbohydrate metabolism and morphogenesis are arrested, nucleotide metabolism continues. Under prolonged anaerobic conditions the adenosine nucleotide pools are

depleted, whereas the concentration of the diguanosine nucleotide, Gp₄G, steadily decreases for several months and the concentration of the diguanosine nucleotide, Gp₃G, initially increases and reaches a plateau which is maintained for several months.

The stability of these embryos during anaerobiosis is interesting not only in that it occurs, but also that it can be maintained for at least 5 months of anoxia. Such an adaptive mechanism of encysted gastrulae of Artemia salina during anaerobiosis is unique. Most embryos will develop normally only in the presence of oxygen (Boell, 1955) and malformation or death usually results from extended periods of anoxia (Stocco et al., 1972).

That the absence of oxygen should have such profound effects on a developing organism is understood in light of the need of the embryo to execute specific events at specific times in order to properly undergo morphogenesis. The embryo possesses insufficient stored energy to undergo morphogenesis without producing its own energy. It is for this reason that cleavage and blastulation will occur in the frog embryo during anaerobiosis but gastrulation is incomplete and neurulation is impossible in the absence of oxygen (Needham, 1942). Also, in sea urchins and ascaris embryos, cell division is blocked immediately by oxygen lack but the zygote can undergo activation and associated cortical changes in the absence of oxygen (Boell, 1955).

Artemia embryos under anaerobiosis possess a mechanism which successfully restricts the embryos from committing themselves to a process which they cannot complete appropriately and thereby are not subject to the deleterious effects of such an action.

It was felt that such an adaptive mechanism entails the stabilization of cellular components such as membranes and enzyme systems. Several experiments were performed on aerobic, anaerobic and anaerobic cysts (recovered) for 9.5 h in air, to evaluate the influence of anaerobic conditions on the stability of selected structures and the persistence of the stability in recovered embryos. This was accomplished by measuring in the three groups of cysts the structural integrity of the yolk platelets and the visible spectral properties of the carotenoid-protein complex (Warner et al., 1972) found in the yolk platelet and the role played by the carotenoids in the structural integrity and spectral properties. Also, the activity of membrane-associated and cytosol enzymes of various fractions was measured in aerobic, anaerobic and recovered cysts. The results of these experiments indicated that anaerobiosis conferred some stability on these cellular components with the carotenoids playing a central role in this stability, and that in some instances, stability was still present in the cysts after a 9.5 h recovery period in air compared to cysts maintained aerobically.

Prior investigation (unpublished) in this laboratory on the cytosol during gel filtration chromatography revealed a shift in the elution position of the macromolecular compounds to a higher molecular weight range with increased time under anaerobic conditions. In an attempt to elucidate this observation, the distribution of the protein, lipid, carotenoids and inorganic phosphate in three subcellular fractions was investigated in aerobic, anaerobic and recovered cysts.

Of the various strategies used by organisms to overcome the problems of anaerobiosis, the one employed by Artemia salina is especially

successful. The attempt will be made to demonstrate that an integral part of this successful strategy is the complexing of carotenoids to selected cellular structures of the embryo.

II. MATERIALS AND METHODS

The experimental results reported here were obtained from three separate batches (designated numbers 4, 7 and 9) of dried encysted embryos (cysts) of the brine shrimp Artemia salina harvested from the salterns in Utah (Canadian Aquarium Supply Co., Ltd.). Batch 4 was used for the study of the solubility properties of the carotenoid-protein complex from yolk platelets. Batch 7 was used for the experiments on the subcellular distribution of protein, lipid, carotenoid and inorganic phosphate. Batch 9 was used for the remainder of the experiments.

A. Sterilization, N₂ - Purging and Incubation Procedures

The cysts were sterilized by immersion in 7% antiformin solution for 30-45 minutes in an ice-bath (Nakanishi et al., 1962). Following the removal of debris and floating cysts by suction, the cysts were washed with distilled water until the washings were neutral to pH paper. The cysts were then suspended in ice cold 1% urea for 30 minutes to remove any residual antiformin after which the cysts were washed with water as outlined above. The sterilized cysts were collected on a fritted-glass funnel and divided into two portions. One portion was frozen immediately and served as control (C) embryos, while the other was purged with N₂ as described below.

Ten-gram portions of sterilized cysts were placed in 50 ml ampules (Wheaton Gold Band funnel top ampules, Wheaton Glass and Plastics,

Brantford, Ontario) and covered with approximately 50 ml of sterile sea water (Warner and McClean, 1968). The ampules were placed in an ice-bath and purged for at least 30 minutes with purified N₂ (Stocco et al., 1972).

Following the N₂ treatment the ampules were sealed and stored at room temperature. At the desired times the ampules were broken open and the cysts collected on a fritted-glass funnel and washed with distilled water. In some cases the cysts were divided into two portions; one portion was frozen immediately and served as the experimental (E) embryos while the other was divided into 1.5-2.0 g portions which were placed in petri dishes and covered with artificial sea water containing penicillin (1000 units/ml) and streptomycin sulphate (100 µg/ml) (Warner and McClean, 1968) and incubated for 9.5 h at 30°C. These cysts were then collected on a fritted-glass funnel washed with water and then stored at -20°C. Based on a previous study (Stocco et al., 1972), these embryos should have fully recovered from the effects of the N₂ treatment, at least with respect to their nucleotide profile, and they were denoted as (R).

Storage of hydrated cysts at -20°C results in the freezing of the water around the cysts causing the entire population of cysts to form a solid mass. Consequently, sample taking becomes difficult and leads to certain problems, i.e., the cysts either need to be thawed in air or a stream of water needs to be forced into the mass of cysts dislodging the required amount. In order to eliminate this difficulty the C, E and R embryo samples from batch 9 were lyophilized for 24 h and stored at -20°C.

The percentage of embryos that emerged and hatched after the above treatments was determined as described previously (McClellan and Warner, 1971).

B. Collection of Subcellular Fractions

In this study four subcellular fractions were obtained; these were total homogenate (TH), yolk platelets (YP), mitochondrial-rich fraction (MRF) and post-mitochondrial fraction (PMF). The four fractions were obtained sequentially by differential centrifugation in a refrigerated centrifuge (Sorvall, RC-2B) as described below.

Batches of 5-10 grams of cysts were hydrated overnight in 2.5% NaCl at 4°C. The cysts were then collected and washed on a fritted-glass funnel then suspended in 20-30 ml of 0.3 M sucrose containing 0.2 mM EDTA and 0.03 M Tris-HCl, pH 7.4, and homogenized by stirring vigorously for 2.0-3.5 h at 0°C using a magnetic stirring device. The mixture was then filtered through a nylon cheesecloth nylon filter. The filtrate represented the total homogenate. Aliquots were removed from the filtrate and the remainder was centrifuged at 700 g for 15 minutes. The 700 g supernatant fraction was removed avoiding the lipid material adhering to the sides of the centrifuge tube. The 700 g pellet was washed several times with the homogenization buffer and the 700 g supernatant fractions were pooled. The final 700 g pellet containing primarily yolk platelets was suspended in a small volume of homogenization buffer.

The pooled 700 g supernatant fractions were centrifuged at 12,100 g for 15 minutes and the resulting pellet (MRF) was washed with homogenization buffer until there was no lipid material adhering to the sides of

the centrifuge tube. The MRF was suspended in a small volume of homogenization buffer and either used immediately or stored at 20°C for future use. The pooled 12,100 g supernatant fractions represented the post-mitochondrial fractions (PMF) and they were stored as above until needed.

C. Isolation of Lipids, Carotenoids and Protein, and Determination of Inorganic Phosphate

Aliquots were taken from each subcellular fraction, one each for the determination of protein, lipid and carotenoid content and the other for the determination of inorganic phosphate.

For the determination of lipid, carotenoid and protein, the subcellular fractions were extracted according to the method of Bligh and Dyer (1959) as outlined below. Sufficient redistilled methanol and chloroform (made 1% with respect to ethanol as a preservative) were added to an aliquot of the subcellular fraction so that the final volumes of chloroform, methanol and water were in a ratio of 1:2:0.8, respectively. The precipitate that formed upon extraction was collected by centrifugation (at room temperature) and re-extracted using a mixture of chloroform, methanol and water in the ratios described above until the precipitate became colourless (3-4 times). The precipitate was saved for protein determination (Lowry et al., 1951).

The chloroform-methanol-water soluble fractions were transferred to a stoppered polyethylene centrifuge tube and stored at 0°C. To the chloroform-methanol-water soluble fractions sufficient chloroform and 0.05% NaCl (Folch et al., 1954) were added to bring the volume of chloroform, methanol and water to a ratio of 2:2:1.8, respectively, and the

two layers were separated by centrifugation. The lower phase contained the lipids, pigments and some non-lipid material which was removed by washing with a volume of chloroform, methanol and 0.3% NaCl (3:48:47 by volume) equal to the discarded upper phase. This is a modification of the Folch upper phase which contains 0.02% CaCl_2 , 0.017% MgCl_2 , and 0.29% NaCl (Folch et al., 1957). The presence of NaCl in the wash enhances the distribution coefficient of some lipids so that they remain in the lower phase minimizing their loss during washing (Folch et al., 1957). After thorough mixing the two phases were resolved by centrifugation and the lower chloroform phase containing the semi-purified lipids and pigments was saved.

The carotenoid content of the chloroform phase was determined spectrophotometrically as described below (Beckman, Model DB-G). For the total lipid content an aliquot of the chloroform layer was concentrated at 37°C using an Evapomix Apparatus (Buchler Industries, Ft. Lee, N.J., U.S.A.). Aliquots of the lipid concentrate were transferred to tared circular aluminum foil discs (10-20 mg, 2.0 cm in diameter), and the chloroform was evaporated by placing the discs on a hot plate set at 54°C, and the samples were weighed on a Cahn Electrobalance (Cahn Instrument Company, Paramount, Calif., U.S.A.).

For the determination of inorganic phosphate, aliquots of each subcellular fraction were made 2 N with respect to HClO_4 at 0°C. The precipitate was removed by centrifugation at 12,100 g for 15 minutes and the supernatant fraction was used for the determination of phosphate according to the method of Ernster et al. (1950).

D. Analysis of Carotenoids of Subcellular and Various Yolk Platelet Fractions

Washed cysts (2-5 g wet weight) were homogenized in 0.03 M Tris-HCl, pH 7.4, containing 0.3 M sucrose according to Warner (1975) except that the brine cyst paste was suspended in 25 ml of buffer. The homogenate was filtered as described above and the filtrate was centrifuged at 1,500 g for 15 minutes. The subcellular fractions were the same as those described above except that the mitochondrial-rich pellet was excluded.

The purified yolk platelets (12.7 mg protein/ml for the control and 12.9 mg protein/ml for the experimental) were suspended in 0.03 M Tris-HCl, pH 7.4, and the preparation was divided into two equal portions. One portion (6 ml) was made 1 M with respect to NaCl by adding the appropriate amount of 0.03 M Tris-HCl, pH 7.4, containing 2 M NaCl and the carotenoid-protein complex (CPC) was extracted for 1.5 h at 0°C. The second portion (6 ml) was treated with 10 µg/ml trypsin (lyophilized; Worthington Biochemical Co.). The solubilized material from both preparations was obtained by centrifugation at 7,500 g for 15 minutes. The carotenoids from the soluble fractions and from whole cysts were extracted according to the Bligh and Dyer extraction procedure described above. The chloroform was removed by evaporation at 30°C facilitated by a stream of nitrogen and the oily residue was dissolved in petroleum ether (30°C-60°C). Samples were streaked across a thin-layer plate of silica gel (Eastman Kodak, type 6061) previously activated at 115°C for 30 minutes and the thin-layer plate was developed as recommended by Davies (1963) using petroleum ether and diethyl ether (1:1 by volume) as the solvent (Foppen, 1971). This procedure resolved four visible bands. Each band was scraped off the thin-layer plate, extracted from

the silica gel with chloroform, then analyzed in a spectrophotometer (Beckman Acta MVI). Two of the carotenoids, one red and the other yellow in the solvent system employed here, comprised 95% of the total visible pigments. The red carotenoid presumed to be canthaxanthin was quantitated using the extinction coefficient:

$$E_{1 \text{ cm}}^{1\%} = 220 \text{ at } 482 \text{ nm}$$

This coefficient was determined using a pure sample of canthaxanthin from F. Hoffman-LaRoche and Co., Ltd. The yellow pigment was transferred from chloroform to petroleum ether (30°C-60°C) as described previously. This pigment thought to be echinenone was quantitated using the following extinction coefficient (Foppen, 1971):

$$E_{1 \text{ cm}}^{1\%} = 2168 \text{ at } 458 \text{ nm}$$

These two carotenoids were analyzed further by thin-layer chromatography and spectral analysis in several solvents before and after saponification (Davies, 1965) but only the red carotenoid was compared to a known standard.

E. Purification of Mitochondria for Enzyme Studies

Hydrated cysts were homogenized in a cold mortar and pestle with 0.3 M sucrose containing 1.0 mM EDTA and 0.3 M Tris-HCl, pH 7.4. The mitochondrial-rich fraction (MRF) was prepared by the same manner as described in the collection of subcellular fractions except that the homogenate was filtered through a cheesecloth-glass wool-cheesecloth filter and the filtrate centrifuged at 1,500 g for 15 minutes. The MRF pellet was suspended in 15% sucrose (w/v) containing 0.1 mM EDTA and 0.01 M Tris-HCl, pH 7.4, then purified on sucrose gradients as described below.

The mitochondria were purified on a linear sucrose gradient using one of two centrifugation procedures. The first procedure utilized a 15% to 65% linear sucrose (w/v) gradient containing 0.1 mM EDTA and 0.01 M Tris-HCl, pH 7.4, and the samples were centrifuged at 30,000 rpm for 1.0 h in a SW 41 rotor (Beckman L-55 ultracentrifuge) (Young and Zimmerman, 1973). The second procedure utilized 15% to 60% linear sucrose (w/v) in the same buffer and the centrifugation was at 25,000 rpm for 45 minutes. In both cases the mitochondrial band was removed with a pasteur pipette, diluted with the homogenization buffer and centrifuged at 12,100 g for 15 minutes to pellet the mitochondria. The resulting mitochondria were either suspended in the appropriate buffer and used immediately for the enzyme assays or stored as a pellet at -10°C .

F. Assay of Mitochondrial Malate Dehydrogenase (E.C. 1.1.1.37) Activity

The malate dehydrogenase (MDH) assay was performed at 30°C - 31°C according to England and Siegl (1969). The reduction of NAD^{+} was followed at 340 nm for 3 minutes using a Beckman Spectrophotometer (Model DB) connected to a recorder (Photovolt Corp., New York, N.Y.). The initial velocity was obtained from the slope of the straight portion of the recorder tracing. The reaction volume in both cuvettes was 1 ml containing the following components: 96 mM glycine-NaOH, pH 10, in both cuvettes; 84 mM L-malate, pH 7.5, in both cuvettes; 1.875 mM NAD^{+} , pH 6.5, in the sample cuvette; and the appropriate concentration of the mitochondrial suspension in both cuvettes. Activity is defined as $\mu\text{moles NAD}^{+}$ reduced per min per mg protein at 30°C .

G. Assay of Cytochrome c Oxidase (E.C. 1.9.3.1) Activity

The substrate cytochrome c was prepared in the reduced form as follows. A sample (20 mg) of cytochrome c was dissolved in 0.01 M potassium phosphate buffer, pH 7.0. For assay of the first mitochondrial preparation 11.6 mM cytochrome c, of which 62% had been reduced with potassium ascorbate (prepared according to Holland (1948)) was used. For assay of the second mitochondrial preparation 5.2 mM cytochrome c of which 98% had been reduced with dithiothreitol was used. The reductant was separated from the reduced cytochrome c by gel filtration (Yonetani, 1966) on Sephadex G-25 (Pharmacia) previously equilibrated with 0.01 M potassium phosphate, pH 7.0. The concentration of the reduced cytochrome c in all preparations was determined spectrophotometrically by oxidation with 0.1 M $K_3Fe(CN)_6$ using the following extinction coefficients (Margoliash, 1954):

$$550 \text{ nm (oxidized)} = 9.2 \text{ mM}^{-1} \times \text{cm}^{-1}$$

$$550 \text{ nm (reduced minus oxidized)} = 18.5 \text{ M}^{-1} \times \text{cm}^{-1}$$

The cytochrome c oxidase assay was performed according to the method of Wharton and Tzagoloff (1967). The reaction volume in both cuvettes was 1 ml containing the following components: 80 mM potassium phosphate, pH 7.0, in both cuvettes; the appropriate concentration of cytochrome c in both cuvettes as mentioned above; 5 mM potassium ferricyanide in the reference cuvette; and the appropriate concentration of mitochondrial suspension in the sample cuvette. The reaction rates were followed by measuring the decrease in absorption at 550 nm in a Beckman spectrophotometer connected to a recorder (Photovolt Corp., New York, N.Y.). The initial velocity was expressed as nmoles cytochrome c oxidized per min per mg protein at 30°C.

H. Determination of Post-Mitochondrial Fraction Malate Dehydrogenase (E.C. 1.1.1.37) (MDH) Activity

Hydrated cysts were homogenized in 0.015 M Tris-HCl containing 0.15 M NaCl, pH 7.4, using a pre-chilled mortar and pestle. The homogenate was filtered through a cheesecloth-glass wool-cheesecloth filter and the filtrate centrifuged at 12,100 g for 15 minutes. The 12,100 g supernatant was subjected to gel filtration and the ultra-violet-absorbing material which eluted in the void volume was pooled and used directly as the crude enzyme preparation (CEP). The assay procedure for MDH activity in the CEP is the same as that described for the mitochondrial MDH assay.

I. Determination of Glycerophosphate Dehydrogenase (E.C. 1.1.1.8) Activity in the Post-Mitochondrial Fraction

The crude enzyme preparation (CEP) described previously was also used for the determination of the glycerophosphate dehydrogenase activity according to the method of Beisenherz et al. (1955). Both cuvettes contained 1 ml with the following components: 42 mM TEA-HCl, pH 7.4; 92 μ M dihydroxyacetone phosphate; NADH (125 μ M), in the sample cuvette only and a suitable amount of enzyme. Incubation was at 30°C and the reaction rates were followed by measuring the change in optical density at 340 nm in a Beckman spectrophotometer (Model DB-G) connected to a recorder (Photovolt Corp., New York, N.Y.). The dihydroxyacetone phosphate was prepared according to the Sigma Chemical Company (Circular No. 383-1).

J. Determination of Trehalase (E.C. 3.2.1.28) Activity in the Post-Mitochondrial Fraction

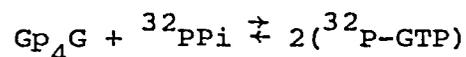
The CEP was adjusted to 60% with respect to ethanol, stirred for 15 minutes in an ice-bath, and the insoluble material was collected by centrifugation at 5,900 g for 10 minutes. The precipitate was suspended in a small volume of 0.005 M Tris-HCl, pH 7.4, and used as the enzyme source (Ewing, personal communication). The assay was performed according to Hill and Sussman (1963). The reaction mixture contained in 8 ml final volume: 125 mM maleic acid plus 125 mM NaOH, pH 6.5; 14.6 mM trehalose; and a suitable amount of enzyme preparation. Incubation was at 40°C and the addition of the enzyme preparation was used to start the reaction. Samples were taken at various times and the reaction terminated by boiling the sample for 4 minutes with 1.0 ml of distilled water (previously boiled for 30 seconds). The heat-treated samples were centrifuged at 3,000 g for 10 minutes and an aliquot of the supernatant fraction was analyzed for glucose (Somogyi, 1952).

K. Determination of Diguanosine Tetraphosphatase (E.C. 3.6.1.17) Activity

For the determination of diguanosine tetraphosphatase activity the CEP was concentrated using a Minicon B 15 macrosolute concentrator (Amicon Corp.). The assay was performed according to Warner and Finamore (1965). The reaction mixture contained in 1.5 ml final volume: 107 mM Tris-HCl, pH 8.0; 2 mM Gp₄G; 10 mM MgCl₂, and a suitable amount of the concentrated CEP. Incubation was at 40°C and the concentrated CEP was used to start the reaction. The reaction was terminated by boiling an aliquot of the reaction mixture for 4 minutes.

L. Determination of GTP:GTP Guanylyltransferase Activity in Yolk Platelets and Post-Mitochondrial Fraction

The CEP was made 40% with respect to ammonium sulphate and stirred in an ice-bath for 45 minutes. The insoluble material was collected by centrifugation at 6,800 g for 10 minutes, suspended in 0.015 M NaCl containing 0.015 M Tris-HCl, pH 7.4, and dialyzed against the same buffer for 6 h. Yolk platelets were prepared by cysts homogenized in a chilled mortar and pestle as described previously, except that the buffer contained 0.1% Tween 80, and the yolk platelets were suspended in distilled water prior to the enzyme assay. The reaction shown below was measured according to Warner and Huang (1974):



Each reaction mixture contained in 1.5 ml final volume: 1 mM Gp_4G ; 1 mM ${}^{32}\text{PPI}$ (S.A. $2.8\text{--}13.2 \times 10^6$ cpm/ μmole); 60 mM TEA-acetic acid, pH 5.75; 10 mM MgCl_2 ; 20 mM dithiothreitol; and a suitable amount of enzyme source.

M. Determination of Yolk Protein Solubility in NaCl and Sensitivity Towards Trypsin

Yolk platelets for the determination of yolk platelet solubility in NaCl were isolated as described above. The concentration of yolk platelets in these suspensions was determined turbidometrically at 500 nm then adjusted to equal concentrations by the addition of an appropriate amount of 0.03 M Tris-HCl, pH 7.4. The yolk platelet suspension for C, E, and R samples were each divided into three equal portions and NaCl containing 0.03 M Tris-HCl, pH 6.9, was added to each portion to a final concentration of 0.5, 1.0 and 2.0 molar, respectively. All samples treated in this way were kept in an ice-bath and shaken periodically.

Aliquots were taken at various times, centrifuged at 12,000 g for 15 minutes, and the protein content of the supernatant fraction was determined as described previously.

In an attempt to evaluate whether more than one type of carotenoid-protein complex was present in the yolk platelet, the carotenoid content was measured from carotenoid-protein complexes extracted by various NaCl concentrations. The assumption was made that different NaCl concentrations in the extraction medium would select different carotenoid-protein complexes that would differ in their carotenoid content. The final NaCl concentration of the extracting media was 0.5, 1.0 and 2.0 molar, and each contained 5 mM EDTA and 0.015 M Tris-HCl, pH 6.9. From the resulting carotenoid-proteins the carotenoids were extracted by the Bligh and Dyer (1959) lipid extraction procedure. The quantity of carotenoid in the complex was measured spectrophotometrically (Beckman DB-G).

In order to determine the sensitivity of yolk platelet protein to trypsin, equal quantities of yolk platelets were suspended in 0.3 M sucrose containing 0.03 M potassium phosphate, pH 7.4. Each yolk platelet preparation (6.0 ml, 14.0 mg protein/ml for the control and 14.3 mg protein/ml for the experimental) was pre-incubated for 5 minutes after which 21 μ g trypsin was added to each preparation and incubated in a Dubnoff metabolic shaking incubator (Precision Scientific Co.) at 37°C. Periodically, aliquots were taken and added to centrifuge tubes containing 13.1 μ g ovomucoid (Worthington Biochemical Co.) at 0°C. The tubes were centrifuged at 6,800 g for 5 minutes and the supernatant fluid was analyzed for protein without prior delipidation (Lowry et al., 1951).

N. Determination of Spectral Properties of Various Yolk Platelet Fractions

In these experiments purified yolk platelets were suspended in 0.03 M Tris-HCl, pH 7.4. Each suspension was divided into two portions: one portion was made 1 M with respect to NaCl by adding the appropriate amount of 0.03 M Tris-HCl, pH 7.4, containing 2 M NaCl and the CPC extracted for 1 h at 0°C. The second portion was treated with trypsin for 3 h at 37°C (in the manner as outlined previously) and centrifuged at 7,500 g for 15 minutes. UV-visible spectra were obtained on the supernatant fluid of each sample using a Beckman recording spectrophotometer (Acta MVI).

The supernatant fraction of each sample was subjected to a sequential fractionation using ammonium sulphate until the CPC precipitated. The ammonium sulphate precipitated material was solubilized in either 0.5 M Tris-HCl, pH 7.4, or 0.03 M Tris-HCl, pH 7.4, and their absorption spectra were determined.

O. Assay for Heat Sensitivity of Homogenate and Carotenoid-Protein Complex

The total homogenate after filtration as described above was incubated at 45°C in a Dubnoff metabolic shaking incubator in the dark. Aliquots were taken at various times, delipidated and the total carotenoids measured spectrophotometrically in chloroform. The CPC from the yolk platelets was suspended in 0.03 M Tris-HCl, pH 7.4, containing 2 M NaCl and incubated in the same manner as the total homogenate. Aliquots were taken at various times and their UV-visible spectra recording using a Beckman spectrophotometer (Model DB-G) attached to a 10" Beckman recorder.

P. Treatment of Carotenoid-Protein Complex from Yolk Platelets with Urea

Yolk platelets were treated with 2 M NaCl containing 5 mM EDTA and the mixture was centrifuged. The CPC in the supernatant was removed and analyzed spectrophotometrically. The CPC was then made 2 M with respect to urea by adding an equal volume of 4 M urea, stirred in an ice-bath for 1 h under N₂ and then frozen at -20°C. Before analysis the samples were thawed, centrifuged at 6,800 g for 15 minutes to remove the insoluble material and the supernatant fraction analyzed spectrophotometrically.

Q. Extractability of Carotenoids with Various Organic Solvents

The four subcellular fractions were isolated as previously described. These fractions were extracted with acetone and water until no pigment was visible in the supernatant fluid after centrifugation (approximately three extractions). The combined acetone-water extracts were pooled and the pigment transferred to petroleum ether (Lee, 1966). The remaining pigment in the pellet was extracted by the Bligh and Dyer lipid extraction method as described above.

R. Statistical Analysis

The pooled t-test or unpaired t-test was utilized for the statistical analysis. The pooled t-test was utilized in these circumstances since samples were drawn randomly from their respective populations instead of pairs drawn randomly from a single population as would be the case for the utilization of the paired t-test.

The value of t for the pooled t-test was evaluated by:

$$t = \frac{\bar{Y}_1 - \bar{Y}_2}{s_p \sqrt{\frac{1}{n_1} + \frac{1}{n_2}}}$$

where, \bar{Y}_1 represents the mean of the first group of samples, \bar{Y}_2 represents the mean of the second group of samples, and s_p represents the pooled standard deviation and is given by:

$$s_p = \sqrt{\frac{(n_1 - 1) s_1^2 + (n_2 - 1) s_2^2}{n_1 + n_2 - 2}}$$

where, n_1 represents the number of samples in the first group of samples, n_2 represents the number of samples in the second group of samples, s_1^2 represents the variance of the first group of samples, and s_2^2 represents the variance of the second group of samples.

The results of the statistical analysis were primarily reported at the 0.05 level of significance but other levels were also reported when it was felt that their presence would clarify the argument at hand.

III. RESULTS

A. Distribution of Protein, Lipids, Carotenoids and Inorganic Phosphate

The distribution of protein, lipid, carotenoids and inorganic phosphate and the corresponding ratio with protein in the subcellular fractions was determined for the control, experimental and recovered embryos. The method of evaluating the distribution of these subcellular constituents in a particular fraction was to measure the percent of the total of that constituent in that particular fraction. For each subcellular constituent in a group of embryos the total amount used for this calculation is derived by summing the amount of the constituent found in the fractions. The reason for choosing the sum of the fractions as the total was to minimize the chance of error. If the total homogenate was used as the total in the calculations an error in this value would result in an error in each of the fractions. If the sum of the fractions was used as the total amount for each constituent an error in the value of one subcellular constituent would not result in as large an overall error.

1. Distribution of protein among various embryo fractions of treated and untreated embryos.

The distribution of protein in the three subcellular fractions in embryos subjected to various periods of anaerobic conditions is depicted in Table 1. There is no significant difference between these values except for the MRF of the recovered embryos compared to the

TABLE 1. Distribution of protein among the subcellular fractions of Artemia embryos exposed to N₂/air for various periods of time.

Fraction ^a	Period Under Anoxia/Air	Percent of Total ^b
C-TH	NIL	100.0
C-YP	NIL	54.5 ± 9.5
C-MRF	NIL	4.3 ± 0.8
C-PMF	NIL	41.4 ± 9.6
E-TH	1 week under N ₂	100.0
E-YP	"	62.3 ± 2.3 (I)
E-MRF	"	4.8 ± 0.9 (I)
E-PMF	"	32.6 ± 1.4 (I)
E-TH	2 months under N ₂	100.0
E-YP	"	52.5 ± 6.4 (I)
E-MRF	"	5.3 ± 0.5 (I)
E-PMF	"	42.3 ± 6.9 (I)
R-TH	2 months under N ₂ , then	100.0
R-YP	9.5 h incubation in air	52.2 ± 11.8 (I)
R-MRF	at 30°C	6.1 ± 0.8 (S) ^c
R-PMF		41.7 ± 12.6 (I)

^aA control (washed cysts stored at -20°C) was run with each experimental group. The control values reported here are the average of all three control values (6). The letter in brackets after the number refers to the statistical significance. The (I) denotes a value which is not significantly different from the control value at the 0.05 level of significance and the (S) denotes a value significantly different from the control value at the 0.05 level of significance.

^bThe total is derived as the sum of the YP, MRF and PMF fractions.

^cAlthough this value was significantly different from the control value at the 0.05 level, it was not at the 0.02 level of significance. Also, it was not significantly different from the values of the MRF fraction of the other groups of cysts at the 0.05 level of significance.

control. In the MRF of the recovered embryos there is a significant difference at the 0.05 level but not at the 0.02 level of significance. Also, this value of the MRF of the recovered embryos is not significantly different from the other MRF values at the 0.05 level.

From Table 1 it can be seen the majority of the protein of the embryo is localized in the yolk platelet fraction (from 52 to 63%). The PMF fraction is responsible for approximately 32 to 42% of the protein with the MRF representing approximately 4 to 6% of the total.

2. Distribution of lipid among various embryo fractions of treated and untreated embryos.

The percent distribution of the lipid in the three subcellular fractions depicted in Table 2 demonstrates that there is no significant difference at the 0.05 level of significance as compared to the corresponding control values. Of interest is the lipid to protein ratio of the total homogenate of the embryos exposed to two months of anaerobiosis. Although the value of this ratio is not significantly different at the 0.05 level of significance from the corresponding control value it is significantly different from the corresponding lipid to protein ratios of the other two embryo groups. This discrepancy is primarily due to the greater variation in the value of the total lipid to total protein of the control embryos. With respect to the lipid to protein ratio the largest is found in the PMF: an approximate 1:1 ratio. The second largest lipid to protein ratio (0.7:1 to 0.95:1) is found in the MRF. The yolk platelet fraction has the lowest lipid to protein ratio (0.11:1 to 0.18:1). The average overall lipid to protein ratio of the embryo, depicted by the total homogenate, is 0.5:1.

TABLE 2. Distribution of lipid among the subcellular fractions of Artemia embryos exposed to N₂/air for various periods of time.

Fraction ^a	Period Under Anoxia/Air	Percent of Total ^b	Lipid (mg)/Protein (mg) ^c
C-TH	NIL	100.0	0.53 ± 0.13
C-YP	NIL	18.9 ± 1.2	0.18 ± 0.08
C-MRF	NIL	7.8 ± 2.6	0.95 ± 0.26
C-PMF	NIL	73.3 ± 3.6	1.09 ± 0.23
E-TH	1 week under N ₂	100.0	0.45 ± 0.02 (I)
E-YP	"	15.8 ± 3.0 (I)	0.11 ± 0.02 (I)
E-MRF	"	8.9 ± 4.5 (I)	0.80 ± 0.23 (I)
E-PMF	"	75.3 ± 7.4 (I)	1.04 ± 0.19 (I)
E-TH	2 months under N ₂	100.0	0.63 ± 0.01 (I) ^d
E-YP	"	16.9 ± 2.8 (I)	0.21 ± 0.06 (I)
E-MRF	"	5.9 ± 1.1 (I)	0.70 ± 0.07 (I)
E-PMF	"	77.2 ± 1.8 (I)	1.16 ± 0.21 (I)
R-TH	2 months under N ₂ , then 9.5 h incuba-	100.0	0.49 ± 0.04 (I)
R-YP	tion in air at	14.5 ± 4.1 (I)	0.13 ± 0.03 (I)
R-MRF	30°C	10.1 ± 1.6 (I)	0.80 ± 0.16 (I)
R-PMF		75.5 ± 3.5 (I)	0.93 ± 0.32 (I)

a and b As designated in Table 1.

^cThis ratio for the TH is derived by dividing the sum of the lipid of the YP, MRF and PMF fractions by the sum of the protein of the YP, MRF and PMF fractions.

^dAlthough this value is not significantly different from the control value it is significantly different from the other two total homogenate, lipid to protein ratios at the 0.05 level of significance.

Table 2 also demonstrates that approximately 15 to 19% of the lipid is localized in the yolk platelet fraction with the majority (approximately 73-77%) localized in the PMF. The MRF represents approximately 6 to 10% of the total lipid in the embryo.

As seen from Tables 1 and 2 there does not seem to be a major redistribution of the lipid or protein in the embryos due to anaerobiosis.

3. Distribution of carotenoids among various embryo fractions of treated and untreated embryos.

The influence of anaerobic conditions on the distribution of carotenoids in the encysted gastrulae is shown in Table 3. Here the data suggest that during anaerobiosis there is a redistribution of the carotenoids from the yolk platelet fraction into the other fractions (MRF and PMF). For example, with increased time under anaerobiosis, the percent of the total carotenoids represented by the yolk platelet fraction decreases and this is significant at the 0.01 level of significance. In the control embryos the yolk platelet fraction represents $82.1 \pm 10.6\%$ of the total carotenoids, whereas, in the embryos exposed to anaerobiosis for 1 week or 2 months and in the recovered embryos that yolk platelet fraction represents $60.0 \pm 2.7\%$, $49.5 \pm 4.2\%$ and $49.9 \pm 9.9\%$, respectively, of the total carotenoids. Of interest is the observation that although the percent of the total carotenoids of the recovered embryos represented by the yolk platelet fraction is not significantly different from the corresponding value of the two experimental embryos at the 0.05 level of significance it is significantly different from the control value at the 0.01 level.

TABLE 3. Distribution of carotenoids among the subcellular fractions of Artemia embryos exposed to N₂/air for various periods of time.

Fraction ^a	Period Under Anoxia/Air	Percent of Total ^b	Carotenoids (O.D. 482)/Protein (mg) ^c
C-TH	NIL	100.0	0.20 ± 0.05
C-YP	NIL	82.1 ± 10.6	0.30 ± 0.05
C-MRF	NIL	0.5 ± 0.5	0.03 ± 0.02
C-PMF	NIL	17.5 ± 10.4	0.09 ± 0.07
E-TH	1 week under N ₂	100.0	0.35 ± 0.03 (S)
E-YP	"	60.0 ± 2.7 (S) ^d	0.34 ± 0.03 (I)
E-MRF	"	3.0 ± 0.8 (S)	0.22 ± 0.04 (S)
E-PMF	"	37.1 ± 1.7 (S) ^e	0.40 ± 0.04 (S)
E-TH	2 months under N ₂	100.0	0.37 ± 0.00 (S)
E-YP	"	49.5 ± 4.2 (S) ^d	0.35 ± 0.01 (I)
E-MRF	"	2.5 ± 0.3 (S)	0.18 ± 0.03 (S)
E-PMF	"	48.1 ± 4.0 (S) ^e	0.43 ± 0.04 (S)
R-TH	2 months under N ₂ , then	100.0	0.34 ± 0.08 (S)
R-YP	9.5 h incubation in air	49.9 ± 9.9 (S)	0.32 ± 0.06 (I)
R-MRF	at 30°C	3.1 ± 0.1 (S)	0.17 ± 0.01 (S)
R-PMF		47.6 ± 9.8 (S)	0.41 ± 0.08 (S)

a and b

As designated in Table 1.

^cAs designated in Table 2.

^dThese values are significantly different from each other at the 0.10 level of significance.

^eThese values are significantly different from each other at the 0.10 level of significance.

With respect to the carotenoids the response of the PMF with increased time under anaerobiosis displays a slightly greater difference than was observed in the YP fraction. With increased time under anaerobiosis the percent of the total carotenoids represented by the PMF increases significantly. In the control embryos, the percent of the total carotenoids represented by the PMF is $17.5 \pm 10.4\%$, whereas in the embryos exposed to anaerobiosis for 1 week or 2 months and in the recovered embryos, the corresponding values are $37.1 \pm 1.7\%$, $48.1 \pm 4.0\%$ and $47.6 \pm 9.8\%$, respectively. As in the case of the YP fraction the value for the PMF of the recovered embryos is not significantly different from the other two experimental groups at the 0.05 level of significance but is significantly different from the control value at the 0.01 level of significance. These changes appear to be due primarily to an overall increase in the carotenoid content of all fractions of the experimental and recovered embryos as suggested by the carotenoid to protein ratio in these fractions.

In all fractions except the YP fraction, the carotenoid to protein ratio is significantly greater (at the 0.05 level) in all embryos exposed to anaerobic conditions as compared to the control. Among the various yolk platelet fractions there is no significant difference in the carotenoid to protein ratio (see Table 3). Of particular significance is the increase in the carotenoid to protein ratio of the PMF of the embryos exposed to anaerobic conditions as compared to the control embryos. Except for the MRF, the PMF showed the highest increase in this ratio. The large increase in the carotenoid content of the MRF of embryos exposed to anaerobic conditions may be

related to the fact that the mitochondria from embryos exposed to anaerobic conditions are "stickier" than mitochondria from control embryos. This quality of the anaerobic mitochondria will be elaborated on in the discussion. Since the carotenoid to protein ratio of the YP fraction is relatively large a small contamination of the MRF with yolk platelets may result in a substantial increase of the carotenoid to protein ratio of the MRF. This "stickiness" of the anaerobic mitochondria may cause a greater adherence of the platelets to the mitochondria and create a greater contamination than is found in the control embryos. However, increase in the carotenoid to protein ratio of the PMF in the experimental embryos is believed to be attributable primarily to changes in the PMF itself. In the control fraction, the carotenoid to protein ratio for the PMF is $0.09 \pm 0.07:1$, whereas for treated embryos the ratios were all around $0.40:1$ (see Table 3).

A general increase in the carotenoid content of the embryos which have experienced anaerobic conditions can be seen by comparing the carotenoid to protein ratio in the total homogenates of control and treated embryos. In the control this ratio is 0.20 ± 0.05 . For the embryos exposed to anaerobiosis the value increases to 0.37 after 2 months of anaerobiosis and remains near that value even after incubation in air for 9.5 h. It should be noted that this increase in ratio is seen in all embryo fractions. The change in the percent of the total carotenoids of the YP and PMF fractions during anaerobiosis can be explained by an increase in the carotenoid content in the PMF of the experimental and recovered embryos. Such a condition would reduce the percent of the total carotenoid represented by the YP fraction and

increase the percent of the total represented by the PMF as is seen in Table 3.

4. Distribution of Pi among various embryo fractions of treated and untreated embryos.

Nucleotide metabolism is active in Artemia embryos under anaerobic conditions (Stocco et al., 1972), therefore, an analysis of the change in distribution, if any, of inorganic phosphate appeared warranted. Table 4 shows an experiment in which the inorganic phosphate content of various embryo fractions was analyzed in relation to the protein content and with respect to the distribution of the Pi among these fractions. In these experiments there appears to be an increase in the Pi content of the embryos exposed to anaerobic conditions. The Pi to protein ratio of the total homogenate fraction from the experimental and recovered embryos is significantly greater at the 0.05 level than the corresponding value of the control embryos. The Pi to protein ratio of the PMF of the embryos exposed to anaerobic conditions for 1 week and 2 months are also significantly different at the 0.05 level from the corresponding value of the control embryos. The Pi to protein ratio of the PMF of the recovered embryos is not significantly different from the control value nor from the two experimental values (at the 0.05 level of significance). However, this apparent lack of correlation appears to be due to the large deviations of the individual sample values used to calculate this ratio. There does not seem to be a relationship between time under anaerobic conditions and Pi content. The values of the Pi to protein ratio of the total homogenate of the embryos exposed to 1 week and 2 months of anaerobic conditions are not significantly different at even the 0.20 level of significance. This

TABLE 4. Distribution of Pi among the subcellular fractions of Artemia embryos exposed to N₂/air for various periods of time.

Fraction ^a	Period Under Anoxia/Air	Percent of Total ^b	Pi (nmoles)/Protein (mg) ^c
C-TH	NIL	100.0	54.5 ± 6.9
C-YP	NIL	23.3 ± 5.0	24.5 ± 10.7
C-MRF	NIL	1.0 ± 0.4	9.4 ± 5.3
C-PMF	NIL	75.7 ± 4.8	111.0 ± 48.7
E-TH	1 week under N ₂	100.0	117.9 ± 2.5 (S)
E-YP	"	5.8 ± 1.2 (S) ^d	11.0 ± 2.4 (I)
E-MRF	"	0.3 ± 0.3 (I) ^d	7.7 ± 4.6 (I)
E-PMF	"	94.4 ± 2.2 (S) ^d	340.4 ± 26.9 (S) ^d
E-TH	2 months under N ₂	100.0	146.8 ± 33.5 (S)
E-YP	"	6.7 ± 3.1 (S) ^d	18.3 ± 6.6 (I)
E-MRF	"	0.4 ± 0.4 (I) ^d	11.5 ± 9.1 (I)
E-PMF	"	92.8 ± 3.5 (S) ^d	335.4 ± 140.5 (S) ^d
R-TH	2 months under N ₂ , then	100.0	101.4 ± 33.0 (S)
R-YP	9.5 h incubation in air	15.1 ± 8.5 (I)	28.1 ± 13.4 (I)
R-MRF	at 30°C	1.1 ± 1.2 (I)	15.4 ± 16.6 (I)
R-PMF		83.8 ± 9.8 (I)	231.8 ± 159.9 (I)

a and b As designated in Table 1.

c As designated in Table 2.

d Although these values are significantly different from the control value they are not significantly different from the corresponding value of the recovered (R) embryos at the 0.05 level of significance.

similarity of values is perhaps due to the large standard deviation of the value of the ratio of the embryos exposed to 2 months of anaerobic conditions ($146.8 \pm 33.5:1$). The Pi to protein ratios of the YP and MRF fractions are not significantly different from the control values at the 0.05 level of significance. It would seem that the increase in the Pi to protein ratio of the PMF in the embryos exposed to anaerobic conditions is responsible for the overall increase of the Pi to protein ratio of the embryos as evidenced by the Pi to protein ratio of the total homogenate.

One manifestation of the increased Pi to protein ratio of the PMF is a significant (0.05 level) increase in the percent total Pi that is found in the PMF as compared to the PMF of the control embryos. In the recovered embryos, the percent of the total Pi found in the PMF is not significantly different from the corresponding control value. At the 0.05 level of significance, however, this value is also not significantly different from the two corresponding values of the experimental embryos. Such a condition exists because of the relatively large error in the value of the PMF of the recovered embryos as evidenced by the standard deviation, $83.8 \pm 9.8\%$. The percent of the total Pi represented by the YP fraction is significantly smaller in the experimental embryos compared to the control embryos. In the recovered embryos, the same situation exists as was found with the PMF. Thus, during anaerobiosis there seems to be considerable metabolism of a phosphate rich substrate(s) resulting in an increase in the Pi. This increased Pi seems to be located mainly in the PMF. Whether such an increase in Pi is maintained in the recovered embryos is difficult to say, since the

diversity in the values used to arrive at the distribution of Pi and Pi to protein ratio is sufficiently great to make meaningful statistical inference difficult.

Generally, a redistribution of the subcellular components of the Artemia embryo under anaerobic conditions does not seem likely as evidenced by the data from Tables 1 and 2. The main differences between the control and experimental embryos as shown in Tables 3 and 4 are due to an increase in the carotenoid and Pi content of these embryos and not to a redistribution of these components.

B. Enzyme Activity in Various Subcellular Fractions

The relative metabolic quiescence of Artemia embryos exposed to anaerobic conditions warranted an analysis of the activity of various enzymes in order to determine the extent and nature of this quiescence.

The carbohydrate reserve is not utilized to any appreciable extent in Artemia embryos exposed to anaerobic conditions (Dutrieu and Chrestia-Blanchine, 1966). The major carbohydrate reserve of Artemia embryos is in the form of trehalose and in an attempt to understand the nature of the lack of carbohydrate utilization during anaerobiosis, the activity of trehalase was measured.

Although the major route of carbohydrate metabolism does not seem to be operative (Ewing and Clegg, 1969) in Artemia embryos during anaerobiosis, alternative processes may be operative under these conditions. These alternative processes are generally referred to as "shuttles." Two examples of such "shuttles" are the NAD requiring enzymes glycerophosphate dehydrogenase and cytoplasmic malate dehydrogenase.

Also of interest was the activity of certain mitochondrial enzymes in embryos maintained under anaerobic conditions. The response of the mitochondria to anoxia was studied by determining the activity of malate dehydrogenase in an attempt to evaluate whether the malate shuttle was operational. The respiratory potential of the mitochondria was evaluated by measuring the activity of cytochrome c oxidase.

As mentioned previously, nucleotide metabolism, especially diguanosine nucleotide metabolism, is not quiescent in Artemia embryos exposed to anaerobic conditions. Therefore, enzymes associated with diguanosine nucleotide metabolism (diguanosine tetrphosphatase and Gp₄G synthetase) were assayed in the yolk platelet and PMF fractions.

1. Activity of selected enzymes in the post-mitochondrial fraction.

A summary of the activity of these enzymes from embryos exposed to various N₂ treatments is shown in Table 5.

Trehalase activity in the PMF does not appear to be influenced by anaerobic conditions. In fact, there is no significant difference (at the 0.05 level) between the embryos exposed to 1 month of anoxia, even after re-incubation for 9.5 h, compared to the control embryos.

The glycerophosphate dehydrogenase activity of the PMF is also shown in Table 5. In this case, anaerobiosis appears to promote an increase of the activity of this enzyme as compared to the control value at the 0.05 level of significance. In the recovered cysts, the activity is also greater than the control at the 0.05 level of significance. A comparison of the activities of the experimental and recovered embryos reveals that they are similar at the 0.05 level of significance but different at the 0.10 level of significance. The implication in this

TABLE 5. Assay of several enzymes in the post-mitochondrial fraction.

Sample	Period Under Anoxia/Air	Trehalase Activity (μ moles glucose generated/h/mg protein)	Glycerophosphate Dehydrogenase Activity (μ moles NADH oxidized/min/mg protein)	MDH Activity (μ moles NAD ⁺ reduced/min/mg protein)	Diguanosine Tetraphosphatase Activity (μ moles Gp ₄ G hydrolyzed/h/mg protein)
C	NIL	3.46 \pm 0.83	0.91 \pm 0.053	1.95 \pm 0.062	0.083 \pm 0.015
E	1 month under N ₂	3.53 \pm 0.63	1.27 \pm 0.066 ^{Sa}	2.04 \pm 0.066 ^I	0.103 \pm 0.014 ^I
R	1 month under N ₂ , then 9.5 h incubation in air	3.80 \pm 1.30	1.13 \pm 0.086 ^{Sa}	2.16 \pm 0.087 ^S	0.099 \pm 0.054 ^I

^I These values are not significantly different from their respective control values at the 0.05 levels of significance.

^S This value is significantly different from its respective control value at the 0.05 level of significance but not significantly different from the experimental value (E) at the 0.05 level of significance.

^{Sa} These values are significantly different from their control value at the 0.05 level of significance and are similar to each other at the 0.05 level of significance but different at the 0.10 level of significance.

situation is that the activity of the recovered embryos is approaching that of the activity of the control embryos.

The activity of cytoplasmic MDH does not seem to be influenced by exposure of embryos to anoxia for 1 month as compared to the control embryos at the 0.05 level of significance. However, the MDH activity of embryos recovering from anoxia (9.5 h in air) is significantly greater than the control value at the 0.05 level of significance, but not significantly different from the N₂-treated embryos at the 0.05 level of significance. This implies that these values may be quite similar and that a cytoplasmic MDH "shuttle" most likely is not operational during anaerobiosis but may be operational during the recovery phase.

The activity of the diguanosine tetraphosphatase in the PMF was found to be very similar in all the cysts tested. The activities of the experimental and recovered cysts are not significantly different from the activity of the control value at the 0.05 level of significance nor are these two values significantly different from each other at the 0.05 level of significance.

2. Selected enzymes of the mitochondrial fraction.

In another series of experiments the activity of two mitochondrial enzymes was studied in embryos exposed to various periods of anoxia. For these experiments mitochondrial preparations were obtained from different samples of the same batch of cysts. Mitochondria prepared from one sample of cysts were used immediately for the enzyme analysis while mitochondria prepared from a second sample were frozen for 12 h then thawed prior to the enzyme analysis. The results of these studies are shown in Table 6.

TABLE 6. Malate dehydrogenase and cytochrome c oxidase activities of two mitochondrial preparations from Artemia embryos.

Mitochondrial Preparation	Period Under Anoxia/Air	MDH Activity ^c (μ moles NAD ⁺ reduced/ min/mg protein)	Cytochrome Oxidase Activity (nmoles cytochrome c oxi- dized/min/mg protein)
Preparation 1 ^a			
C	NIL	2.04 \pm 0.039	185.4 \pm 11.5
E	1 month under N ₂	2.01 \pm 0.084 ^I	146.5 \pm 3.0 ^S
R	1 month under N ₂ plus 9.5 h in air	2.10 \pm 0.269 ^I	179.2 \pm 3.6 ^I
Preparation 2 ^b			
C	NIL	1.66 \pm 0.061	402.8 \pm 11.5
E	1 month under N ₂	1.20 \pm 0.057 ^S	313.1 \pm 9.9 ^S
R	1 month under N ₂ plus 9.5 h in air	1.47 \pm 0.067 ^{Sa}	392.8 \pm 19.5 ^I

^aMitochondria purified on a 15% to 65% linear sucrose (w/v) gradient and centrifuged at 30,000 rpm for 1.0 h, and used as soon as possible for the enzyme assays.

^bMitochondria purified on a 15% to 60% linear sucrose (w/v) gradient and centrifuged at 25,000 rpm for 45 minutes, frozen for 12 h, thawed, and then used for the enzyme assays.

^cThe mitochondria were isolated from a sample of embryos different from the embryos used for the MDH activity of the PMF (see Table 6).

^IThese values are not significantly different from their respective control values at the 0.05 level of significance.

^SThese values are significantly different from their respective control values at the 0.05 level of significance.

^{Sa}This value is significantly different from the control value at the 0.05 level of significance but not at the 0.02 level of significance. This value is also significantly different from the experimental value at the 0.05 level of significance.

Using the first sample of embryos, the mitochondrial MDH activity of the experimental and recovered embryos was found to be similar to the MDH activity of control embryos at the 0.05 level of significance. In fact, the activity of the mitochondrial MDH from all three groups is quite similar to the activity of the cytoplasmic MDH from all three groups.

Mitochondria stored frozen prior to analysis showed reduced MDH activity compared to the first preparation, and the activity of the mitochondria from the experimental cysts displayed a reduction in MDH activity compared to the control value at the 0.05 level of significance. The value of the MDH activity from the recovered embryos is significantly smaller than the control embryos at the 0.05 level of significance but not at the 0.02 level of significance.

The major distinction between these two mitochondrial preparations is that the second preparation was frozen prior to the assay. It seems that freezing reduces the MDH activity of mitochondria especially in embryos exposed to anaerobic conditions.

The cytochrome c oxidase activity from both samples of cysts display the same pattern; the activity of the experimental embryos was significantly less than the activities of the control and recovered cysts at the 0.05 level of significance. The activity of the recovered cysts was not significantly different from the activity of the control cysts.

The cytochrome c oxidase activities from the second sample of cysts (frozen prior to assay) were greater than the activities of the first sample of cysts by approximately 2.1 times in all three cases.

3. An enzyme of diguanosine tetraphosphate metabolism.

Next, the activity of an enzyme which is found in both the PMF and yolk platelets was studied. The enzyme Gp₄G synthetase (GTP:GTP guanylyltransferase) is found in both the PMF and yolk platelets and it may be functional during anoxia since Gp₄G is metabolized during this treatment. The effect of exposure of embryos to anaerobic conditions on PMF Gp₄G synthetase activity is shown in Table 7. The PMF Gp₄G synthetase activity from embryos exposed for 1 month of anaerobiosis and embryos re-incubated for 9.5 h in air are significantly less than their control value at the 0.05 level of significance 3 days after freezing. These two values, however, are not significantly different from each other at the 0.05 level of significance.

The enzymes were subsequently refrozen and then thawed the next day for evaluation of activity. The activities of the experimental and recovered embryos are significantly less than their control value at the 0.05 level of significance. Freezing and thawing once more (the next day) caused a reduction in the enzyme activity from control embryos to the point where the enzyme activities from the experimental and recovered cysts are not significantly different from the control value.

It should be noted that the enzyme from the control embryos is sensitive to freezing and thawing, whereas the same enzyme from the experimental embryos is stable to freezing and thawing. The enzyme activity of the control embryos after three successive freezings and thawings is less than the activity (at the 0.10 level) after a single freezing and thawing. Throughout the three days of freezing and thawing the activities of the experimental and recovered embryos were similar at the 0.05 level of significance.

TABLE 7. The effect of storage conditions on the GTP:GTP guanylyltransferase^a (Gp₄G synthetase) from the PMF.

Number of Days ^b After Initial Freezing	Sample	Period Under Anoxia/ Air of Intact Cysts	Gp ₄ G Synthetase Activity (nmoles Gp ₄ G transformed/h/mg protein)
3	C	NIL	48.2 ± 9.1
	E	1 month under N ₂	22.3 ± 9.9 ^S
	R	1 month under N ₂ , then 9.5 h incubation in air	22.8 ± 12.7 ^S
4	C	NIL	40.3 ± 5.0
	E	1 month under N ₂	20.6 ± 7.7 ^S
	R	1 month under N ₂ , then 9.5 h incubation in air	25.1 ± 4.9 ^S
5	C	NIL	31.8 ± 5.0
	E	1 month under N ₂	25.6 ± 7.9 ^I
	R	1 month under N ₂ , then 9.5 h incubation in air	24.5 ± 6.9 ^I

^aThe crude enzyme preparations (CEP) were made 40% with respect to ammonium sulphate. The insoluble material was dissolved in 0.015 M NaCl containing 0.015 M Tris-HCl, pH 7.4, dialyzed, and frozen.

^bThree days after the freezing of the ammonium sulphate purified enzymes, the enzymes were thawed and the enzyme assays performed. The same procedure was repeated twice more.

^SThese values are significantly different from their respective controls but are similar to each other and other E and R values, at different days, at the 0.05 level of significance.

^IThese values are not significantly different from their respective controls and are similar to the other E and R values, at different days, at the 0.05 level of significance.

In contrast to the reduced level of Gp₄G synthetase activity in the PMF of cysts exposed to anaerobic conditions for one month, this enzyme in the yolk platelets is unaffected by storage under N₂. The results of this experiment are shown in Table 8. The activities of the experimental and recovered embryos are not significantly different from the control value. The values of the activities of the Gp₄G synthetase from the yolk platelets are of the same order of magnitude as the activities of the Gp₄G synthetase from the PMF.

The initial assumption regarding the metabolic quiescence of Artemia embryos exposed to anaerobic conditions seems to be warranted to some extent. Cytochrome c oxidase activity and Gp₄G synthetase activity of the PMF from embryos exposed to anoxia are reduced. However, with the possible exception of the glycerophosphate dehydrogenase activity, the activities of the other enzymes in the PMF that were tested from embryos exposed to anoxia are quite similar to their respective controls.

C. Identification of Carotenoids in Artemia Cysts

The thin-layer chromatogram of the two main carotenoids of Artemia embryos is seen in Fig. 1. The data in Table 9 summarize the spectral properties of these two carotenoids in several solvents before and after saponification. On the basis of their chromatographic and spectral properties the two main carotenoids in Artemia cyst appear to be canthaxanthin and echinenone. Also, it should be noted that saponification does not influence the chromatographic behavior of either carotenoid on silica gel in the solvent system used.

TABLE 8. GTP:GTP guanylyltransferase activity in various yolk platelet preparations.

Source of Yolk Platelets ^a	Period Under Anoxia/ Air of Intact Cysts	Gp ₄ G Synthetase Activity (nmoles Gp ₄ G transformed/h/mg protein) ^b
C	NIL	32.2 ± 4.4
E	1 month under N ₂	33.1 ± 5.1 ^I
R	1 month under N ₂ , then 9.5 h incubation in air at 30°C	27.4 ± 1.9 ^I

^aYolk platelets were homogenized in 0.3 M sucrose containing 0.2 M EDTA, 0.1% Tween 80 and 0.03 M Tris-HCl, pH 7.4. Prior to the enzyme assay, the yolk platelets were centrifuged at 700 g for 15 min and then resuspended in distilled water. The yolk platelets in distilled water were used as the enzyme source.

^bThe method employed for the measurement of enzyme activity was the same as that employed in Table 8 except for the enzyme source.

^IThese values are not significantly different from the control value at the 0.05 level of significance.

FIG. 1. Thin-layer chromatography on silica gel of the two main carotenoids from the total homogenate of Artemia embryos (before and after saponification) using petroleum ether-diethyl ether (1:1, v/v) as the solvent. Developed for 1.5 h.

1. canthaxanthin standard;
2. the red pigment (see Materials and Methods) unsaponified;
3. the red pigment saponified;
4. the yellow pigment (Materials and Methods) unsaponified;
5. the yellow pigment saponified.

The dotted circles are representations of trace amounts of pigment.

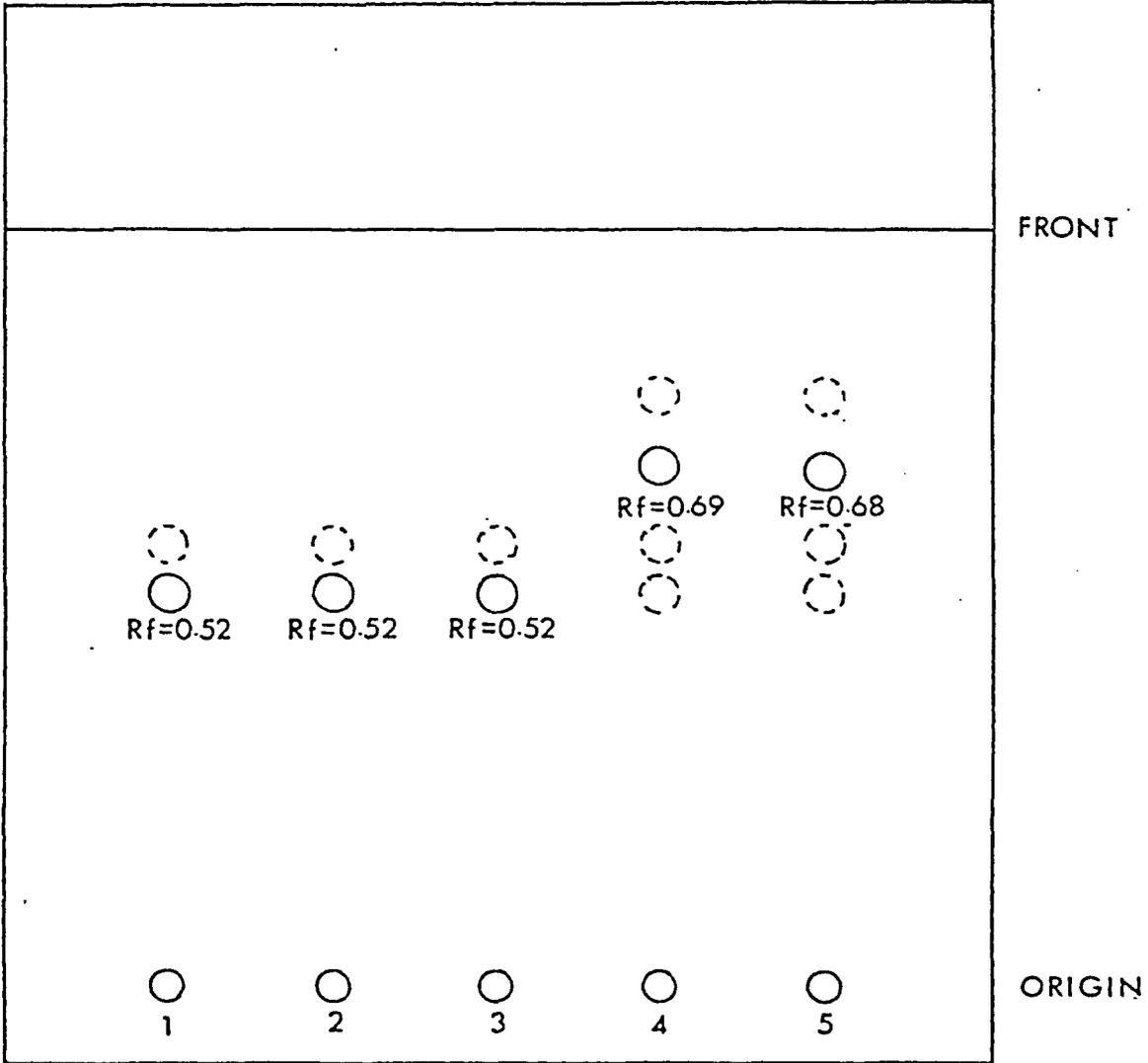


TABLE 9. Summary of absorption maxima of the two main carotenoids of Artemia embryos in several solvents before and after saponification.

Pigment	Absorption Maxima (nm)					
	Petroleum Ether		Chloroform		Benzene	
	Before saponi- fication	After saponi- fication	Before saponi- fication	After saponi- fication	Before saponi- fication	After saponi- fication
Red	460	460	482	482	480	480
Yellow	454 (355) ^a	456 (355)	469 (363)	472 (372)	471 (367)	474 (367)
Canthaxanthin standard	460	ND ^b	482	ND	ND	ND

^aValues in parentheses are minor maxima.

^bNot done.

The spectra of the red pigment are unchanged by saponification and the absorption maxima are identical to the canthaxanthin standard. A comparison of the spectral maxima for the red pigment with the literature reveals that the red pigment is similar to canthaxanthin (Lee, 1966; Foppen, 1971). Although absorption maxima of the red pigment in petroleum ether (460 nm) and benzene (480 nm) are approximately 2-5 nm shorter than the literature values, these findings may be explained by the fact that drying carotenoid extracts (which was necessary in order to transfer the carotenoid to petroleum ether and to benzene from chloroform) may cause the isomerization of the carotenoid resulting in lower absorption maxima (Lee, 1966).

The yellow pigment has been designated as echinenone, on the basis of a survey of the literature and its behaviour on thin-layer chromatography and absorption maxima in different solvents. Thin-layer chromatography has revealed that the unsaponified and saponified yellow pigment are indistinguishable (Fig. 1). Furthermore, co-chromatography of this material in the same solvent system showed no separation of the unsaponified and saponified yellow pigment.

The absorption maxima of the major peaks of the unsaponified yellow pigment were 2-3 nm shorter than the expected values for echinenone found in the literature (Czeczuga, 1971; Lee, 1966; Foppen, 1971). However, upon saponification, the absorption maxima of the major peaks were observed to be identical to the literature values (Table 9). Such a spectral shift after saponification is an indication of the presence of a carotenoid ester. The structure of echinenone however does not lend itself to being esterified. Although minor absorption maxima have been

reported for echinenone (Foppen, 1971), the minor absorption maxima observed in this study do not coincide with the literature values. Circumstantially the yellow pigment isolated in this study is most likely echinenone. For example, Krinsky (1965) and Hsu et al. (1970) found canthaxanthin and echinenone to be the major pigments of Artemia embryos. Also, it has been shown that Artemia salina is capable of converting β -carotene into echinenone and canthaxanthin (Davies et al., 1965; Hata and Hata, 1969; Davies et al., 1970).

At this time there is no explanation for the spectral shift of the yellow pigment due to saponification nor for the values of the minor absorption maxima. It is, however, felt that these observations may not be an intrinsic property of this pigment but perhaps some artifact due to the preparation and purification.

D. Distribution of the Two Main Carotenoids in Artemia Embryos

The distribution of the two main carotenoids among the various subcellular fractions is represented by the results in Table 10. The overall pattern as reflected by the total homogenate, yolk platelet and PMF fractions supports the data in Table 3 (which shows total carotenoids) and demonstrates that the cysts exposed to anaerobic conditions have a greater amount of carotenoids.

In the control cysts, canthaxanthin represents the greatest proportion of the carotenoids in each fraction. In the total homogenate, canthaxanthin is 2.7 times more abundant than echinenone (73% canthaxanthin and 27% echinenone) and a similar pattern is seen in the PMF. In the yolk platelets, the canthaxanthin:echinenone ratio is 1.56:1.

TABLE 10. Analysis of carotenoid content of various *Artemia* embryo subcellular fractions and yolk platelet sub-fractions.

Sample	Period Under Anoxia/Air	µg Canthaxanthin per mg protein	µg Echinenone per mg protein	Total µg Carotenoids per mg protein	% Canthaxanthin	% Echinenone
C-TH	NIL	0.27	0.10	0.37	73.0	27.0
C-YP	"	0.64	0.41	1.05	61.0	39.0
C-PMF ^a	"	0.63	0.23	0.86	73.3	26.7
C-NaCl ^b	"	0.63	0.60	1.23	51.2	48.8
C-TRYPSIN ^c	"	0.86	0.64	1.50	57.3	42.7
E-TH	1 month under N ₂	0.78	0.29	1.07	72.9	27.1
E-YP	"	0.53	0.70	1.23	43.1	56.9
E-PMF ^a	"	0.94	0.32	1.26	74.6	25.4
E-NaCl ^b	"	0.51	0.68	1.19	42.9	57.1
E-TRYPSIN ^c	"	0.75	0.73	1.48	50.7	49.3

^aThe post-mitochondrial fraction was isolated from a preparation that was different from the preparation used to isolate the other fractions.

^bThese preparations of CPC were made from yolk platelets using 1 M NaCl containing 0.03 M Tris-HCl, pH 7.4.

^cThese preparations of CPC were made by treating a yolk platelet suspension with 10 µg/ml trypsin and obtaining the solubilized fraction.

In the cysts exposed to anaerobiosis, the total homogenate and PMF have carotenoid ratios nearly identical to the corresponding controls, whereas the carotenoid ratio in the yolk platelet fraction is different from the control. In this fraction echinenone is present in excess of canthaxanthin, whereas the ratio of canthaxanthin to echinenone in yolk platelets from control cysts is 1.56:1 the ratio is 0.76:1 in the yolk platelets from anaerobic cysts. When subfractions of the yolk platelets were analyzed, similar differences in the carotenoid ratios were observed.

In Table 11, the carotenoid to protein ratio of the carotenoid-protein complex as extracted by various salt concentrations is seen. The general greater carotenoid to protein ratio of the embryos exposed to anaerobic conditions is seen.

The salt concentration of the extracting medium does not seem to influence the carotenoid to protein ratio. If there were various carotenoid-protein complexes with different carotenoid to protein ratios generated during anaerobic conditions, extraction of the yolk platelets with different salt concentrations may have resolved such complexes.

One apparent discrepancy between Table 10 and Table 11 is seen in a comparison of the control and anaerobic carotenoid to protein ratio of the carotenoid-protein complex extracted with 1 M NaCl. In Table 10 there seems to be very little or no difference in this ratio between the control and anaerobic embryos; however, in Table 11 such a comparison between control and anaerobic embryos does seem to reveal a difference.

TABLE 11. Carotenoid to protein ratio of carotenoid-protein complex of yolk platelets extracted by various NaCl solutions.^a

Sample	NaCl Concentration (M)	Period Under Anoxia/Air	Carotenoid (O.D. 482)	Protein (mg)	Carotenoid (O.D. 482)/Protein (mg)
C	0.5	NIL	1.46	8.4	0.174
C	1.0	"	2.05	11.5	0.178
C	2.0	"	2.38	13.7	0.174
E	0.5	3 months under N ₂	0.90	4.4	0.205
E	1.0	"	2.08	9.8	0.212
E	2.0	"	2.49	10.9	0.228

^aThe various salt concentrations, pH 6.9, all contained 5 mM EDTA and 0.015 M Tris-HCl.

A reconciliation of such a condition may be found in the observation as seen in Table 3 that the carotenoid to protein ratio of the yolk platelets of the control and embryos exposed to anaerobic conditions for two months are not significantly different. The anaerobic embryos used for the experiment shown in Table 11 were exposed to anoxic conditions for three months and the anaerobic embryos exposed to anoxic conditions in Table 10 were exposed to anoxic conditions for one month. Perhaps more extended periods of anoxia are needed before major differences become apparent in the carotenoid to protein ratio of the carotenoid-protein complex.

There is an approximately 17% increase in the carotenoid to protein ratio of the yolk platelets of the anaerobic embryos as seen in Table 10. In Table 3, an approximately 17% increase in this same ratio of the yolk platelets of the anaerobic embryos is also seen; however, there is no significant difference between the carotenoid to protein ratio of the yolk platelets of the control and anaerobic embryos seen in Table 3. The increase in the carotenoid content of Artemia embryos exposed to anaerobic conditions is due primarily to an increase in the carotenoid content in the PMF (Table 3). This is also reflected in Table 10 where an increase in the carotenoid to protein ratio of the PMF of approximately 47% is seen due to anaerobic conditions.

E. Solubility of Carotenoids from the Various Subcellular Fractions of Artemia Embryos in Various Organic Solvents

Extraction of the various subcellular fractions with 50% acetone does not solubilize all the carotenoids in the total homogenate and yolk platelet fractions (see Table 12). Complete extraction requires the use of a more efficient lipid extraction solvent such as chloroform-methanol. When this two-step procedure was tested with cysts kept under anaerobic conditions it was observed that the carotenoids from the total homogenate of N_2 -treated cysts were more resistant to extraction than from the controls. Whereas, 77.3% of the carotenoids from the total homogenate were extracted from control cysts with 50% acetone, only 68.7% were extracted from the total homogenate of anaerobic cysts. From the yolk platelet fraction of the control cysts 70.1% of the carotenoids were extracted with 50% acetone and 58.3% were extracted from anaerobic cysts. The carotenoids from the MRF and the PMF of both the control and anaerobic cysts were extractable with 50% acetone. It appears that during anaerobiosis the complex between the carotenoids and protein has been altered in such a way as to decrease their solubility in acetone. Perhaps this same pattern would have been observed in the MRF and PMF fractions if a lesser concentration of acetone were used.

F. Influence of Heat on the Carotenoid-Protein Complex of Artemia Embryos

It was felt that carotenoids were more stable in cysts subjected to anaerobic conditions than in control cysts. The carotenoid to protein ratio of the total homogenate and the peak ratios of the spectra of the carotenoid-protein complex were the parameters chosen to test

TABLE 12. Extraction of the carotenoids from the various subcellular fractions with various organic solvents.

Sample	Period Under Anoxia/Air	Total Carotenoids Extracted ^a (O.D. 458 plus O.D. 482)	% of Carotenoids in Petroleum Ether	% of Carotenoids in Chloroform
C-TH	NIL	6.9	77.3	22.75
C-YP	"	24.5	70.1	29.90
C-MRF	"	1.3	100.0	0.00
C-PMF	"	4.0	100.0	0.00
E-TH	1 month under N ₂	6.2	68.7	31.30
E-YP	"	22.3	58.3	41.70
E-MRF	"	1.4	100.0	0.00
E-PMF	"	4.2	100.0	0.00

^aA suitable aliquot from each subcellular fraction was taken for carotenoid extraction. After the carotenoids were extracted from the subcellular fractions with acetone-water (1:1, v/v), they were transferred to petroleum ether and their optical density measured at 458 nm. The pigment remaining in the pellet was extracted using the Bligh and Dyer lipid extraction procedure and the optical density of the chloroform layer was measured at 482 nm.

this hypothesis. One attempt to measure such a difference was to incubate the total homogenate and the carotenoid-protein complex at 45°C and sample at various times.

Such an experiment using the total homogenate is seen in Table 13. Incubation of the total homogenate from both control and N₂-treated cysts for 5 h at 45°C resulted in very little change in the carotenoid protein ratio, although there seems to be a slight decrease in the ratio for N₂-treated total homogenate. However, in both cases, the carotenoid to protein ratio rises slightly before declining. This may be due to partial disruption of the carotenoprotein complex increasing the efficiency of carotenoid extraction in organic solvents.

A comparison of the difference between the control and experimental carotenoid to protein ratio of the total homogenate as seen in Tables 3, 10 and 13, reveals that the difference is least in Table 13. Various preparations can reveal different quantities of carotenoids depending on the method and time of preparation and extraction of the tissue. What is common to each situation is that the carotenoid to protein ratio of the total homogenate of the N₂-treated cysts is consistently greater than the ratio of the control.

When the carotenoid-protein complex from yolk platelets (solubilized in 1 M NaCl) was incubated for various periods of up to 9 h at 45°C results similar to those found with the total homogenate were observed. These results are seen in Table 14. Initially, the ratio of the spectral maxima in the carotenoid-protein complex from anaerobic cysts was larger than the ratio from the control cysts. Within a 9 h incubation both preparations approached the same spectral ratio. During

TABLE 13. The effect of incubating the total cyst homogenate at 45°C on the stability of the carotenoids.^a

Sample	Hours of Incubation	Period Under Anoxia/Air	Carotenoids (O.D. 482) per Protein (mg) x 10 ⁻²
C	0.0	NIL	17.2
	0.5	"	18.8
	1.0	"	19.0
	2.0	"	17.7
	3.0	"	17.5
	5.0	"	16.5
E	0.0	1 month under N ₂	24.1
	0.5	"	24.0
	1.0	"	25.5
	2.0	"	24.5
	3.0	"	23.5
	5.0	"	21.3

^aAliquots of the incubated homogenates were taken at various times; their carotenoids were extracted using the Bligh and Dyer (1959) lipid extraction procedure and measured spectrophotometrically at 482 nm.

TABLE 14. The effect of incubating the carotenoid-protein complex from yolk platelets at 45°C on the spectral properties of the complex.^a

Hours of Incubation	Control	Experimental ^b
	Ratio of the O.D.'s of the Absorption Maxima 370-372/460-465 nm	Ratio of the O.D.'s of the Absorption Maxima 370-372/460-465 nm
0.0	1.49	1.58
1.0	1.45	1.51
2.0	1.45	1.50
4.5	1.44	1.49
6.5	1.45	1.49
9.0	1.44	1.45

^aA 1 M NaCl CPC was extracted from the yolk platelets (see Materials and Methods). Aliquots of the incubated CPC were taken at various times and their spectra recorded. The ratio of the two absorption maxima was measured in each case. Refer to Fig. 5a.

^bOne month under N₂.

this period the ratio of the controls decreased by 3%, whereas that of the N₂-treated group decreased by 8%.

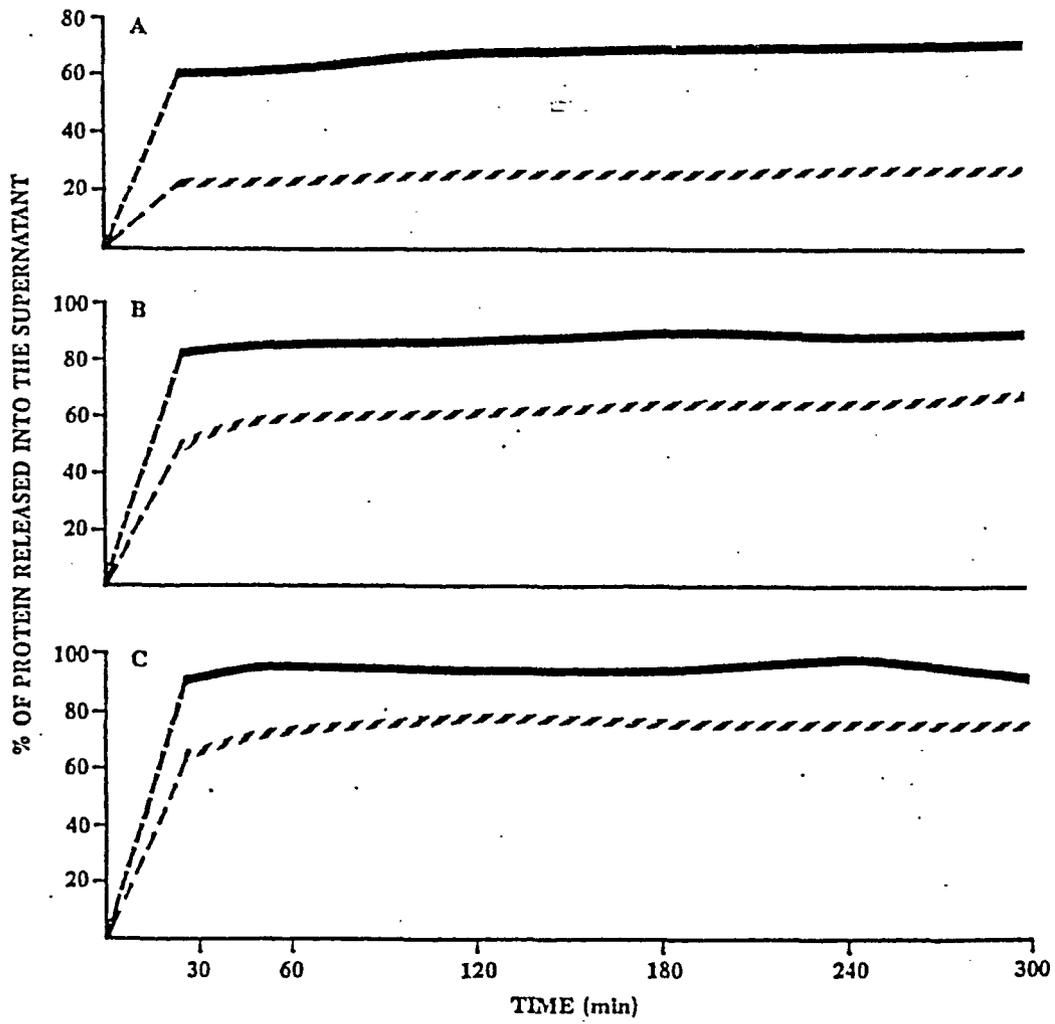
With respect to incubation at 45°C, the carotenoid content does not seem to change drastically for the control and experimental embryos; however, a change seems to occur in the ratio of the spectral maxima upon incubation at 45°C for 9 h. The ratio of the N₂-treated cysts decreases and approaches that of the control group. The ratio of the spectral maxima of the carotenoid-protein complex is an indication of the degree of interaction of the carotenoid(s) and protein in the complex. It may be that the interaction of the carotenoid(s) and protein in the carotenoprotein complex of N₂-treated cysts is modified due to incubation at 45°C to a point where it mimicks the interaction of the carotenoid(s) and protein in the carotenoid-protein complex of the aerobic embryos.

G. Extraction of the Carotenoid-Protein Complex from Yolk Platelets by NaCl

The solubility of the yolk platelet carotenoid-protein complex from control and N₂-treated cysts in different concentrations of NaCl is seen in Fig. 2. At each NaCl concentration tested (0.5 to 2.0 M) more protein was released from the yolk platelets of the control cysts than from yolk platelets of cysts exposed to anaerobic conditions. With increasing NaCl concentrations the difference between the control and anaerobic systems became less. In the controls, the maximum yolk protein solubilized by NaCl is approximately 90%, whereas with the N₂-treated cysts the maximum amount of yolk protein solubilized by NaCl is about 75%.

FIG. 2. The solubility of the carotenoid-protein from yolk platelets using different concentrations of NaCl. To 15 ml aliquots of a yolk platelet suspension in water was added 15 ml of 0.03 M Tris-HCl, pH 7.4, containing various NaCl concentrations. The preparations were maintained at 0°C and at various times 4 ml aliquots were removed for the determination of protein released. The interpolation to zero time is depicted by— ——. This interpolation was performed since no sample was taken prior to the addition of NaCl; the determination of protein prior to the addition of NaCl was felt to be unnecessary since yolk platelets are quite stable in low ionic strength media (Warner et al., 1972). Control ———; 3 months under N₂ ■■■■.

- A. Yolk platelet suspension in 0.5 M NaCl. 5.5 mg/ml protein for the control yolk platelet suspension and 6.0 mg/ml for the experimental.
- B. Yolk platelet suspensions in 1.0 M NaCl. 6.0 mg/ml protein for the control yolk platelet suspension and 5.7 mg/ml for the experimental.
- C. Yolk platelet suspensions in 2.0 M NaCl. 5.5 mg/ml protein for the control yolk platelet suspension and 5.7 mg/ml for the experimental.



H. Digestion of Yolk Platelets by Trypsin

The response of the yolk platelets to trypsin treatment is shown in Fig. 3. In this experiment there seems to be no difference in the sensitivity to trypsin between the control and anaerobic cysts. In both cases between 33-34% of the available protein in the yolk platelets is released into the supernatant after 30 minutes incubation at 37°C.

During the investigation of the response of yolk platelets to trypsin the stability of yolk platelet preparations to incubation at 37°C in the absence of trypsin was also studied. The results of this experiment are shown in Fig. 4. From these data it is apparent that there is a greater rate of release of protein into the supernatant from the yolk platelets of the control cysts than from the yolk platelets of anaerobic cysts. After 30 minutes of incubation, approximately 30% more protein has been released from platelets of control cysts than from yolk platelets of anaerobic cysts.

Although the yolk platelets from control and anaerobic cysts are equally susceptible to digestion by trypsin, they are not equally susceptible to the influence of incubation at 37°C. Susceptibility to trypsin is a reflection of the availability of the carboxyl groups of lysine and arginine, whereas susceptibility to heat is an indication of the overall stability of the yolk platelet.

I. Spectra of Carotenoid-Protein Complex from Artemia Yolk Platelets

The carotenoid-protein complex extracted from the yolk platelets of control cysts has a brown colour while the carotenoid-protein complex from the yolk platelets of anaerobic cysts has a green colour. In an

FIG. 3. The sensitivity of yolk platelets to trypsin at 37°C. To 6.0 ml of each yolk platelet suspension 21 μ g of trypsin were added in 0.01 ml, resulting in a yolk platelet protein to trypsin ratio of approximately 4000 to 1. At various times, 1.0 ml samples were taken and added to centrifuge tubes containing 13.1 μ g ovomucoid in 0.5 ml; the ovomucoid to trypsin ratio was 3.75 to 1. Control, 14.0 mg/ml of protein in the yolk platelet suspension **————**; 1 month under N₂, 14.3 mg/ml of protein in the yolk platelet suspension **■■■■**.

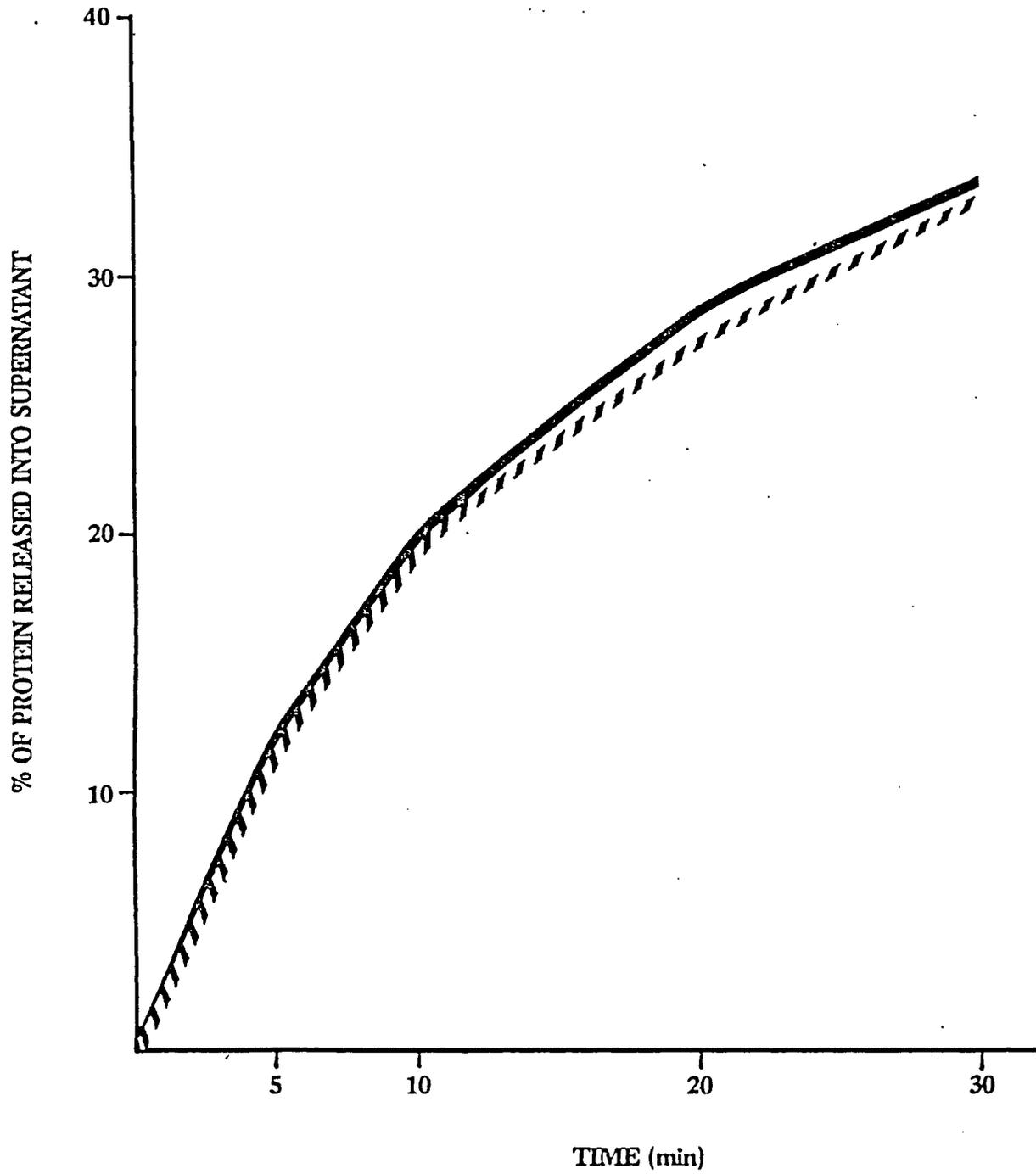
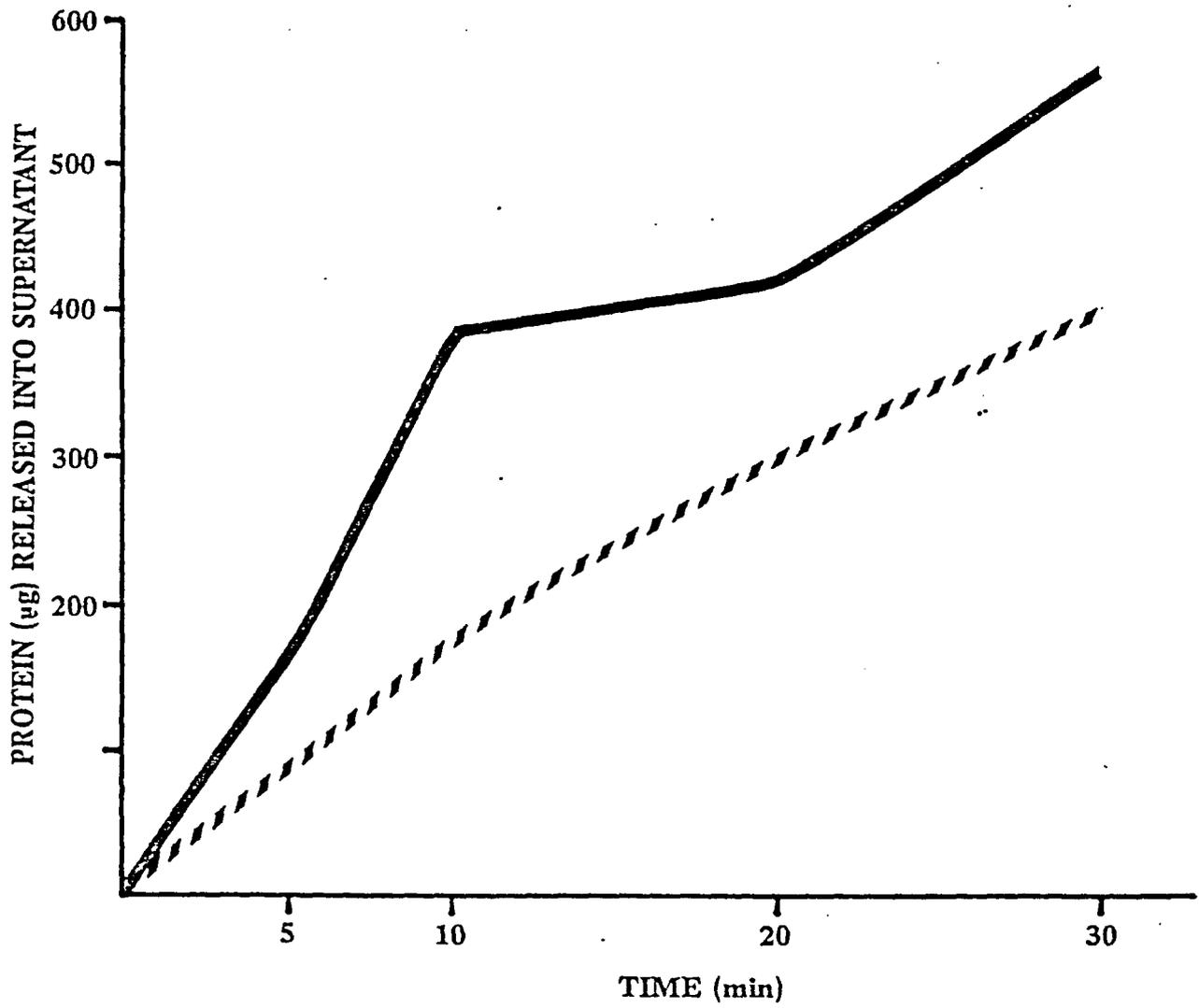


FIG. 4. Lysis of yolk platelets during constant shaking at 37°C in a 0.03 M potassium phosphate buffer, pH 7.4. The yolk platelet preparation used here was the same as used in Fig. 3. These yolk platelets were the controls, i.e., no trypsin added. Control ; 1 month under N₂ .



attempt to analyze and quantitate these colour differences, the spectra of the carotenoproteins were recorded.

Two types of carotenoid-proteins were obtained based on the method of extraction. The first type was extracted by NaCl (for spectrum see Fig. 5a) and the second type was released by the action of trypsin on intact yolk platelets (for spectrum see Fig. 6a).

The results in Table 15 summarize the spectral data of the carotenoid-protein complex obtained by NaCl extraction. The absorption maxima of peak I and peak II of the carotenoid-protein complex extracted by NaCl from N_2 -treated cysts are 461-463 nm and 370-371 nm, respectively. The corresponding control maxima of peak I and peak II are 463-465 nm and 370-371 nm, respectively. It can be seen that the absorption maximum of peak I of the carotenoid-protein complex extracted by NaCl from N_2 -treated cysts is approximately 2 nm shorter than the corresponding control. For peak II, the absorption maximum of the carotenoid-protein is similar in both the control and experimental groups. Of particular interest is the observation that in all experiments the ratio of absorption maxima (peak II/peak I) consistently revealed a higher value for the carotenoid-protein complex from the N_2 -treated cysts compared to the control. The actual value for these ratios was found to depend on the conditions of the preparation and elapsed time before the spectra were taken; the ratio would decrease with a harsher extraction procedure and prolonged exposure to air and light. Nevertheless, the peak II/peak I ratio for the N_2 -treated carotenoid-protein complex was higher than the control ratio from the carotenoid-protein complex.

FIG. 5a. Visible spectra of the NaCl extracted carotenoid-protein from yolk platelets. The yolk platelets were suspended in 0.03 M Tris-HCl, pH 7.4, containing 1 M NaCl for 1 h at 4°C. The mixture was centrifuged at 12,000 g for 15 min. The spectra were performed on the supernatant using a Beckman recording spectrophotometer MVI set at 4 nm/sec scan speed and 50 nm/inch chartpaper drive. Control  ; 1 month under N₂ .

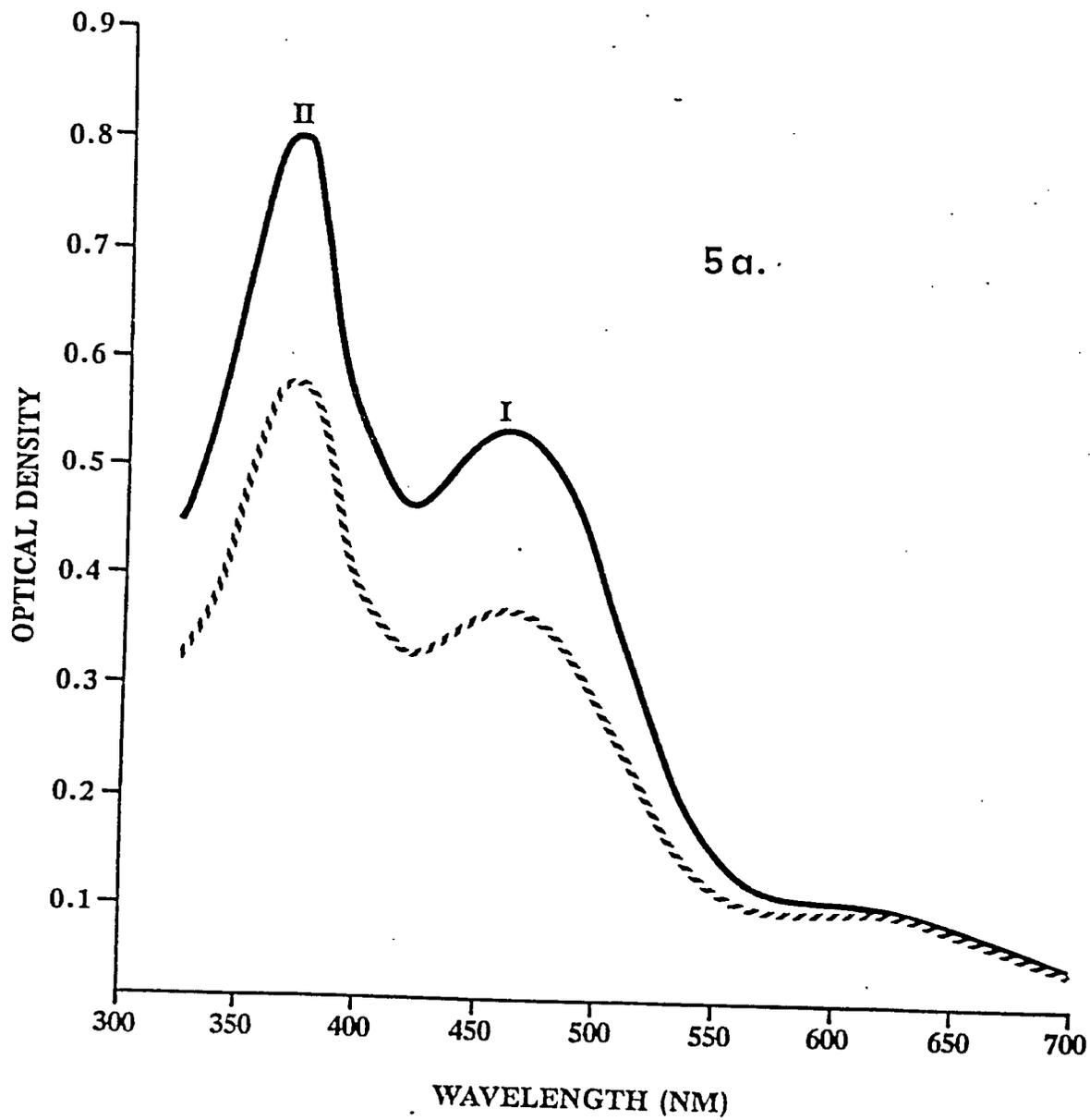


FIG. 5b. Visible spectra of the ammonium sulphate precipitated carotenoid-protein complex extracted from Artemia yolk platelets by NaCl. To the carotenoid-protein in 0.03 M Tris-HCl, pH 7.4, containing 1 M NaCl (Fig. 5a) solid ammonium sulphate was added until the solution was 50% with respect to ammonium sulphate. The resulting precipitate was centrifuged at 6,700 g for 10 min. The sediment was suspended in 0.03 M Tris-HCl, pH 7.4, for 1.5 h at 0°C, then centrifuged at 700 g for 15 min. The 700 g sediment was dissolved in 0.5 M Tris-HCl, pH 7.4, and the spectra were performed. The spectrophotometer and recorder were set as described in Fig. 5a. Control ——— ; 1 month under N₂ - - - - - .

FIG. 5c. UV-visible spectra of the ammonium sulphate precipitated carotenoid-protein complex depicted in Fig. 5a. The carotenoid-protein complex was diluted in 0.5 M Tris-HCl, pH 7.4, buffer so as to include the ultraviolet spectra. The spectrophotometer and recorder were set as described in Fig. 5a. Control ——— ; 1 month under N₂ - - - - - .

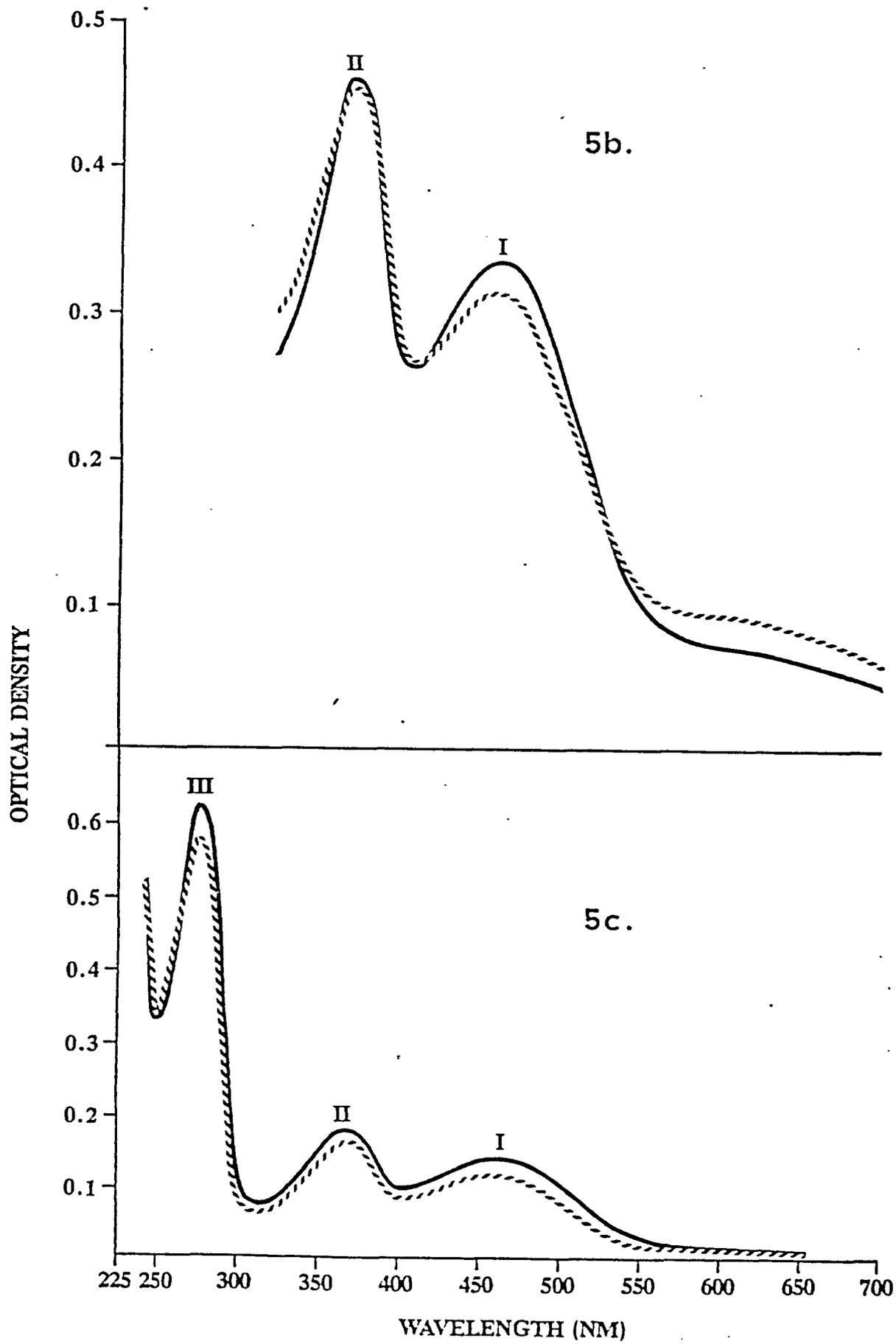


TABLE 15. Summary of spectral properties of the carotenoid-protein complex from yolk platelets extracted by NaCl.

<u>Artemia</u> <u>Embryo</u>	Period Under N ₂	Buffer	Absorption Maximum Peak I (nm)	Absorption Maximum Peak II (nm)	Absorption Maximum Peak III (nm)	O.D. Peak II/ Peak I	O.D. Peak II/ Peak III
C	NIL	1 M NaCl in 0.03 M Tris-HCl pH 7.4	463-465	370-371	ND ^a	1.49	ND
E	1 month	"	461-463	370-371	ND	1.53	ND
C	NIL	0.5 M Tris-HCl pH 7.4 ^b	459-462	368-370	280	1.37	0.284
E	1 month	"	455-457	368-370	280	1.45	0.287

^aNot done.

^bThis fraction was initially precipitated by 50% ammonium sulphate.

The carotenoid-protein complex extracted with 1 M NaCl was partially purified by 50% ammonium sulphate precipitation and the carotenoid-protein complex precipitate redissolved in 0.5 M Tris-HCl, pH 7.4. The results of such an experiment were analyzed spectrophotometrically and are shown in Fig. 5b and summarized in Table 15. The absorption maxima of peak I, peak II and peak III of the carotenoid-protein complex from N_2 -treated cysts are 455-457 nm, 368-370 nm and 280 nm, respectively. The corresponding values from the control cysts are 459-462 nm, 368-370 nm and 280 nm, respectively. As is seen, the absorption maximum of peak I of N_2 -treated cysts is 4-5 nm shorter than the corresponding control. A comparison of peak II reveals that the absorption maximum of the N_2 -treated and control cysts is similar; the same can be said for the absorption maximum of peak III. The peak II/peak I ratio of the N_2 -treated cysts revealed a higher value than the controls and is consistent with the finding previously made with the 1 M NaCl extracted carotenoid-protein complex. A comparison of the peak II/peak III ratio reveals that there does not seem to be a difference in this ratio between the N_2 -treated cysts and the control cysts.

A comparison of the absorption maxima of the 1 M NaCl extracted carotenoid-protein complex before and after partial purification by 50% ammonium sulphate precipitation reveals that the absorption maximum of both peak I and peak II shift to shorter wavelengths by approximately 2-3 nm due to ammonium sulphate precipitation and a reduction in the peak II/peak I ratios.

As mentioned above, another type of carotenoid-protein complex can be generated by the digestion of intact yolk platelets by trypsin

FIG. 6a. Visible spectra of the trypsin generated carotenoid-protein complex from intact yolk platelets. Yolk platelets were incubated in 0.03 M Tris-HCl, pH 7.4, containing 10 $\mu\text{g/ml}$ trypsin at 37°C for 3 h. The reaction was terminated with an ovomucoid concentration five times that of the trypsin concentration. The mixture was centrifuged at 12,000 g for 15 min. The spectra were performed on the supernatant. The Beckman recording spectrophotometer MVI was set at 4 nm/sec scan speed and 50 nm/inch chartpaper drive. Control  ; 1 month under N₂ .

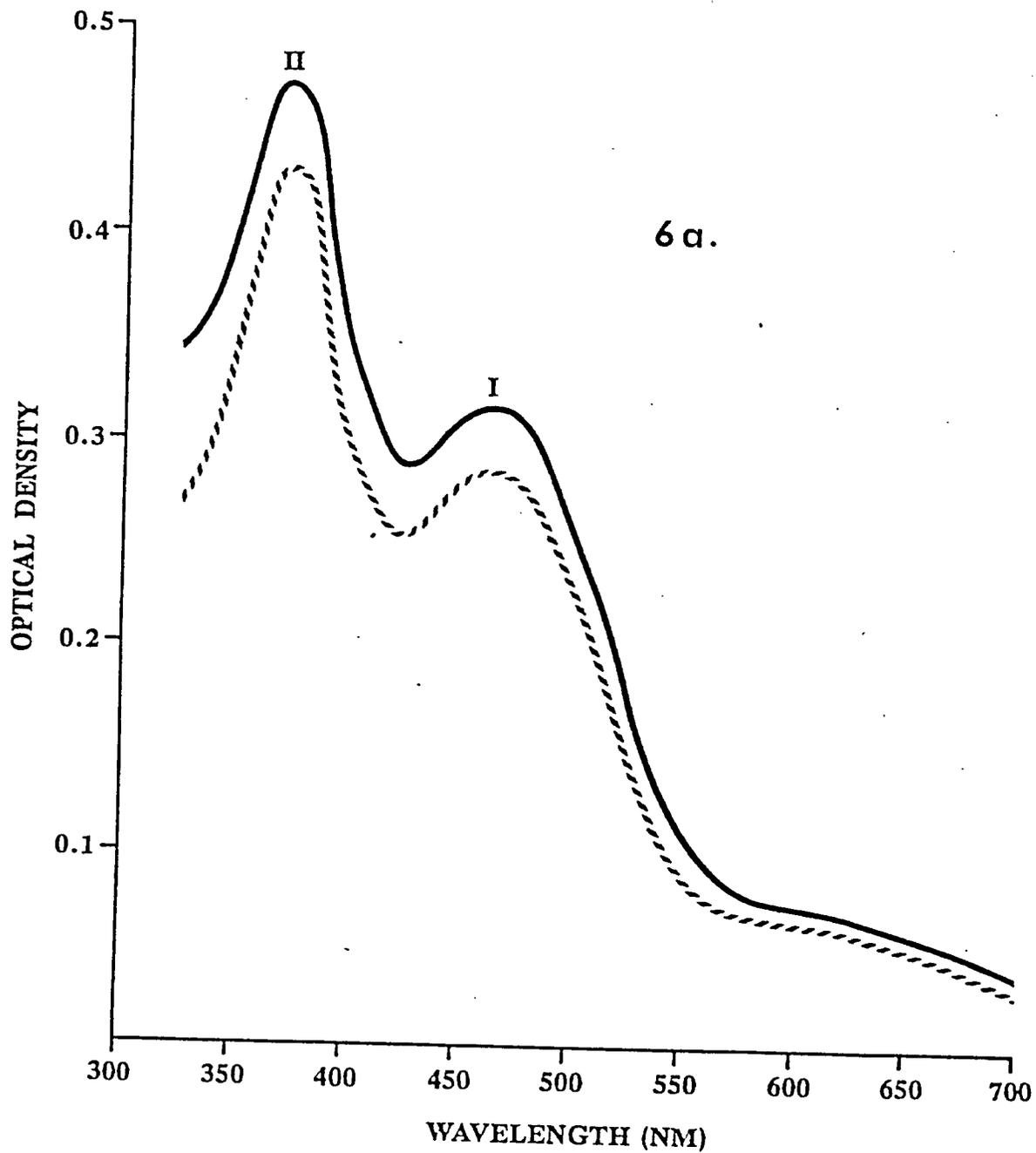
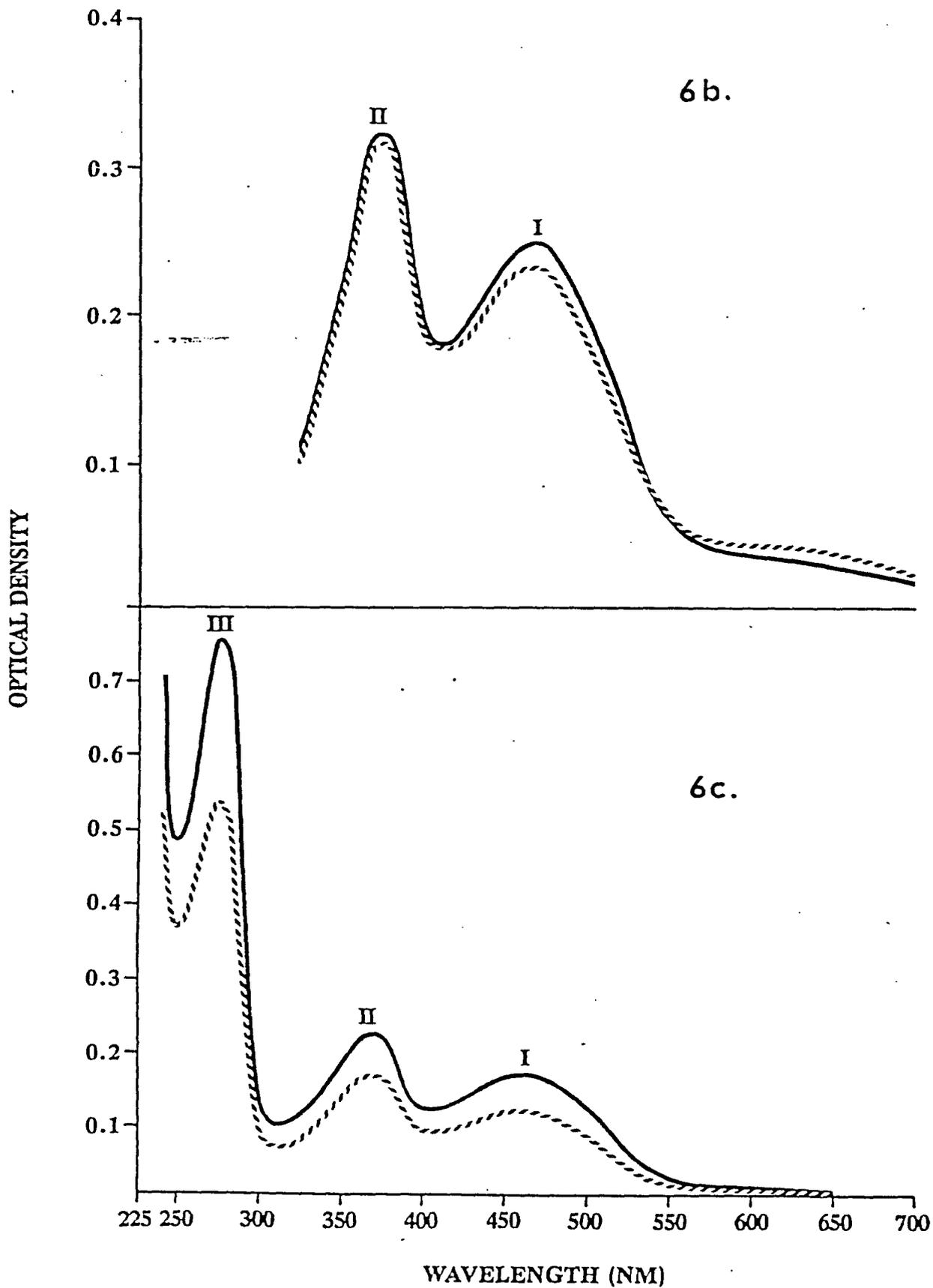


FIG. 6b. Visible spectra of the ammonium sulphate precipitated carotenoid-protein complex generated by the action of trypsin on intact yolk platelets. To the carotenoprotein in a 0.03 M Tris-HCl, pH 7.4, solid ammonium sulphate was added until the solution was 70% with respect to the ammonium sulphate. The sediment was dissolved in 0.03 M Tris-HCl, pH 7.4, and the spectra of this solution was taken. The spectrophotometer and recorder were set as described in Fig. 6a. Control  ; 1 month under N₂  .

FIG. 6c. UV-visible spectra of the ammonium sulphate precipitated carotenoid-protein complex depicted in Fig. 6b. The carotenoid-protein was diluted in 0.03 M Tris-HCl, pH 7.4, so as to include the ultraviolet spectra. The spectrophotometer and recorder were set as described in Fig. 6a. Control  ; 1 month under N₂  .



(Fig. 6a). The absorption maxima of this carotenoid-protein complex is summarized in Table 16. The absorption maximum of peak I of this carotenoid-protein complex from N_2 -treated and control cysts appears to be the same (463-465 nm). Similarly, the absorption maximum of peak II of the N_2 -treated and control cysts appears to be the same (370-371 nm). A comparison of the peak II/peak I ratio between the N_2 -treated and control cysts reveals that this ratio is similar.

The partial purification of the trypsin generated carotenoid-protein complex by precipitation with 70% ammonium sulphate and subsequent solution of the precipitate in 0.03 M Tris-HCl, pH 7.4, resulted in no distinction in the absorption maxima of peak I, peak II and peak III between the N_2 -treated and control cysts. The absorption maxima of peak I, peak II and peak III of both groups are 464-466 nm, 370-371 nm and 275 nm, respectively. A slight increase in the absorption maximum of peak I exists in the ammonium sulphate purified carotenoid-protein compared to the carotenoid-protein prior to ammonium sulphate purification of the trypsin generated carotenoid-protein. A comparison of the peak II/peak I ratio of the ammonium sulphate purified carotenoid-protein of the N_2 -treated cysts reveals a slight increase as compared to the control cysts. The peak II/peak III ratio of the N_2 -treated and control cysts is quite similar.

The carotenoid-protein complex extracted by 1 M NaCl (Table 15) reveals greater differences between the N_2 -treated and control cysts than the trypsin generated carotenoid-protein complex (Table 16). The carotenoid-protein complex acquired by trypsin digestion, even after precipitation by 70% ammonium sulphate, with respect to the absorption

TABLE 16. Summary of spectral properties of the carotenoid-protein complex released from yolk platelets by the action of trypsin.

<u>Artemia</u> <u>Embryo</u>	Period Under N ₂	Buffer	Absorption Maximum Peak I (nm)	Absorption Maximum Peak II (nm)	Absorption Maximum Peak III (nm)	O.D. Peak II/ Peak I	O.D. Peak II/ Peak III
C	NIL	0.03 M Tris-HCl pH 7.4	463-465	370-371	ND ^a	1.52	ND
E	1 month	"	463-464	370-371	ND	1.52	ND
C	NIL	0.03 M Tris-HCl pH 7.4 ^b	464-466	370-371	275	1.30	0.298
E	1 month	"	464-466	370-371	275	1.36	0.313

^aNot done.

^bThis fraction was initially precipitated by 70% ammonium sulphate.

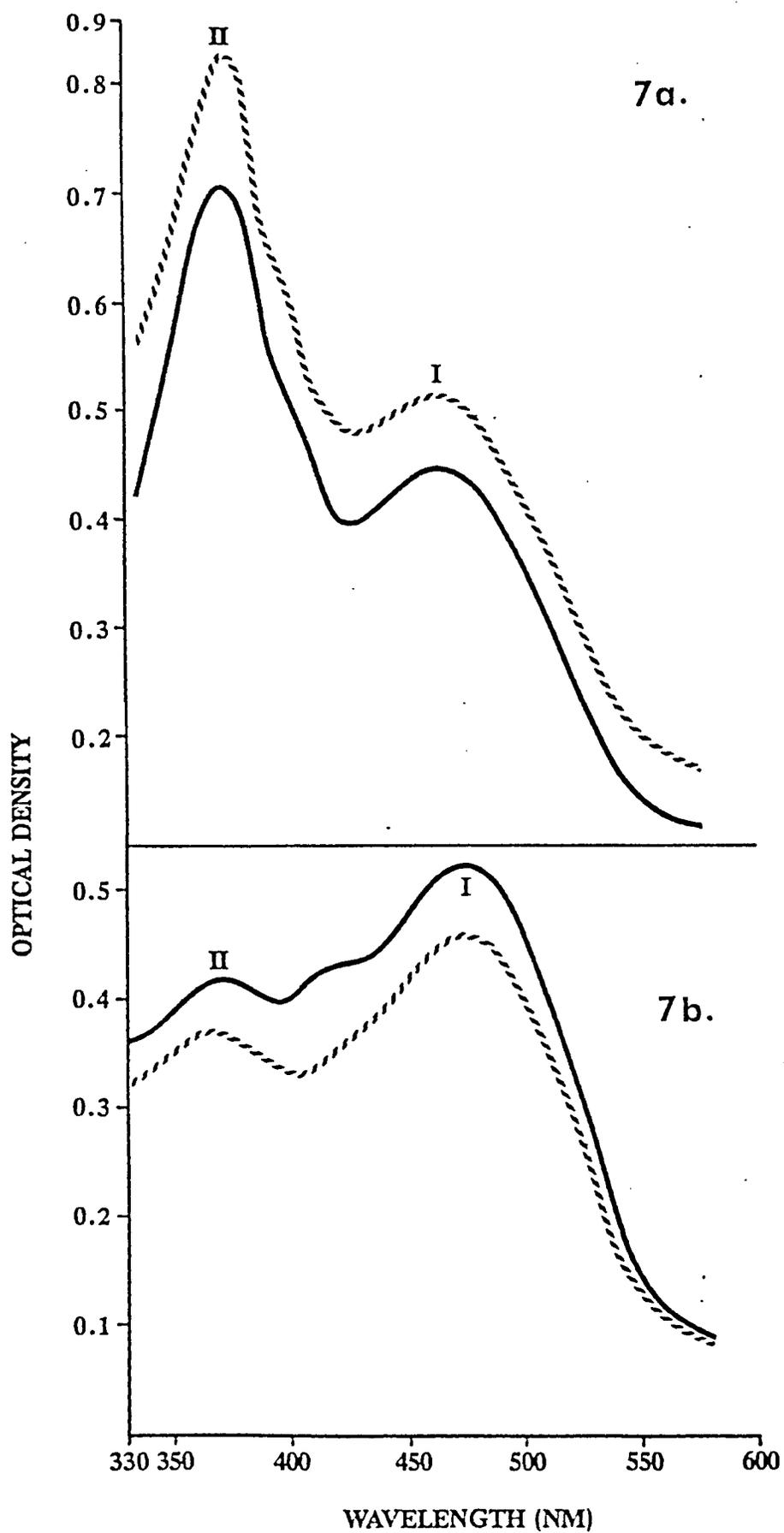
maxima of peak I and peak II is most like the carotenoid-protein complex from the control cysts extracted by 1 M NaCl. A difference between these two types of carotenoid-protein complexes is their absorption maximum in the ultraviolet: 280 nm for the 1 M NaCl extracted carotenoid-protein and 275 nm for the carotenoid-protein released by trypsin digestion of intact yolk platelets.

J. Effect of Urea on the Spectral Characteristics of the Carotenoid-Protein Complex from Artemia Cysts

In an attempt to separate the carotenoids from the apoprotein of the carotenoid-protein complex, the carotenoid-protein complex was exposed to 2 M urea. The spectra which resulted from this treatment are shown in Fig. 7b and compared to the untreated carotenoid-protein in Fig. 7a. The absorption maxima of the carotenoid-protein isolated from control and experimental cysts are similar in the presence or absence of urea. The absorption maxima of peak I and peak II of the untreated carotenoid-protein complex are 465 and 372 nm, respectively. Also, in the non-urea treated preparations the peak II/peak I optical density ratio for the control carotenoid-protein complex is 1.59 and for the experimental it is 1.61. After 72 h in 2 M urea (at -10°C) it can be seen that there is a disruption of the spectrum of both the control and experimental carotenoid-protein complexes. Concomitant with this disruption in spectra there is a change of the characteristic colour of the complex from brown-green to red. The absorption maxima for peak I is now about 475 nm and the optical density is increased slightly. Treatment of the carotenoid-protein complex with urea greatly reduced the optical density of peak II for both the control and N_2 -treated cysts.

FIG. 7a. Visible spectra of the carotenoid-protein complex extracted by NaCl from Artemia yolk platelets. The yolk platelets were suspended in 0.03 M Tris-HCl, pH 7.4, containing 2 M NaCl and 5 mM EDTA. The carotenoid-protein was collected by centrifugation at 12,000 g for 15 min. The spectra was taken of the supernatant in a Beckman spectrophotometer (Model DB-G) and the wavelength to optical density relation was recorded manually. Control————; 3 months under N₂ - - - - .

FIG. 7b. Effect of urea on the visible spectra of the carotenoid-protein complex described in Fig. 7a. The carotenoid-protein was made 2 M with respect to urea by adding an equal volume of 4 M urea. The contents were stirred for 1 h under N₂ and then frozen at -10°C for 72 h. After thawing the contents were centrifuged at 6,800 g for 15 min to remove the insoluble material and the supernatant analyzed spectrophotometrically. Control————; 3 months under N₂ - - - - - .



A consequence of the change in optical densities is the change in the peak I/peak II ratio; again, the control and N₂-treated cysts respond in a like manner. The peak II/peak I ratio prior to urea treatment is approximately 1.6 and after urea treatment it is approximately 0.80.

K. Viability of Artemia Embryos

The third batch of cysts was analyzed for viability before and after treatment with N₂. The data, summarized in Table 17, was obtained from control and N₂-treated cysts part of which were lyophilized as outlined in Materials and Methods. The maximum viability for the unlyophilized cysts after 77 h of incubation is: 38.0% for the control, 11.5% for the N₂-treated cysts and 49.1% for the recovered cysts.

The control and recovered cysts respond in such a manner that the recovered cysts were approximately 9.5 h ahead of the control cysts especially after 40 h of incubation. The relatively high viability of the recovered cysts in all the time periods analyzed as compared to the control cysts reveals that the plateau for emergence and hatching has not been reached; this viability of the recovered cysts implies that the low viability of the experimental cysts is most likely due to some storage problem occurring at some later time than the partitioning of the cysts to generate the recovered cysts.

The lyophilized cysts (control and N₂-treated) displayed a similar degree of viability beginning at 40 h of incubation with the N₂-treated cysts lagging behind by approximately 15 h. The maximum viability of the lyophilized cysts after 77 h of incubation is 20.3% for the control cysts and 26.6% for the N₂-treated cysts. For any given time of

TABLE 17. The viability of Artemia embryos after exposure to anaerobiosis and recovery.^a

Artemia Embryos	Treatment	% Emerged Plus % Hatched					
		16 h ^b	25 h	40 h	51 h	60 h	77 h
C	Unlyophilized	2.1	19.8	22.1	26.4	36.0	38.0
E	"	less than 1%	less than 1%	1.2	2.4	4.6	11.5
R	"	7.0	22.4	31.6	36.4	41.7	49.1
C	Lyophilized ^c	less than 1%	2.2	9.1	11.5	11.3	20.3
E	"	less than 1%	less than 1%	6.2	15.9	19.2	26.6

^aThird batch.

^bA zero time was not deemed necessary because a visual inspection of the cysts after suspending in the artificial sea water revealed no obvious hatching or emergence.

^cAfter all the experiments were performed there was no longer any recovered cysts available for the measurement of their viability.

incubation, the lyophilized cysts displayed a lower % hatching plus % emergence than the unlyophilized cysts. For example, measurable percentages of emergence and hatching (6.2 to 9.1%) occurred at 40 h of incubation for the lyophilized cysts as compared to 2.1 to 7.0% at 16 h of incubation for the unlyophilized cysts. The results with the lyophilized cysts supports the notion that the N₂-treated unlyophilized cysts experienced some condition(s) that resulted in a lower viability.

DISCUSSION

The encysted gastrulae of Artemia salina display some remarkable adaptive mechanisms enabling them to survive in a relatively harsh environment. In most cases the environment is restricted to saline lakes and salterns found throughout the world (Clegg, 1974). The primary adaptive mechanism is the ability of these gastrulae to achieve some form of dormancy.

The usual fate of the early embryo, after being encased by a shell and subsequently released into the environment, is desiccation. Desiccation presumably has been selected as a means of enabling the embryo to resume development in a more favourable environment.

The work in this thesis, however, focused on a different form of dormancy. Rather than desiccation, the absence of oxygen was used as the trigger to induce a state of dormancy (Dutrieu and Chrestia-Blanchine, 1966). Though this form of dormancy is not as complete as desiccation its characteristics are such as to warrant such a classification. The studies performed in this thesis were initiated in an attempt to elucidate the properties and hopefully the mechanism of this form of dormancy.

A. Metabolic Quiescence and the Anaerobic State

Due to the relative quiescent state of the anaerobic cysts of Artemia, it was felt that the enzyme activities as compared to the controls would be lower; however, what was generally observed were enzyme

activities similar to the controls. The reconciliation of this situation initially involves a consideration of the control cysts which have been sterilized and washed without allowing development to begin. The enzymes in the control cysts are initially masked due primarily to their desiccated state. Given this consideration a more appropriate question concerning the enzyme activities of anaerobic cysts is: Why haven't the enzyme activities increased? These anaerobic cysts are no longer desiccated. The immediate answer is the activities of these enzymes need oxygen directly or indirectly. Then it would seem the more appropriate interpretation of the enzyme activities of anaerobic cysts where the activities are similar to the control cysts is that the structural integrity of the cysts has been maintained under anaerobiosis. The anaerobic adaptive mechanism of Artemia embryos is such that metabolic activities are generally not allowed to begin. Since the control embryos are in a truly quiescent state, the enzyme activities measured in this state are a reflection of activity compatible with such a state.

The enzymes of carbohydrate metabolism generally reflect the situation presented in the above. Ewing and Clegg (1969) observed no appreciable change in trehalose content after eight hours of anaerobiosis; however, Dutrieu and Chrestia-Blanchine (1966) stated that after two months of anaerobiosis there was a 7% to 12% increase in trehalose content in the embryo. Along with this increase Dutrieu and Chrestia-Blanchine observed an increase in trehalase activity which after 48 hours of anaerobiosis reached the level found in newly hatched nauplii. However, the work in this thesis shows that the trehalase activity is not influenced by anaerobic conditions. There seems to be no significant

difference in the activity between the embryos exposed to one month of anoxia compared to control embryos. The results in this study are compatible with the study conducted by Ewing and Clegg (1969). Since Ewing and Clegg (1969) evaluated the content of trehalose for only eight hours of anaerobiosis, a direct comparison with Dutrieu and Chrestia-Blanchine (1966) who evaluated trehalose content after two months of anaerobiosis is not possible. However, it is possible that the trehalose content may have risen 7% to 12% over a two month period. At such a rate of increase, a detectable increase of trehalose may not be measurable after eight hours. With respect to the trehalase activity, at least, the results in this study appear to be inconsistent with those of Dutrieu and Chrestia-Blanchine (1966).

The observation in this study regarding the trehalase activity is consistent with the observation made by Stocco et al. (1972) of the anaerobic embryos hatching at the same percentage but not necessarily at the same rate. Ewing and Clegg (1969) have shown that after eight hours of aerobic incubation of the encysted gastrulae, trehalose concentration decreases approximately 50%; an increase in trehalase activity during pre-emergence development as compared to the control embryos is implied and it would seem that a certain amount of time (at least 9.5 h, as seen in this study) is necessary for the embryos, recovered for 9.5 h, to marshal their resources before an increase in trehalase activity is observed.

B. Response of Mitochondria to Anaerobic Conditions

The response of the mitochondria of an aerobic organism subjected to anaerobic conditions was studied to determine the effect of the lack of oxygen on mitochondrial integrity. Two enzymes, malate dehydrogenase and cytochrome c oxidase were analyzed. During the mitochondrial studies, it was observed that mitochondria from anaerobic embryos were consistently difficult to resuspend after centrifugation at 12,000 g (15 minutes). The mitochondria would clump and adhere to the sides of the centrifuge tube and, in general, resist dispersion until they were washed in buffers containing EDTA. The mitochondria from aerobic embryos were more easily suspended after centrifugation. A quantitative assessment of this behaviour was attempted by pumping mitochondrial suspensions from aerobic and anaerobic embryos through columns of very small glass beads. The results of this experiment showed no difference between mitochondria from aerobic and anaerobic embryos with respect to adherence to glass beads under these conditions. Perhaps the extensive washing with EDTA sufficiently changed the adherence character of the mitochondria. The adherence may have been caused by the presence of bivalent ions such as Ca^{++} and Mg^{++} which were removed with EDTA buffers.

Plattner et al. (1970) and Groot et al. (1971) have stated that anaerobically grown Saccharomyces cerevisiae contain incomplete mitochondria (promitochondria) that lack a functional respiratory chain. Paltauf and Schatz (1969) have demonstrated profound changes in the lipid composition of the promitochondria showing a greatly lowered level of ergosterol and a simple and unusual fatty acid composition. Schmitt et al. (1973) have reported that the mitochondria of the encysted

gastrulae of Artemia were found to be devoid of cristae and to possess a relatively low respiratory capability. The promitochondria of anaerobically grown Saccharomyces are similar morphologically to the mitochondria of the encysted gastrulae of Artemia. Subjecting the encysted gastrulae of Artemia to anaerobic conditions may dedifferentiate the mitochondria to a degree where their membranes also have a modified lipid composition. In an attempt to study this problem and evaluate the metabolic competence of the mitochondria, mitochondrial malate dehydrogenase and cytochrome c oxidase activities were measured.

Mitochondrial malate dehydrogenase is located in the inner compartment of the mitochondria (Lehninger, 1970) and mitochondria are generally impermeable to NAD^+ (Lehninger, 1970). For this reason, buffers at pH 10.0 are used in the assay of mitochondrial malate dehydrogenase since this pH facilitates the entry of NAD^+ . It appears that the activity of this enzyme from experimental and recovered embryos is not significantly different from the activity of the control embryos. However, samples of mitochondria frozen and then thawed prior to the measurement of the activity, displayed a different pattern. The activities of this enzyme in this group of mitochondria (frozen and then thawed) in each case was less than the enzyme activities of the mitochondria from the embryos that were assayed immediately upon isolation (not frozen). Also it was observed that the anaerobic embryos incubated for 9.5 h in air had a mitochondrial MDH activity intermediate between the control and unincubated anaerobic embryos with the activity from the anaerobic embryos lower than the control activity. An obvious interpretation of these results is that the malate dehydrogenase from

mitochondria of anaerobic embryos has been inactivated to a greater degree than the control and recovered mitochondrial malate dehydrogenase. Unfortunately, I have no interpretation for this discrepancy and further experimentation would be necessary to resolve this point.

The influence of anaerobiosis on the cytochrome c oxidase is different than that observed for mitochondrial malate dehydrogenase. In both groups of mitochondria (unfrozen and frozen) the cytochrome c oxidase activity of mitochondria from embryos exposed to anaerobic conditions was significantly lower than the control and recovered embryos. The "stickiness" of these anaerobic mitochondria plus the stability of yolk platelets under anaerobic conditions leads me to believe that a rearrangement in membrane configuration is possible for anaerobic mitochondria, with the lipid of the membranes playing an important role as mentioned above for promitochondria.

Smith and Camerino (1963) have stated that swollen mitochondria may have a very active cytochrome c oxidase. During anaerobiosis the P_i concentration increases and Lehninger (1970) has stated that P_i in the medium can cause the swelling of the mitochondria. The modifications of the mitochondria suggested here may be in response to increased P_i concentration observed in Artemia embryos during anaerobiosis and suggests that mitochondria of anaerobic cysts are in a less swollen state. Such potentially adverse effects must be guarded against in order to maintain the viability of the embryo under anaerobic conditions. The decreased cytochrome c oxidase activity in the mitochondria under anaerobic conditions may be explained in terms of a transport phenomenon. If the mitochondrial membrane has been modified then it may be possible that the rate of entry of the exogenous cytochrome c into mitochondria of

anaerobic embryos is less than that for the mitochondria of the control embryos. A change in permeability toward cytochrome c may also explain the reduced cytochrome c oxidase activity in the promitochondria of anaerobically grown Saccharomyces. Groot et al. (1971) supported the statement that anaerobically grown Saccharomyces contain mitochondria lacking a respiratory chain by evaluating the cytochrome c oxidase activity using the change of rate of reduced cytochrome c to oxidized cytochrome c. The decreased cytochrome c oxidase activity of the promitochondria as compared to mitochondria from aerobic Saccharomyces may be due to a difference in transport across mitochondrial membranes. In a partial support of this idea Plattner and Schatz (1969) presented electron micrographs of aerobic and anaerobic yeast cells which were "physically" fixed by freeze-etching rather than chemical fixation. They observed a double-layered envelope in the promitochondria with typical cristae resembling aerobic yeast mitochondria.

The increased cytochrome c oxidase activities observed in aerobic and anaerobic embryos using mitochondria that were frozen prior to the assay compared to mitochondria used immediately can be explained by the different cytochrome c concentrations used in each case. Smith and Camerino (1963) have demonstrated that the kinetics of the oxidation of reduced cytochrome c are unusual in that the rate constant decreases with increasing concentration of the total cytochrome c (oxidized plus reduced) in the reaction mixture. The total cytochrome c concentrations in the case where the mitochondria were used immediately and in the case where the mitochondria were frozen prior to use were 11.6 mM and 5.2 mM, respectively, resulting in a 2.2:1 ratio. The cytochrome c oxidase

activities of the mitochondria of the first preparation (11.6 mM cytochrome c) were less than the activities of the mitochondria of the second preparation (5.2 mM cytochrome c) by a factor of 2.1. The decreased total cytochrome c concentration of the second preparation seems to explain the increased cytochrome c oxidase activities observed as compared to the activities observed for the first preparation with a higher total cytochrome c concentration. If this is true then freezing does not seem to influence the cytochrome c oxidase activities in the mitochondria used in this study; therefore, I suspect that if the total cytochrome c concentration had been the same in both the assays the observed results of the two mitochondrial preparations would have been indistinguishable. It is surprising that freezing and thawing had not resulted in a loss of cytochrome c oxidase activity. This observation may be accounted for by the fact that cytochrome c oxidase, being situated in the inner membrane, may derive stability from this location whether the mitochondria are aerobic or anaerobic.

Since the sensing system of the cytochrome c oxidase used in this study is the rate of change of reduced cytochrome c to oxidized cytochrome c, the cytochrome c oxidase activity is dependent on the rate of diffusion of the oxidized cytochrome c out of the mitochondria. Of interest is the cytochrome c oxidase activity of the recovered embryos. This activity is not significantly different from the activity of the control embryos. Such a pattern is seen also in the malate dehydrogenase activity using mitochondria that were frozen prior to assay. This observation is similar to the aerobic adaptation of anaerobically grown Saccharomyces cerevisiae. Plattner et al. (1970) have stated that the

respiratory chain is restored upon aeration. In variable oxygen tension it is not surprising that mitochondria are sensitive indicators of such changes.

The maintenance of structural integrity in the anaerobic Artemia embryo, it would seem, would involve the mitochondria. Such a complex organelle would be subject to some deterioration if it were not stabilized. The qualitative observation regarding the "stickiness" of the anaerobic mitochondria and the results with two mitochondrial enzymes especially cytochrome c oxidase suggests a modification of the mitochondrial membrane and it is proposed that such a modification confers some degree of stability on the mitochondrial membrane of Artemia embryos during anaerobic conditions.

C. The Role of Yolk Platelets in the Energy Metabolism of the Anaerobic Artemia Embryo

At least 92% of the Gp₄G of the Artemia embryo is found in the platelets (Warner et al., 1972). The hydrolysis of Gp₄G during anaerobiosis would be mediated by the enzyme diguanosine tetrphosphatase (Warner and Finamore, 1965) or by the reverse reaction utilizing Gp₄G synthetase (Warner and Huang, 1974). The activity of the diguanosine tetrphosphatase in the experimental (and recovered embryos) is not significantly different from the activity of the control embryos despite the fact that this enzyme is responsible for the hydrolysis of Gp₄G during anaerobiosis. The hydrolysis products of Gp₄G catalyzed by diguanosine tetrphosphatase are GMP and GTP in equimolar amounts (Warner and Findamore, 1965). Furthermore, the GTP released from Gp₄G may be hydrolyzed relatively rapidly to GMP and this may explain the increased

concentration of GMP during the first two weeks of anoxia (Stocco et al., 1972). It is proposed that Gp₄G leaves the anaerobic yolk platelets and enters the cytosol. This increased cytosol Gp₄G could stimulate increased enzyme activity without an increase in enzyme molecules by virtue of increasing the substrate concentration. In support of the proposition that Gp₄G leaves the yolk platelet during anaerobiosis I would like to cite several observations from this study concerning the yolk platelets from embryos exposed to anaerobic conditions. The yolk platelets from embryos maintained anaerobically respond to solubilization by NaCl differently than yolk platelets from control embryos (Fig. 2). Yolk platelets from anaerobic embryos require a greater NaCl concentration to solubilize the yolk protein compared to yolk platelets from control embryos. Also, the appearance of the yolk platelets from embryos exposed to anaerobic conditions is green while the yolk platelets from control embryos are brown. The spectrum of the extracted carotenoid protein from anaerobic embryos is slightly different from the spectrum of the carotenoid protein from control embryos.

The various properties of the diguanosine nucleotide enzymes and the observation concerning the reduced activity of the Gp₄G synthetase activity in the PMF during anaerobiosis are consistent with the above proposal. One result of the hydrolysis of Gp₄G during anaerobiosis with the support of other nucleotide phosphatases would be the increase of inorganic phosphate. One consequence of the increase in Pi concentration is the decrease in pH due to the dissociable protons of the phosphoric acid group. Since the optimal pH of diguanosine tetraphosphatase is approximately 8.0 the increased Pi may serve as a built-in

negative feedback loop to block further hydrolysis by this enzyme. As mentioned by Stocco et al. (1972) 50% of the total available phosphate-bond energy, mainly in the form of Gp_4G was utilized in the first four months of anoxia and of this amount 50% was utilized during the first two weeks. As seen in Table 4, the greatest change in Pi concentration occurs after one week of anaerobiosis. The data concerning Pi in this thesis is consistent with the above proposal.

The reduced pH due to the generation of Pi would result in a more favourable environment (pH 6.0) for Gp_4G synthetase activity and may allow for the conversion of GTP and PPI to Gp_4G ; however, the Gp_4G synthetase activity is significantly reduced in the PMF from anaerobic embryos than that found in control embryos. If this mechanism of Gp_4G hydrolysis by diguanosine tetraphosphatase is true, the conversion of the resulting GTP and PPI to Gp_4G by Gp_4G synthetase would in some way need to be less than the hydrolysis so that some of the available phosphate bond energy would be used for the needs of the anaerobic embryo rather than used for the resynthesis of Gp_4G . This seems to be the case since the resynthesis of Gp_4G to control levels occurs after the anaerobic cysts have been incubated in the air.

It is proposed that part of the adaptive mechanism of Artemia embryos under anaerobic conditions involves the suppression of certain enzymes. One such enzyme might be Gp_4G synthetase. Another mechanism may involve compartmentalization of Gp_4G and its movement into the cytosol for hydrolysis by diguanosine tetraphosphatase.

D. Protein-Carotenoid Complexing in the Post-Mitochondrial Fraction (PMF)

In a preliminary study of Artemia embryos under anaerobic conditions, it was observed that there was a shift to higher molecular weight species of macromolecules in the PMF. To study further this observation experiments were designed based on the assumption that a redistribution of high molecular weight compounds had occurred in the embryo perhaps from the yolk platelet fraction into the PMF. The results of these experiments revealed that no such redistribution had occurred. However, an evaluation of the GTP:GTP guanylyltransferase activity in anaerobic embryos compared to control cysts suggested a possible explanation for this observation. Under various cycles of freezing and thawing no change in the GTP:GTP guanylyltransferase activity was observed. However, the activity of GTP:GTP guanylyltransferase of the control cysts, though initially higher than the activities of the experimental and recovered cysts, decreased steadily with each freezing and thawing cycle. It is proposed that the stability of this enzyme to freezing and thawing (in the experimental and recovered cysts) is due to a complexing of the enzyme with carotenoids, most likely either canthaxanthin or echinenone, and that this association with carotenoids not only confers stability against structural damage due to repeated freezing and thawing but also is responsible for the decreased activity of the enzyme observed in the experimental and recovered embryos compared to the enzyme from control cysts. A comparison of the GTP:GTP guanylyltransferase activity from yolk platelets among the three groups of cysts studied revealed no significant differences. In fact, the activity was quite similar to the activity of the GTP:GTP guanylyltransferase from the PMF of the

experimental cysts, implying that these enzymes may be experiencing a similar environment. The carotenoids in the yolk platelets seem to be organized as carotenoid-protein complexes (Warner et al., 1972). It may be that the GTP:GTP guanylyltransferase of the yolk platelets is associated in some manner with the carotenoids of the carotenoid-protein complex. This may be a possibility since during purification of the GTP:GTP guanylyltransferase from yolk platelets the carotenoid-protein elutes immediately before this enzyme on a calcium phosphate-cellulose column (Warner et al., 1974). With respect to enhanced enzyme stability due to the complexing with carotenoids, further investigation of the observation should be attempted by increasing the number of freezing and thawing cycles to see if the activity of the enzyme from the control cysts falls below the activity observed for the experimental and recovered cysts.

Stability associated with a carotenoid-protein has been described by Cheesman (1958). The carotenoid-protein, ovorubin from the tropical snail, Pomacea canaliculata is not readily coagulated by heat and is resistant to attack by trypsin, whereas its apo-protein is coagulated at temperatures above 70°C and hydrolyzed by trypsin. On the basis of this study Cheesman (1958) has suggested that carotenoids may have an important role in the stabilization of protein molecules in nature. Green (1965) has stated that complexing of a protein to carotenoids may enable a protein to be held in reserve until a specific stage of development. This statement may apply to GTP:GTP guanylyltransferase in the PMF during anaerobiosis. As mentioned previously in a situation where the hydrolysis of Gp₄G is actively occurring (probably for the release of phosphate-bond energy), the re-use of this energy to resynthesize the

initial compound would result in no net release of re-useable energy. The end result in such a situation would be the eventual depletion due to entropic processes of the energy stores of the embryo with no net work performed. The work performed in such an organism as Artemia during anaerobiosis would most likely be to stabilize existing, potentially labile structures against entropic processes. Complexing, in general, tends to stabilize the constituents of a complex and the use of carotenoids in Artemia as a complexing agent seems to have been the strategy selected.

I believe that the above observation suggests an explanation for the shift to higher molecular weights of compounds found in the PMF with increased time under anaerobic conditions as observed by gel permeation chromatography. It may be that the carotenoids located in the PMF exist as two populations: one population associated with lipid globules and the other complexed to proteins. With increasing time under anaerobic conditions a greater proportion of the carotenoids are complexed with the proteins in the PMF. This complexing would increase the apparent molecular weight of the proteins found in the PMF.

E. Increased Concentration of Carotenoids in Artemia Embryos Under Anaerobic Conditions

With regard to the above proposition to explain increased enzyme stability under anaerobic conditions, it should be noted that the total measurable carotenoid content of the cysts increases. Since it is quite unlikely that this increase in carotenoid content is due to de novo synthesis, considering the fact, that the biosynthesis of the carbon skeleton of the carotenoids is outside the realm of animal metabolism (Pullman and Pullman, 1963) the observed increase in the

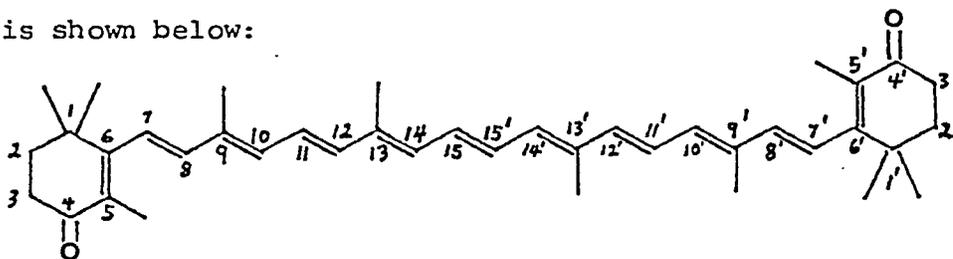
carotenoid level most probably arose by the transformation of a closely related molecule. Certain organisms like Artemia are capable of storing ingested carotenoids intact or transforming them into other carotenoids (Hsu et al., 1970). Also, the increase in carotenoid content in Artemia embryos under anaerobic conditions seems to be due to an increase in canthaxanthin and echinenone content (Table 11). Consequently, it may be interpreted that the increase in carotenoid content is due to a transformation of β -carotene into these two carotenoids since this has been observed to occur (Hata and Hata, 1969). If an increase of canthaxanthin and echinenone occur under anaerobic conditions as a result of the transformation of β -carotene, then a decrease in the β -carotene level would be expected in the experimental embryos compared to the control cysts. Since 90% to 95% of the carotenoids in the control cysts is comprised of echinenone and canthaxanthin it is unlikely that the trace amounts of β -carotene in the cysts are sufficient to account for the appearance of large amounts of these two carotenoids. However, based on the work of Karnaukhov (1971) and Karnaukhov et al. (1977), an explanation is possible. Karnaukhov (1971) has shown the existence of carotenoids in the giant neurons of the gastropod mollusc Lymnae stagnolis, and this author has shown that the carotenoid concentration increases reversibly in these cells due to a decrease in oxygen tension which results from the action of mitochondrial respiratory inhibitors and to an increase in cell activity. This author hypothesized that the conjugated unsaturated double bonds of the carotenoids provide an intracellular stock of oxygen and as a consequence takes part in the oxidative metabolism of the animal. In 1977, Karnaukhov et al. tested nine species of bivalves and

two species of gastropods for their tolerance to environmental pollution. These molluscs were grouped as high, medium or low based on their tolerance to environmental pollution as measured by a decrease in dissolved oxygen and an increase in various toxic substances. Tolerance was determined by comparing the number of particular species surviving in a particular area over a nine year period. During this period all of the areas studied increase in pollution to one degree of another. It was found that some molluscs responded well as evidenced by an increase in the number of specimens per meter of bottom. Others decreased in number. For example, two fresh-water species, Flexopecten ponticus and Donax trunculus inhabit only the mouth of Novorossiusk Bay, whereas previously they inhabited the middle part of the bay in large numbers. In the intervening years pollution increase and silt accumulation has driven these molluscs from the middle part of the bay. One interesting correlation was that the molluscs with high tolerance in every case had a greater total and unsaponifiable carotenoid concentrations than either the molluscs with medium or low tolerance. Also, two species with high tolerance, Cerastoderma glaucum and Fritia retia reticulata, each inhabiting a polluted and a less polluted area were analyzed for their carotenoid concentrations. In both cases the group inhabiting a greater polluted area had a higher carotenoid concentration than the group inhabiting a less polluted area. In another experiment reported by Karnaukhov et al. (1977), the mollusc, Mytilus galloprovinciales, considered to have a high tolerance to environmental pollution was placed in an aquarium which was slowly perfused with sea water spiked with mineral oil. The slow perfusion and mineral oil were to simulate the

decreased oxygen tension and pollutants found in their natural environment. With respect to the carotenoid concentration the most dramatic effect was observed within the first six hours of exposure. During this time an approximate two-fold increase was observed. After 48 h a three- to four-fold increase in carotenoid concentration was observed under these conditions. However, it should be noted that no control for feeding was carried out. It may be that in the environment where there is an increase in population the increased pollution may have favoured a particular food source with relatively high carotenoid content which for various reasons was not favourable to the species that did not survive. The distribution of these molluscial species may be due to the availability of a particular food source which is dependent on the alteration of the environment by pollution and the increase in carotenoid concentration may not be a cause of this distribution, but rather a result of the type of food ingested. However, in support of the hypothesis of the author, the results of the experiment using the slowly perfused aquarium, the molluscs were not feeding during the experiment and the increase in carotenoid concentration may have been due to the re-expression of the chromophoric group of the carotenoids by the re-establishment of the conjugated double bond system. These authors hypothesize that the conjugated unsaturated double bonds of the carotenoids are capable of binding oxygen or an electron-acceptor equivalent to oxygen. The accumulation of oxygen on the carotenoid would nullify its chromophoric nature and render it colourless in the visible region of the spectrum. As a consequence, during times of high oxygen tension the measurable carotenoid content of the organism would be lower than during low or hypoxic conditions. The

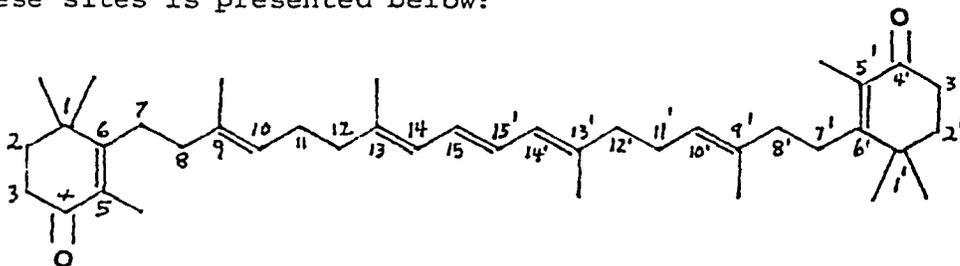
removal of oxygen from the carotenoids during hypoxia would restore the chromophoric nature of the carotenoid and a greater carotenoid content would be observed.

The experimental results in this thesis support the suggestions of Karnaukhov et al. (1977). The reduction and oxidation of carotenoids may be the basis upon which Artemia is able to maintain its viability during prolonged periods of environmental anoxia. It is proposed that such a reduction and oxidation of the carotenoids may involve the enzymes α -glycerophosphate dehydrogenase and cytoplasmic malate-dehydrogenase. The structure of a carotenoid primarily responsible for its colour is the conjugated double bonds. An example of such a structure using canthaxanthin is shown below:



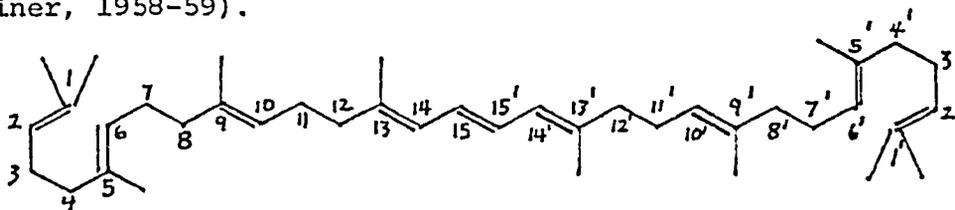
Canthaxanthin (4,4'-diketo- β -carotene)

During aerobic conditions it is proposed that the carotenoid becomes hydrogenated, most likely enzymatically. Pullman and Pullman (1963) have stated that the most reactive sites on the carotenoid are the 7, 7¹ and 11, 11¹ carbon positions. Hydrogenation of canthaxanthin at these sites is presented below:

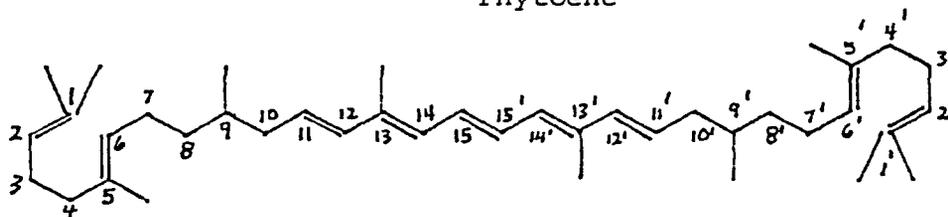


Hydrogenated canthaxanthin

The fully unsaturated canthaxanthin molecule contains 11 conjugated double bonds in the hydrocarbon chain, whereas the hydrogenated canthaxanthin molecule contains 3. This degree of saturation may be sufficient to render the molecule colourless. Phytoene and phytofluene are two naturally occurring carotenoid hydrocarbons which are colourless: they contain three and five conjugated double bonds, respectively (Stainer, 1958-59).

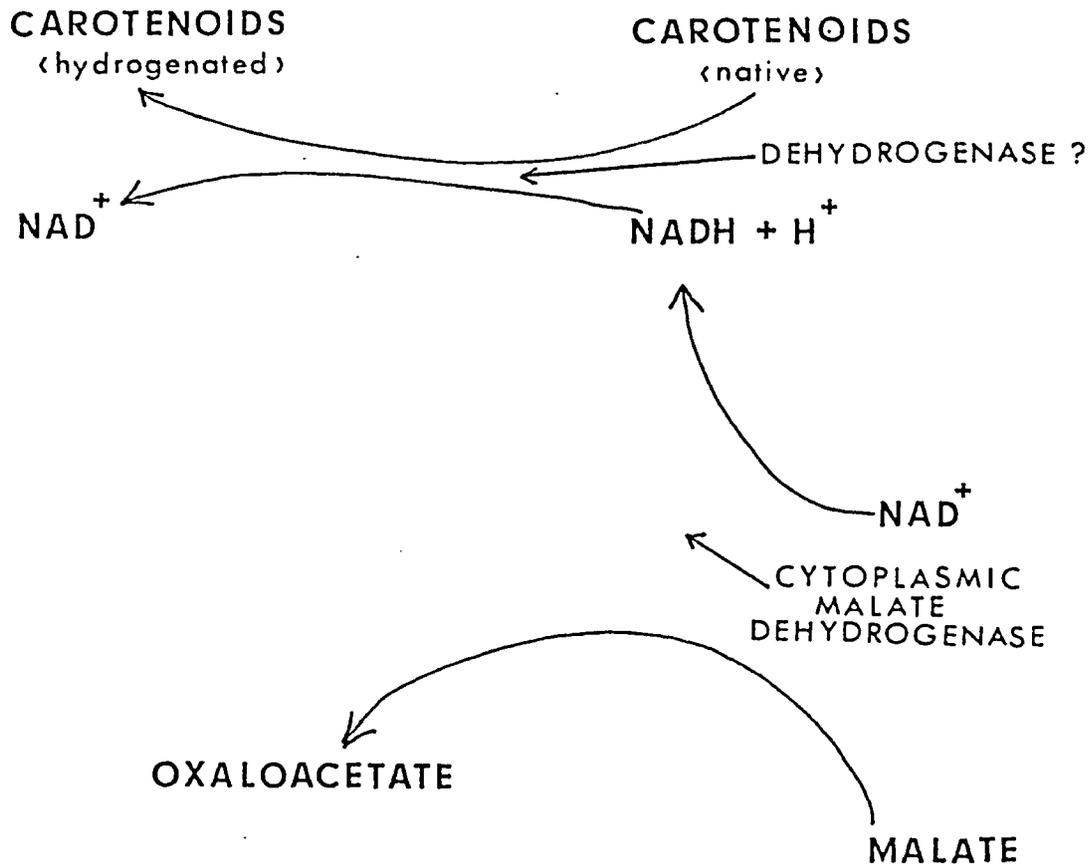


Phytoene



Phytofluene

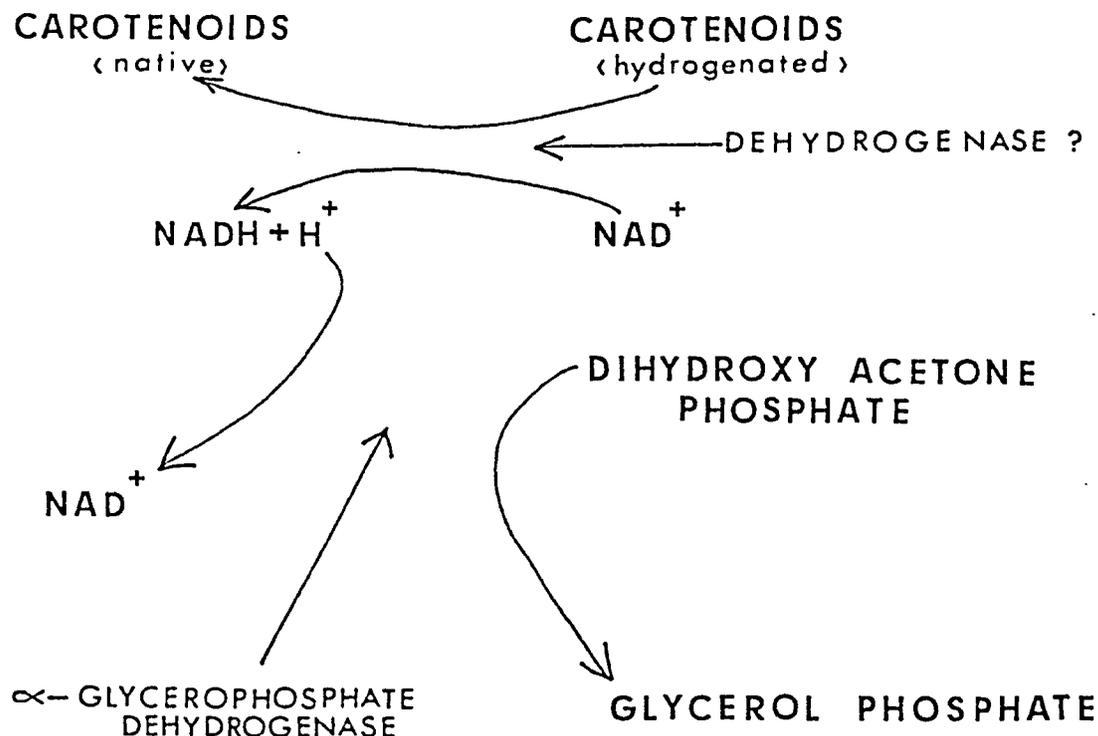
The hydrogenated canthaxanthin molecules presented above possess the same hydrocarbon chain structure of phytoene. It is also proposed that the enzyme involved in the hydrogenation is a dehydrogenase utilizing reduced NAD (NADH) as a coenzyme. Coupled to this hydrogenation is the reduction of NAD by malate by the action of cytoplasmic malate dehydrogenase to yield oxaloacetate and $\text{NADH} + \text{H}^+$. The proposed scheme coupled to the reduction of the carotenoids is presented below.



This scheme can be quite efficient as malate and NAD^+ can be regenerated in an aerobic environment. The primary reason for suggesting cytoplasmic malate dehydrogenase in this scheme is the observed increase in this enzyme in the recovered embryos.

Under anaerobic conditions, it is proposed that α -glycerophosphate dehydrogenase is implicated in the re-establishment of the chromophoric character of the molecule. The dehydrogenase involved in the hydrogenation of the carotenoids would be involved in the dehydrogenation of the carotenoids by a simple reverse reaction; this reverse reaction would be coupled to the reoxidation of NAD by dihydroxy

acetone phosphate by the action of α -glycerophosphate dehydrogenase yielding NAD^+ and glycerol phosphate. This scheme is presented below:



The primary reason for suggesting such a scheme is the observed increase in the α -glycerophosphate dehydrogenase in the anaerobic embryo (Table 5). Also, an increased α -glycerophosphate dehydrogenase is consistent with the observation made by Ewing and Clegg (1969) concerning the lack of the accumulation of lactate in the anaerobic embryo. Under normal aerobic respiration, the NADH produced by glycolysis is shuttled away from lactate dehydrogenase by glycerophosphate dehydrogenase and ultimately the respiratory chain by virtue of their higher affinity for NADH (Lehninger, 1970). It is proposed that under anaerobic conditions α -glycerophosphate dehydrogenase is induced in Artemia embryos to participate in the reoxidation of the carotenoids and a consequence of this induction is the removal of NADH so that interaction with lactate

dehydrogenase is minimized. It may be that the increase in NADH during anaerobiosis is the initial trigger in the adaptive mechanism displayed by Artemia embryos; the increased NADH under anaerobic conditions may be involved in the induction of α -glycerophosphate dehydrogenase of Artemia embryos simply by the Law of Mass Action. The dehydrogenation of the carotenoids may render the carotenoids more reactive so that they may interact with the proteins more readily. The ability of the carotenoids to undergo hydrogenation and dehydrogenation is partially explained by the fact that carotenoids are both excellent electron donors and excellent electron acceptors evidenced by the values of the energy coefficient of their highest occupied and their lowest empty molecular orbitals (Pullman and Pullman, 1963).

F. Carotenoid-Protein Complexes

Wallace et al. (1967) has stated that carotenoid pigments are found ubiquitously in the eggs of Crustacea. These carotenoids are usually associated with yolk bodies. These authors have suggested the term crustacean lipovitellin, analogous to the vertebrate lipovitellins, for the high-density lipoprotein of the yolk. Zagalsky and Gilchrist (1976) have stated that the main storage protein of the yolk of Crustacea is a high-density lipoglycoprotein to which the carotenoid pigment is associated. The majority of the carotenoids in the embryo of Artemia are associated with a lipoprotein in the yolk platelet and is similar to other Crustacean lipoproteins (Warner et al., 1972). This association results in a carotenoid-protein complex which is influenced by anaerobiosis (Warner et al., 1972).

The most obvious effect of anaerobiosis on the Artemia embryo is the change in colour of the yolk platelet preparation. Yolk platelets prepared from aerobic embryo are brown while yolk platelets from anaerobic cysts are olive-green. Such a change in colour is an indication of some alternation of the carotenoid-protein complex. This observation suggests that the change in colour is due to an alteration in the protein-carotenoid association or to a change in the distribution of canthaxanthin and echinenone within the complex.

The influence of urea on the carotenoid protein complex reveals the importance of hydrophobic bonding in the structure of the complex. Hammes and Swann (1967) measured the influence of urea on polyethylene glycol. Upon increasing the urea concentration an increase was found in the intrinsic viscosity of the polyethylene glycol. The viscosity increase indicates a slight expansion of the polyethylene glycol polymer coil, and they attribute this expansion to increased solvation. The increased solvation is supposedly due to the breaking down of the local solvent structure. They suggest that the apparent greater solvation is consistent with the belief that these reagents reduce the unfavourable entropy of hydration of hydrocarbon-like groups and thereby weaken hydrophobic bonding.

Urea was found to alter the spectral pattern of the carotenoprotein complex from both aerobic and anaerobic embryos. The colour of the carotenoid-protein complex in urea is red indicating a disruption of the carotenoid-protein association (Green, 1965; Jencks and Buten, 1964). As a consequence, the loss of the 370 nm peak due to urea may indicate that hydrophobic binding is responsible for this peak. The peak, with a maximum at 460 nm which increases under the influence of urea is quite

close (15-20 nm) to the absorption maxima of carotenoids found in this carotenoprotein complex (see Table 9) suggested that a high degree of disassociation exists in the carotenoid-protein complex in the presence of urea. Wallace et al. (1967) have stated that the carotenoid in the carotenoid-protein complex of crustacean eggs may reside in hydrophobic "pockets" of the protein and not covalently bonded since the carotenoid can be extracted with lipid solvents. However, hydrophobic binding is not the only method of binding of the carotenoids to the protein since the carotenoid and protein were still associated in the urea solutions and it is unlikely that the carotenoids are soluble in aqueous 2 M urea.

Gilchrist (1968) has reported carotenoid-protein complexes containing canthaxanthin as the main carotenoid in three Anostraca: Branchipus stagnalis, Chirocephalus diaphanus, and Fanymastix luccinae. Phosphate buffer extracts of these three Anostraca each resulted in brown solutions which when chromatographed on DEAE cellulose resulted in various fractions. Common to all the chromatograms was a blue-green carotenoid-protein complex having maxima at 275, 370 and 685 nm and varying absorbance in the range 400-500 nm. Lee (1966) reported on the carotenoid-protein complex of the marine isopod Idothea granulosa. The extracted carotenoid-protein was green in colour with absorption maxima at 683-(620)-280 nm and very heavy broad absorption in the 400-500 nm range. The carotenoid-protein complex after precipitation by saturated ammonium sulphate could be redissolved in phosphate buffer. Upon shaking with diethyl ether and subsequent centrifugation, the aqueous phase was bright blue and the diethyl ether phase yellow with a large amount of yellow lipid gathered at the surface. The main fraction of the chromatography of the crude carotenoid-protein complex in DEAE-cellulose was a blue

canthaxanthin carotenoid-protein with a spectrum similar to the crude extract in having absorption maxima at 680-(620)-280 nm but different in that a peak at 375 nm was observed and there was little absorbance in the 400-500 nm range. The last fraction of the DEAE-cellulose column contained the carotenoids removed by the diethyl ether treatment mentioned above. Zagalsky (1976) in a review on carotenoid-protein complexes suggests that the 400-500 nm absorption band represents a relatively loose association of carotenoids in the lipid portion of the apo-lipoprotein of the carotenoid-protein complex since it can be eliminated by mild treatment (diethyl ether). That the apo-protein be designated as apo-lipoprotein is fitting since lipid extraction of the carotenoid-protein complex during experiments in my study revealed a lipid fraction detectable by iodine vapor. Zagalsky (1976) also suggests that the maxima in the 370 and 680 nm regions arises from more firmly bound canthaxanthin. Zagalsky (1976) also states that absorption maxima of carotenoids shifted bathochromically 10-25 nm compared with solutions of the carotenoid in hexane can be explained by refractive index effects associated with dissolution of the carotenoids in the lipid or protein components of the lipoproteins. The maximum absorption of canthaxanthin and echinenone in hexane are 464 nm and 458 nm, respectively. If this suggestion by Zagalsky is correct then it would be seen that the carotenoid(s) responsible for the absorption of the carotenoid-protein of Artemia at 460 nm is associated in a manner different from a simple dissolution of the carotenoid in the lipid or protein component of the apo-lipoprotein of the carotenoid-protein complex. The resulting bathochromic shift of the 460 nm absorption maxima of the carotenoid-protein to 475 nm in the presence of urea implies that hydrophobic binding may also play some role

in the association of the carotenoid(s) and protein resulting in the 460 nm absorption maxima found in the carotenoid-protein of Artemia.

Lee and Zagalsky (1966) studying the specificity of the carotenoid-protein linkage of crustacyanin, the lobster-shell pigment, isolated the apo-protein (apo-crustacyanin) and combined it with canthaxanthin and echinenone. The naturally occurring carotenoid-protein α -crustacyanin is composed of eight subunits of β -crustacyanin containing astaxanthin as the prosthetic group. The association of canthaxanthin with the apo-crustacyanin resulted in a complex that separated into three fractions on DEAE-cellulose. Their absorption spectra were identical consisting of a smooth curve with a maximum at 580 nm, slightly below that of β -crustacyanin (585 nm). Also, these components corresponded both in molecular size (36,000) and in electrophoretic mobility to β -crustacyanin. Evidence for the association of echinenone with apo-crustacyanin was not found. Echinenone is a 4-keto- β -carotene containing one carbonyl group on one of the β -ionone rings, whereas canthaxanthin is a 4,4'-diketo- β -carotene. It was concluded that substitution on both β -ionone rings was probably necessary for binding. Hallenstvet et al. (1978) have demonstrated that echinenone comprises 95% of the carotenoids in the unfed larvae of the sea-urchin. Based on the observation by a different author, that carotenoid-protein complexes were found in the eggs and embryo of a related organism, Hallenstvet et al. (1978) have hypothesized the existence of a carotenoid-protein complex in sea urchin larvae containing echinenone as the prosthetic group. The lack of evidence for binding of echinenone to apo-crustacyanin may be a reflection of the incompatibility of the echinenone and apo-crustacyanin since it is most likely that apo-crustacyanin is a different protein than

the apo-protein of the carotenoid-protein complex of Artemia embryo. In support of this statement, the association of canthaxanthin with apo-crustacyanin can be cited. The canthaxanthin apo-crustacyanin complex did not result in a spectrum resembling the spectrum of the carotenoid-protein of Artemia embryo and α -crustacyanin is soluble in dilute salt solutions (0.05 M potassium phosphate, pH 7.0), whereas the carotenoid-protein complex from Artemia embryos requires salt concentrations of the order of 1 M NaCl.

At the moment, it is unknown whether echinenone is associated with the apo-protein of the carotenoid-protein complex of Artemia embryo in a manner similar to canthaxanthin. It is tempting to suggest that echinenone is associated with the lipid portion and is primarily responsible for the 460 nm absorption peaks but at this time insufficient information is available to resolve this point.

The carotenoid-protein from anaerobic embryos contain a greater proportion of echinenone than the carotenoid-protein from control embryos and this seems to be at the expense of canthaxanthin. Such a pattern is also seen in the yolk platelets and the carotenoid-protein released by the action of trypsin on the yolk platelets (Table 11). As was stated earlier, β -carotene is converted to canthaxanthin via echinenone in Artemia and it may be possible that the enzymes involved in this conversion may in a reverse reaction convert some canthaxanthin to echinenone during anaerobiosis. There is evidence for a correlation of colour with carotenoid composition. Lee and Gilchrist (1972) have compared the colour change in the isopod Idotea resecata. This particular isopod occurs in two distinct colour varieties, brown and green. These organisms also contain a green canthaxanthin-carotenoid protein complex. Ten

carotenoid pigments were isolated from the whole animals and there was a different distribution of these 10 carotenoids. For example, in the brown variety, the percent of the total carotenoids represented by canthaxanthin and echinenone was 9% and 7%, respectively, whereas in the green variety the relative abundance was 6% and 2%, respectively. This pattern is not what has been found in the case with Artemia but does display the role of carotenoid composition and colour.

A particular distinction should be made concerning the color of the yolk platelets from embryos exposed to anaerobic conditions and the extracted carotenoid-protein complex. The green color observed, as compared to the brown color of control embryos, is primarily a function of the architecture of the yolk platelet. The reason for making this statement is that upon extraction of the carotenoid-protein with NaCl the green colour of the solution was quite evident; however, on standing the colour would fade to some extent but would still retain a green tinge. From the results obtained thus far, it appears that the structure of the yolk platelets has been altered by storage in an anaerobic environment and this alteration is somehow reflected or mediated by the change in association or composition of the carotenoids.

The above statement is supported by the stability studies on the yolk platelets from control embryos and embryos exposed to anaerobic conditions. The extraction of the carotenoid-protein complex with NaCl, the extraction of the carotenoids with organic solvents and the release of proteins from yolk platelets incubated at 37°C, have all revealed the increased resistance of the anaerobic yolk platelets to these processes. Once the carotenoid-protein is released from the yolk platelet the resistance to disruptive forces is no longer as evident. This statement

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can be supported somewhat by the experiments where the influence of incubation at 45°C on the ratio of the absorption maxima of the NaCl extracted carotenoid-protein complex was followed with time. No noticeably increased stability of the carotenoid-protein complex from anaerobic embryos was observed as compared to the aerobic embryos and if anything, the ratio of the absorption maxima of the anaerobic carotenoid-protein complex after nine hours at 45°C approached that of the control value. In general, the stability of the anaerobic embryo may be attributed to the architecture of the intact embryo and once this architecture has been distributed (as in homogenization) this stability may not be readily observed.

The spectra of the carotenoid-protein complex extracted by NaCl from anaerobic and control embryos are very similar with some minor differences. The absorption maximum of peak I of the carotenoid protein from N₂-treated embryo is approximately 1-2 nm shifted to shorter wavelengths. After precipitation with 50% ammonium sulphate, the absorption maximum of peak I of N₂-treated embryo shifted approximately 5 nm to shorter wavelengths. Also, the peak II to peak I ratio of the spectra of the carotenoid-protein complex from anaerobic embryos as found in this study is higher than that for the complex from aerobic embryos. This greater ratio of peak II to peak I in the spectra of the anaerobic carotenoid-protein complex is primarily due to a smaller extinction of the maximum at ≈462 nm as compared to the corresponding maximum at ≈462 nm of the aerobic carotenoid-protein complex; this is seen graphically in Figs. 5b and 6b. For example, the extinction coefficient (O.D./mg protein) for the absorption maximum at ≈462 nm of the carotenoid-protein complex from anaerobic cysts is 0.196 as compared to 0.204 for the

corresponding aerobic carotenoid-protein complex. The extinction for the anaerobic and aerobic carotenoid-protein complexes of the absorption maximum at ≈ 370 nm was 0.312 and 0.308, respectively. The ratio of the two absorption maxima for the anaerobic and aerobic carotenoid-protein complexes was 1.53 and 1.49, respectively (Table 16). In one experiment (not reported since it occurred only once to such an extent), the spectrum for the anaerobic carotenoid-protein complex after precipitation with ammonium sulphate and subsequent re-dissolution in 0.5 M phosphate buffer, pH 7.3, the absorption at ≈ 462 nm was not present; that portion of the spectrum was flat.

Of interest to this problem may be the flavin dehydrogenases. In their oxidized form, they are intensely coloured and may be red, brown or green in colour. They usually have absorption peaks near 370 and 450 nm. When they are reduced enzymatically or chemically they undergo bleaching with the loss of 450 nm absorption peak (Lehninger, 1970). Flavin dehydrogenases are involved with the redistribution of electrons and it may be possible that due to their similar absorption spectra, the carotenoid-protein complexes and flavin dehydrogenases share a common function. Whether flavin dehydrogenases are contaminants of the Artemia carotenoid-protein complexes is unknown and further experimentation would be needed to resolve this point.

The two main differences between the spectra of the anaerobic and aerobic carotenoid-protein complexes, (shift of peak I and larger peak II to peak I ratio of the anaerobic carotenoid-protein complex) may be interpreted by proposing that the carotenoid(s) responsible for the absorption at ≈ 462 nm is (are) more firmly bound or associated in the case of the anaerobic carotenoid-protein complex.

The spectra of the carotenoid-proteins released from yolk platelets by the action of trypsin may be used to support the notion that under anaerobic conditions the architecture of the yolk platelet has been modified and that such a modification may not have been transmitted to the solubilized carotenoid-protein complex. The absorption maxima of the anaerobic and aerobic carotenoid-protein complexes released by trypsin are identical and so are the peak ratios (Table 16). Upon precipitation with ammonium sulphate and subsequent re-dissolution in 0.03 M phosphate buffer, pH 7.4, the absorption maxima of the anaerobic and aerobic carotenoid-protein complexes are still identical although the peak ratio of the anaerobic carotenoid-protein is slightly higher than the corresponding control. Also, the absorption maximum of this carotenoid-protein complex in the ultraviolet is 275 nm while the corresponding maximum of the NaCl extracted carotenoid-protein is 280 nm. The tryptic action may have exposed more tyrosine and phenylalanine with absorption maxima at approximately 275 and 254 nm, respectively, than tryptophan which has an absorption maximum at 280 nm (Lehninger, 1970). It is proposed that the carotenoid-protein complex extracted by NaCl and the carotenoid-protein complex released from the yolk platelets by the action of trypsin are different with respect to their molecular weights. This can be seen primarily from the carotenoid content of these two types of carotenoid-proteins as seen in Table 11. In each case, the trypsin released carotenoid-protein complex has a greater carotenoid content per unit of protein. Also, the extinction (O.D./mg protein) at each maxima of the trypsin released carotenoid-protein is greater than the corresponding maxima from the NaCl extracted carotenoid-protein. For example, the extinction of the trypsin released carotenoid-protein complexes at

the absorption maximum of 370 nm of the anaerobic and aerobic embryos are 0.357 and 0.373, respectively, as compared to the corresponding NaCl extracted carotenoid-protein complexes which are 0.312 and 0.308, respectively. Furthermore, the carotenoid-protein complexes released from yolk platelets by trypsin are readily soluble in relatively low ionic strength media (0.03 M phosphate buffer, pH 7.4), whereas the NaCl extracted carotenoid-protein complex require NaCl concentrations in the order of 1 M.

It may be possible that a portion of the carotenoid-protein complex in the yolk platelet which is readily attacked by trypsin, in both anaerobic and aerobic conditions, is responsible for the resistance to solubilization of the complex by NaCl. One characteristic of the carotenoid-protein complex in the yolk platelet is that when acted upon by trypsin a carotenoid-protein is released containing the same complement of carotenoids as is seen when the carotenoid-protein is extracted by NaCl. During anaerobic conditions, a different association between this portion of the complex and the remainder of the complex containing all or most of the carotenoids may occur. One consequence of this new association during anaerobiosis is an increase in the resistance to solubilization of the carotenoid-protein complex from yolk platelets by NaCl. This portion of the complex may contain a large number of hydrophobic amino acids which when sufficient NaCl has been added, may stabilize the charges on the protein and allow solvation to occur (Mahler and Cordex, 1960). The use of EDTA may also contribute to our understanding of the stability of the yolk platelets during anaerobic conditions. The extraction of the carotenoid-protein complex was generally facilitated by the introduction of EDTA into the extraction medium (0.5 M NaCl - 5 mM EDTA,

pH 5.0) used to solubilize the carotenoid-proteins from the ovaries or eggs of Pagurus pollicaris (hermit crabs), Sesarma reticulatum and Libinia emarginata (spider crab) (Wallace et al., 1967). Also, the extraction of the blue carotenoid-protein crustacyanin from the lobster Homarus gammarus utilized an extraction medium containing 10% EDTA (Quarmby et al., 1977). Furthermore, the extraction of the blue canthaxanthin carotenoid-protein complex from Branchipus stagnalis utilized a medium containing 10% NaCl and 0.04 M EDTA (Zagalsky and Gilchrist, 1976). The importance of EDTA in solubilizing the carotenoid-proteins from yolk platelets is that divalent cations may be involved in this stabilization. To what, if any, extent divalent cations play in the stabilization of the yolk platelets needs to be evaluated in further studies. Another consequence of the proposed different association between the portion of the carotenoid-protein complex, which can be removed by trypsin, and the portion of the carotenoid-protein complex containing the carotenoids under anaerobic conditions may be a more favourable association of the carotenoids with the protein resulting in the differences observed in the spectra of the NaCl extracted anaerobic carotenoid-protein complex when compared to the aerobic carotenoid protein spectrum.

As mentioned earlier, there are other organisms with carotenoid protein complexes containing canthaxanthin as the prosthetic group other than Artemia. One of particular interest is the blue canthaxanthin-carotenoid-protein complex from the yolk platelets of Branchipus stagnalis which also contains large amounts of diguanosine nucleotides (Zagalsky and Gilchrist, 1976). Branchipus stagnalis is a fresh water anostracan related to Artemia salina having the carotenoid-protein

complex confined within the yolk platelets. The absorption spectrum of this carotenoid-protein is different from Artemia; it has a maximum at 625 nm and an inflexion at 675 nm with an additional small peak at 375 nm. Although differences exist in the colour of their carotenoid-protein complexes, there are similarities in the organisms which warrant a comparison and which may shed some light on the structure of the carotenoid-protein of Artemia. Zagalsky and Gilchrist (1976) have stated that chloride ions are necessary for the stability of the spectrum and hypothesize that there are specific chloride-ion-binding site(s) in the protein and that these have an influence on the carotenoid-lipovitellin interaction. They have also demonstrated that the apo-protein of B. stagnalis is a lipoglycoprotein.

Zagalsky and Gilchrist (1976) have also shown that within two to three hours of hatching of Branchipus, the blue platelets break down with the liberation of the carotenoid followed by its incorporation into bright orange lipid globules. Zagalsky and Gilchrist (1976) ask whether the carotenoid as well as the protein serve some precise function in embryonic development and also whether the restriction of diguanosine nucleotides and carotenoid-protein within platelets is a common feature of the eggs of Crustaceae. The liberation of the carotenoid towards the end of embryonic development has been reported for various Crustaceae. Green (1965) reported that the green carotenoid-protein (ovoverdin) of the lobster changed in colour to red towards the end of embryonic development. It has been shown that the total carotenoid content of the embryo does not change but the link between carotenoid and protein is broken. Ball (1944) has shown that the goose barnacle eggs contain a blue carotenoid-protein and the nauplii no longer contain

this complex and are pink in colour - the colour of the free carotenoid. Also, the colour of the nauplii of Artemia are pink and it may be quite possible that such a split between carotenoid and protein near the end of embryonic development may occur also. Green (1965) has suggested that the function of the carotenoid-protein is more important in the early stages than in the later stages. He continues by suggesting that there is a possibility that linking a protein with a carotenoid may remove the protein from the possibility of attack by certain enzymes. Also, such a linkage with a carotenoid might enable a protein to be held in reserve until a specific stage of development. When Hata and Hata (1969) fed yeast to Artemia, they found that the eggs in the ovary were white since yeast has little carotenoid. The resulting embryos hatched and appeared to be normal. This finding suggests that the carotenoid-protein complex may serve some function that is more readily encountered by the embryo in its natural environment. One such function of the carotenoids may be the stabilization of the yolk membrane and other structures during anaerobiosis.

The ability of Artemia embryos to withstand anaerobic conditions is an example of the many biochemical adaptations exhibited by this organism (Clegg, 1974). Also, it is primarily at the embryonic stage that this organism displays resistance to anaerobiosis (Ewing and Clegg, 1969). Artemia has evolved a strategy to overcome anaerobiosis. The overall pattern is to achieve a quiescent metabolic stage. This strategy is successful because the organism does not continue to develop in a situation where the energy needed for the establishment of certain structures is not available. Artemia is suitably equipped for such a strategy because it possesses a mechanism which enables it

to stabilize important structures and functions. This may be accomplished by complexing protein with carotenoid pigment for stabilization.

G. Limnology of Artemia salina Environment

Artemia salina inhabits an environment where the depletion of oxygen readily occurs and is readily maintained, necessitating an adaptive mechanism for survival. A discussion of how the physical conditions of the environment influence the properties of an inland water mass is important in understanding how anaerobic conditions are created in the environment of Artemia salina.

At a certain time in the spring, a lake in the temperate zone is at a uniform temperature of maximum density (4°C) from top to bottom (Ruttner, 1963). With the rising spring temperature, the surface of a lake warms faster than the lower levels. The temperature is distributed throughout the entire water mass by the action of winds on the surface. As the warm weather continues, a condition arises where the accelerated heat intake at the surface establishes a vertical temperature gradient and consequently a vertical density gradient. The resulting density gradient is steep enough to resist mixing due to the agitation of the water by winds. During the summer this gradient is well established dividing the lake vertically into three regions: the upper layer known as the epilimnion where the water temperature is uniform and mixing occurs; the middle layer or thermocline where there is a steep drop in temperature per unit of depth; and the lowest region, the hypolimnion where the temperature from the upper limit to the bottom is nearly uniform but cooler than the epilimnion during the summer. The thermocline effectively isolates the waters of the epilimnion from the waters

of the hypolimnion. One consequence of this stratification is that gaseous and other products of decomposition accumulate in the hypolimnion and become only partially discharged (Welch, 1952).

As autumn approaches, the surface waters cool and the density of the water between the epilimnion and the hypolimnion become less distinct to the point where there is not enough resistance to the mixing of the entire water mass caused by the agitation of the surface by winds. This process eventually results in the equalization of the density and temperature of the water in the lake (Welch, 1952). The temperature at this time of year in the entire lake is approximately 4°C. With the onset of winter the temperature of the surface water becomes colder and approaches 0°C: although the surface water is colder than the water of the lower levels, the surface water does not sink owing to the interesting property of water having its highest density at 4°C and its freezing point of 0°C. The surface of the water in early winter is lighter than the water of the lower regions and as a consequence in winter, a layer of ice forms on the surface of the lake isolating the lower regions from the atmosphere. In the spring, the same cycle described above repeats itself; however, there are circumstances found in the environment of Artemia salina that interfere with this cycle.

In spring the water mass is able to circulate due to the relative uniformity in the density of the water. If the density of the water of the lower levels was maintained at a greater density than the upper layers of water, a situation can arise where the mixing of the entire water mass would not occur. Situations as this exist and the lakes exhibiting this property are known as meromictic lakes. The factor that maintains the lower levels at a greater density is a marked

stratification of the salt concentration known as a chemocline (Ruttner, 1963). Such meromictic lakes can have an average salt gradient of approximately 100 g/l at the surface to 400 g/l at the bottom (Anderson, 1958). A salt content of 1 g/l increases the density of water about 0.008; at the same time a difference in density of 0.000008 arises due to a change in temperature from 4°C to 5°C. In order to overcome this difference in density of 0.000008 due to a change in temperature, an increase in salt concentration of only 10 mg/l is needed (Ruttner, 1963). For this reason it is possible to have lakes where underlying layers of water were warmer than layers immediately above. The temperature of these bottom layers of water can be quite warm even in winter, and can carry over into the spring as well: the radiant energy from the sun is stored in the highly concentrated saline layers and released slowly since release of the heat at the surface takes place only by induction and not by circulation (Ruttner, 1963).

Saline lakes where the stratification of the salt concentration can be quite steep can have a water mass which does not mix entirely, resulting in a permanent stratification with very little or no communication between the upper and bottom layers of water. The oxygen content of the bottom layer of uncirculated water is either very low or non-existent due to decomposition of organic material, or due to the low solubility of oxygen in warm water containing a high concentration of salt. For example, at saturation at 15°C, a litre of sea water (at 36% salinity) contains 5.8 cc oxygen per litre of water, whereas a litre of fresh water at the same temperature holds 10.3 cc. Also, the solubility of oxygen decreases about 40% as fresh water warms from freezing to 25°C (Reid, 1961). Once a condition of an oxygen-free bottom layer occurs in

a saline lake, containing sulphate salts, certain bacteria which are capable of reducing sulphate of H_2S can establish themselves and keep the hypolimnion virtually oxygen-free throughout the year. Anderson (1958) describes such a lake in north central Washington called "Hot Lake," and some features of the life history of A. salina in that lake. During the summer months when the salinity of the lake is highest and the oxygen content the lowest, of the three dominant populations of Artemia present, egg, immature (pre-nauplii, and nauplii) and adult, the egg population is predominant. At the end of July the egg population numbered 50 per litre while the immature and adult populations numbered 6 and 8 per litre, respectively. The stable stratification of such a lake effectively diminishes the water mass capable of supporting the immature and adult populations.

The ability of hydrated A. salina embryos to tolerate extended periods of anoxia (at least five months) without utilizing their carbohydrate and lipid reserves to any appreciable extent was first observed by Dutrieu and Chrestia-Blanchine (1966). Subsequently these observations were confirmed and expanded to show that morphogenesis is arrested under anaerobic conditions concomitant with the lack of utilization of carbohydrate and lipid reserves (Ewing, 1968). In 1969, Ewing and Clegg confirmed Dutrieu's and Chrestia-Blanchine's results and also indicated that there was no increase in the lactic acid concentration or lactate dehydrogenase activity of the encysted gastrulae of Artemia during anaerobiosis. Stocco et al. (1972) demonstrate that although carbohydrate metabolism and morphogenesis are arrested, nucleotide metabolism continues and under prolonged anaerobic conditions the adenosine nucleotide pools are depleted and guanosine nucleotide metabolism continues.

A unique nucleotide, diguanosine 5'-tetrphosphate (Gp_4G), was shown to be responsible for this prolonged guanosine metabolism.

The ability of these gastrulae to produce viable nauplii at the same percentage (Ewing and Clegg, 1969) but not necessarily at the same rate (Stocco et al., 1972) as embryos incubated aerobically suggests a conservation of the structural integrity. The structural stability of these embryos during anaerobiosis is interesting not only in that it occurs, but also that it can be maintained for at least five months.

In summary, an investigation into the structural stability of the Artemia embryos under anaerobic conditions was undertaken. The data collected implicates carotenoids in the stabilization of encysted embryos subjected to anaerobiosis. This stabilization seems to occur through the complexing of carotenoids with proteins and through rearrangements of existing proteins or membranes to form stable carotenoid-protein complexes.

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