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ISOLATION AND CHARACTERIZATION
OF YOLK PLATELETS FROM THE BRINE SHRIMP
ARTEMIA SALINA

BY

JULIUS G. PUODZIUKAS

A Thesis
Submitted to the Faculty of Graduate Studies 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

1970

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ABSTRACT

In the brine shrimp, Artemia salina, diguanosine tetraphosphate (GP₄G) is localized primarily in the 700 g sediment (98 %), whereas the 700 g supernatant fluid contains very little (2 %) of this compound. Purification of the 700 g sediment has revealed that GP₄G is localized in the yolk platelets and not in the nuclei. In addition, Artemia yolk platelets have been found to contain 74.1 % protein, 8.1 % lipid, 4.0 % free carbohydrate and trace amounts of nucleic acids. The molecular weight of the lipoprotein fraction is 220,000 and the lipid moiety appears to be mainly canthaxanthin. The major carbohydrate component is trehalose and very little, if any, glucose and glycogen are present. The purified platelets are relatively stable in H₂O, 0.25 M sucrose and 20 % glycerol but are unstable in NaCl solutions greater than 0.1 M.

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INTRODUCTION

The acid-soluble nucleotides comprise about 3.0 % of the dry weight of encysted embryos of the brine shrimp, Artemia salina, and P^1, P^4 -diguanosine 5'-tetraphosphate (GP_4G) represents about 50 % of the nucleotide composition of the cyst (Warner and Finamore, 1967). The exact role of GP_4G in Artemia development has yet to be ascertained, but it has been implicated as the sole source of purines for development, and in particular, as the primary source of DNA adenine (Finamore and Clegg, 1969). The inability of Artemia to synthesize purines de novo at any stage of its life cycle (Clegg et al., 1967; Warner and McClean, 1968) strongly supports the notion that GP_4G can be converted to adenine-containing nucleotides. That such a large quantity of GP_4G , an important anhydride, should remain in solution in the cyst seems highly unlikely and efforts were directed to investigate the possible compartmentalization of this unique nucleotide.

Preliminary cell fractionation experiments indicated GP_4G to be associated with the 700 g pellet and attempts were made to isolate in pure form the GP_4G -containing particles. In this thesis a study of yolk platelets of the dormant cyst of Artemia salina is presented. The yolk platelets were isolated with a minimum of contamination, according to light microscope criteria, and an investigation of their chemical composition and physical stability was made. The amount of protein, lipid, carbohydrate, nucleotides and nucleic acids was determined in purified preparations, and experiments were performed to more fully elucidate the qualitative nature of these entities. Also, the molecular weight of the major yolk platelet protein was determined and the phosphoprotein phosphorous content ascertained.

METHODS AND MATERIALS

Isolation of Artemia Yolk Platelets

In all experiments, dried dormant cysts of the brine shrimp, Artemia salina, (Sanders Brine Shrimp Company, Ogden Utah), were used as the starting material. The cysts were suspended in ice-cold 0.25 M sucrose containing 0.1 % Tween 80 (10 mls sucrose per gram of cysts dry weight) and stirred vigorously for 4 hours at 3-5° C using a magnetic stirring device. The mixture was then filtered twice through a cheese-cloth-glass wool-cheesecloth filter and the filtrate, free of unbroken cysts was centrifuged at 700 g for 10 minutes. The supernatant fluid was adjusted to 0.5 N with respect to HClO₄ and the precipitate that formed removed by centrifugation. The soluble fraction was deacidified by passage through a 1.5 X 3 cm column of charcoal (Clegg et al., 1967) and the nucleotides recovered by elution with a mixture of ethanol-H₂O-NH₄OH (2:2:1, v/v/v) were stored at -20° C until needed. The 700 g pellet was resuspended in ice-cold 0.25 M sucrose solution and homogenized gently with a loose fitting Dounce homogenizer to ensure a homogenous suspension.

The suspension in 0.25 M sucrose containing 0.1 % Tween 80 was layered on top of a sucrose gradient from 0.88 M to 2.2 M containing 0.1% Tween 80 in 25 mls final volume, and centrifuged for 30 minutes at 41,000 g in a Sorval (RC2B) centrifuge. The pellet was resuspended in 0.25 M sucrose containing the Tween 80 and the procedure repeated once. The final pellet was either used immediately or stored at -20° C for future use.

The degree of nuclear contamination of the yolk platelet

preparation was determined microscopically and the size of the yolk platelets determined using a hemocytometer (AO Spencer). The ratio of nuclei to yolk platelets was determined at all stages of the purification procedure by counting the number of nuclei present for every 2500 platelets in the wet preparations. To facilitate identification of the nuclei, all preparations were stained with methyl green. All photographs were made using either Kodak Ektachrome Tungsten or Ektachrome Daylight film.

Chemical Analysis of Purified Artemia Yolk Platelets

All purified yolk platelet preparations were washed 3 times with distilled water before chemical analysis was carried out. In some cases, however, chemical analyses were carried out on crude fractions prepared using H₂O only. Excess H₂O was removed using a filter paper wick and the platelet preparation was weighed. The preparation was then dried to a constant weight at 37° - 40° C and the wet weight to dry weight ratio determined. Pure platelet preparations were fractionated using four separate procedures and each step of each procedure was analysed. The initial steps in procedures 1,2 and 3 are the same and as follows: A dry yolk platelet preparation was extracted repeatedly at 45° C with ethanol-ether (3:1 v/v) until only a pure white powder remained. The ethanol-soluble fraction was taken to dryness, weighed, and considered to be mostly lipid. However, some carbohydrate material was detected here using the anthrone method (Zill, 1956) and the amount determined was subtracted from the total dry weight. In Procedure 1, the dried ethanol-ether insoluble material was extracted with ice-cold 0.5 N HClO₄ and the acid soluble fraction was assayed for carbohydrate (Zill, 1956) and protein (Lowry et al., 1951). The acid-insoluble fraction was heated for 10 minutes at 100° C in 0.4 N

PROCEDURE 1

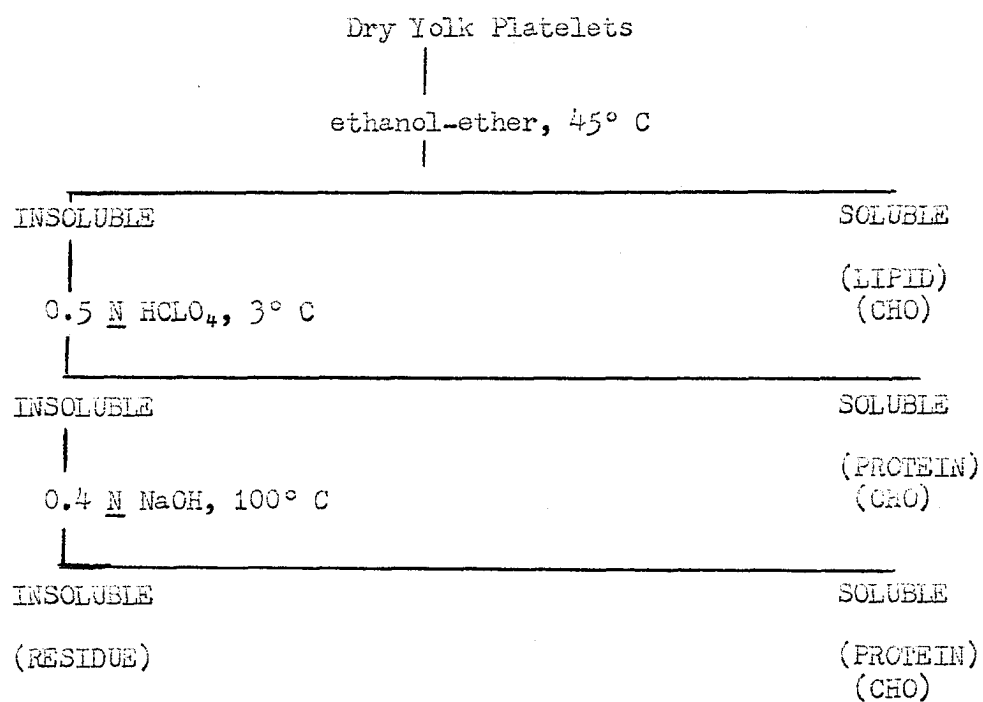


Figure 1. Fractionation of Artemia Yolk Platelets
for Chemical Analysis.

NaOH and the soluble fraction assayed for protein and carbohydrate as before. The insoluble material was dried, weighed, and considered to be residue.

In Procedure 2, the white powder remaining after ethanol-ether treatment was heated with 0.4 N NaOH to 100° C for 10 minutes and the soluble fraction removed by centrifugation.

The insoluble residue was then heated with 0.5 N HClO₄ at 100° C for 10 minutes and the acid-soluble fraction collected by centrifugation.

The soluble fractions for both the hot alkaline and acid treatments were assayed for protein and carbohydrate as before.

Using Procedure 3, freshly prepared yolk platelets were acidified with 0.5 N HClO₄ and the nucleotide content determined by chromatography on DEAE-cellulose (see Appendix A).

In Procedure 4, the ethanol-ether insoluble powder was extracted with 2 M NaCl at 90° C (Tyner et al., 1953) and the soluble fraction collected by centrifugation. A sample was applied to a column of G-75 Sephadex (1 x 40 cm.) and the column was developed with H₂O. The contents of the first peak containing the nucleic acids were pooled and the amount of DNA was determined by the method of Ceriotti (1952) and the RNA was calculated by difference.

In a separate experiment the carbohydrate content of yolk platelets was determined on the 700 g pellet obtained by using ice-cold H₂O as the fractionation medium. The 700 g sediment was obtained exactly as before except that H₂O was used instead of the sucrose - Tween 80 medium. The partially purified 700 g sediment was extracted with ethanol-ether then ice-cold 0.5 N HClO₄ as for Procedure 2 and the carbohydrate content was determined on each extract using the anthrone method of Zill

PROCEDURE 2

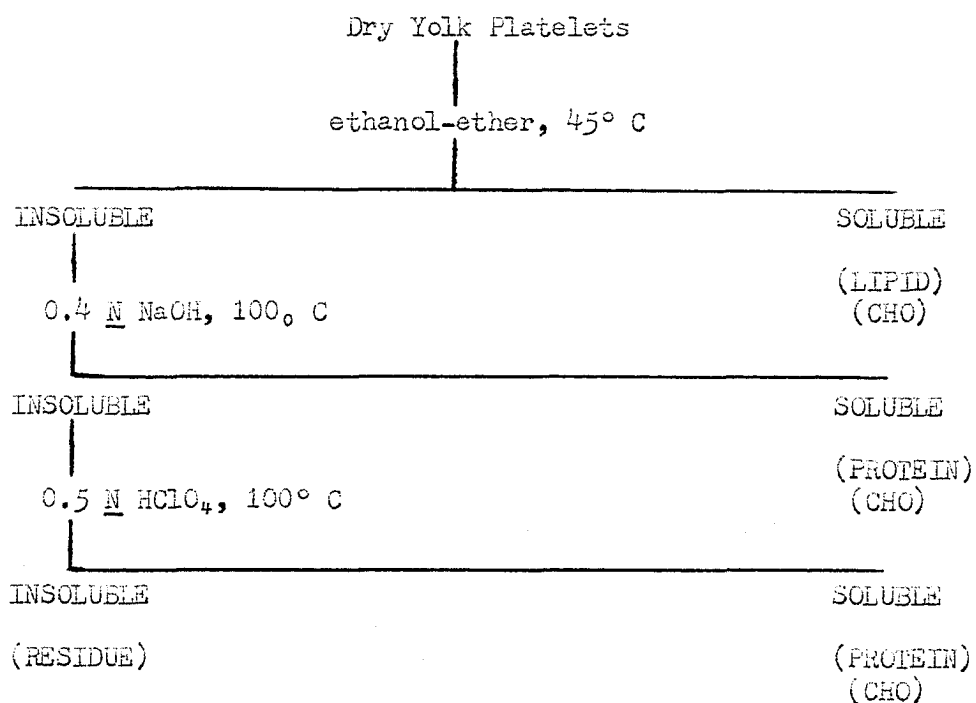


Figure 2. Fractionation of Artemia Yolk Platelets
for Chemical Analysis.

PROCEDURE 3

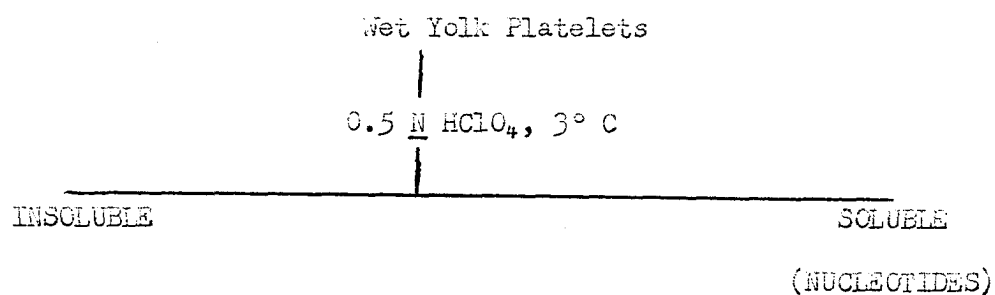


Figure 3. Fractionation of Artemia Yolk Platelets for Chemical Analysis.

PROCEDURE 4

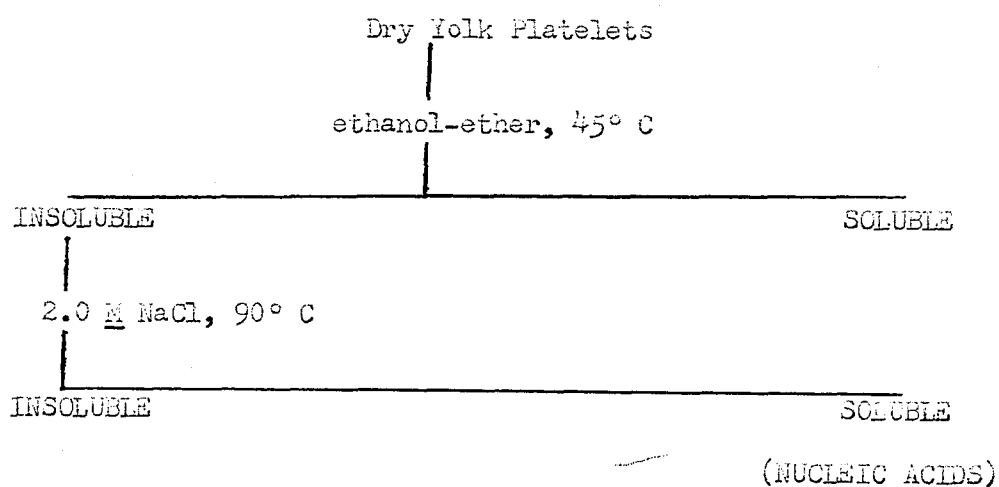


Figure 4. Fractionation of Artemia Yolk Platelets for Chemical Analysis.

(1956). In addition, the acid-soluble fraction was percolated through a charcoal column (1.5 x 3 cm.) to remove nucleotide pentoses, and the free carbohydrate which passed through the column was measured using the anthrone method.

The ethanol-ether soluble fraction was analysed spectrophotometrically between 310 and 620 nanometers (nm) in a Beckman spectrophotometer (model DB) and compared to spectra of several known carotenoid compounds.

The identity of the carbohydrate(s) present in the charcoal column effluent was determined by ascending paper chromatography (Whatman No. 1) using a butanol-ethanol-water (4:1:1.9, v/v/v) mixture (Fairbairn, 1958). The chromatogram was developed by dipping in an aqueous solution of silver nitrate, then dried at 100° C. The dried paper was sprayed with 0.5 N NaOH in aqueous ethanol and excess silver oxide was removed by dipping the paper in 6 N NH₄OH. The paper was then washed for 1 hour in running tap water and dried at 100° C (Trevelyan et al., 1950). Glucose and trehalose were run at the same time as references.

Isolation and Fractionation of Acid-Soluble Nucleotides on DEAE-Cellulose

The purified yolk platelet preparation was homogenized directly in ice-cold 0.5 N NClO₄ and the acid-soluble fraction was collected by centrifugation at 21,000 g for 10 minutes. The pellet was washed with a small volume of 0.5 N HClO₄ and the soluble fraction pooled with the first extract. The pooled fractions were deacidified by shaking with N alamine (see appendix A) then applied to a 1 x 50 cm. column of DEAE - cellulose (Whatman DEAE 11) prepared according to Peterson and Sober (1956) and previously converted to the bicarbonate form with NH₄HCO₃, pH 8.6. The sample was washed on the column with 0.002 M NH₄HCO₃, pH 8.6, and the nucleotides eluted using a linear gradient of NH₄HCO₃, pH 8.6,

from 0.002 M (1500 ml) to 0.25 M (1500 ml) according to Warner and Finamore (1967).

The fractions were identified by their 280/260 ratios and on the basis of their elution position as previously determined (Warner and Finamore, 1967). The amount of each nucleotide in the preparation was determined using the proper extinction coefficients (Volkin and Cohn, 1950; Finamore and Warner, 1966).

Isolation and Characterization of Artemia Yolk Protein

Isolation of yolk platelet protein was carried out on purified preparations as follows. A wet preparation was suspended in 3 volumes of ice-cold 1.0 M NaCl containing 0.025 M ethylene diamine tetraacetate (EDTA), pH 5.0, and extracted with stirring for 30 minutes. The extract was centrifuged at 41,000 g for 30 minutes and the pellet was washed once with one volume of the NaCl-EDTA medium. The supernatant fractions were combined and when necessary passed through Whatman No. 1 filter paper to remove the fatty film on the surface. The clear orange-coloured extract was applied directly to a 2 x 56 cm column of Sepharose 6B (Pharmacia, Montreal) previously equilibrated with 1.0 M NaCl containing 0.025 M EDTA, pH 5.0, and the protein was eluted with the same solution. Each column fraction was assayed for material absorbing at 260, 280 and 480 nm then assayed for protein using the method of Lowry et al., (1951). In a separate experiment, the material soluble in the NaCl-EDTA medium was treated with 2 volumes of saturated $(\text{NH}_4)_2\text{SO}_4$, and the precipitate that formed on standing overnight at 5° C was collected, resuspended in the extracting medium, and applied to a freshly prepared column of Sepharose 6B (2 x 56 cm.) The column was washed with the NaCl-EDTA solution and each column fraction was assayed as before. The molecular weight of the protein

fraction was determined using 4 globular proteins as standards (see appendix B)

The phosphoprotein phosphorous content was determined by extracting a purified preparation of yolk platelets with 1.0 N NaOH (Wallace et al., 1966) and measuring the amount of Pi liberated (Ernster et al., 1950) by the alkalai when heated at 100° C for 15 minutes. As before the protein content was estimated by the method of Lowry et al., (1951).

Stability of Artemia Yolk Platelets in Various Media

To test the stability of the yolk platelets in various media the following experiment was carried out. A purified yolk platelet preparation was suspended in 0.25 M sucrose and 2 ml aliquants were dialysed for 2.5 days against 100 mls of the following solutions: H₂O, 0.25 M sucrose, 20% glycerol, 0.1 M NaCl, 0.25 M NaCl, 0.5 M NaCl and 1.0 M NaCl. All fractions were kept at 5° C and periodically, the dialysate was assayed spectrophotometrically between 220 and 290 nm for dialysable components. Also, the amount of acid-soluble material in the yolk platelet preparation was determined by extracting an aliquant with 0.5 N HClO₄ and measuring the ultraviolet-absorbing material in the soluble fraction.

In a separate experiment, a purified yolk platelet preparation was aged in H₂O at 0° - 5° C for periods up to 12 days. At various times the acid-soluble nucleotides were extracted and fractionated on columns of DEAE-cellulose, and the quantity of each nucleotide determined.

RESULTS

Isolation, Purification, and Characterization of Yolk Platelets from Artemia

Isolation of highly purified yolk platelets from the cysts of Artemia salina was achieved using 0.25 M sucrose containing 0.1 % Tween 80 in the fractionating medium. It was observed that in the presence of the detergent, cytoplasmic contamination was virtually eliminated as determined by light microscope examination. Also, it was observed that purification of the yolk platelets required centrifugation through a very dense sucrose gradient (0.88 M---~~2.2~~ M). Less dense sucrose gradients were found to produce preparations heavily contaminated with nuclei. Figure 1 contains photographs of purified yolk platelets. When the nuclear contamination at various stages in the purification scheme was determined microscopically, the data in Table I were obtained. From these data it can be seen that repeated centrifugation of the 700 g sediment through a 0.88 M to 2.2 M sucrose gradient reduces the nuclear contamination about 290-fold and that the final preparation contains only 0.003 % nuclei in terms of particle number.

Freshly prepared yolk platelets appear to be oval in shape and range in size from 2.5 to 3.2 μ in width and 3.1 to 5.2 μ in length. Upon ageing in H_2O , however, the platelets become spherical and clump readily as they decrease in size. In comparison, the nuclei found in the extracts of Artemia cysts are larger than yolk platelets and range in diameter from 6.9 to 14 μ . From the ratio of nuclei per cyst (4000 according to Nakanishi et al., 1962), there appears to be 5.16×10^4 yolk platelets per encysted embryo.

TABLE I

Nucleus / Yolk Platelet Ratios for Various Isolation Procedures

Procedure	Suspension	12,100 g Sediment	27,000 g Sediment
Preparation I ^a	0.0775		
Preparation II ^b	0.0650		
700 g Sediment (0.25 <u>M</u> Sucrose)	0.0440		
700 g Sediment in 0.25 <u>M</u> Sucrose layered on top of following gradients			
0.88 <u>M</u> → 0.88 <u>M</u>		0.0170	0.0084
0.88 <u>M</u> → 1.6 <u>M</u>		0.0136	0.0263
0.88 <u>M</u> → 2.0 <u>M</u>		0.0050	0.0038
0.88 <u>M</u> → 2.2 <u>M</u>		0.0028	0.0010
Final Preparation ^c			0.0003

^aCysts just broken with teflon ball homogenizer, 3 strokes.

^bCysts ground with automatic grinder.

^cThe yolk platelets were prepared as described in Methods and Materials using 0.25 M sucrose containing 0.1 % Tween 80 and the sediment resulting from centrifugation through the 0.88 M to 2.2 M sucrose gradient was centrifuged again using the same gradient.

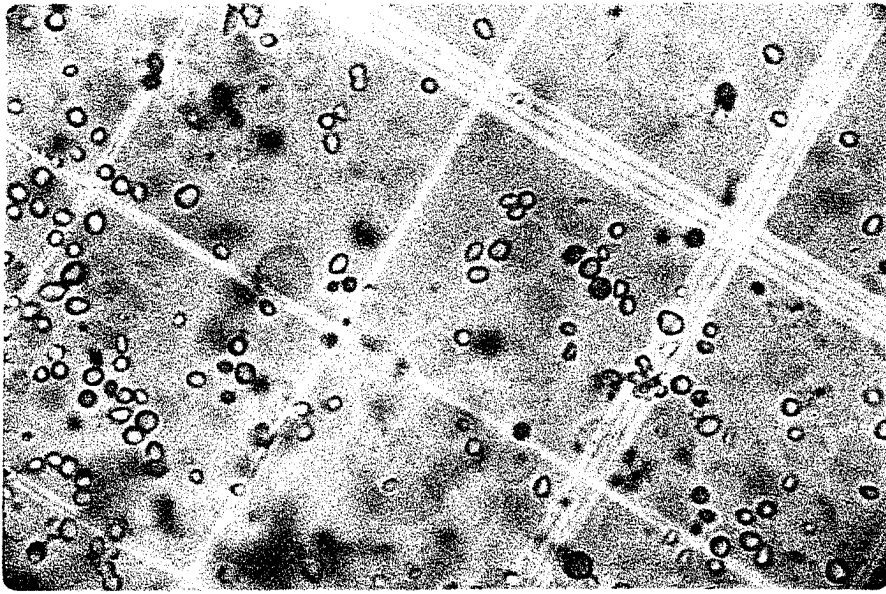


Figure 5. Photographs of Artemia Yolk Platelet Preparations.
 (a) Crude 700 g pellet, nuclei and yolk platelets, unstained, (760 X).

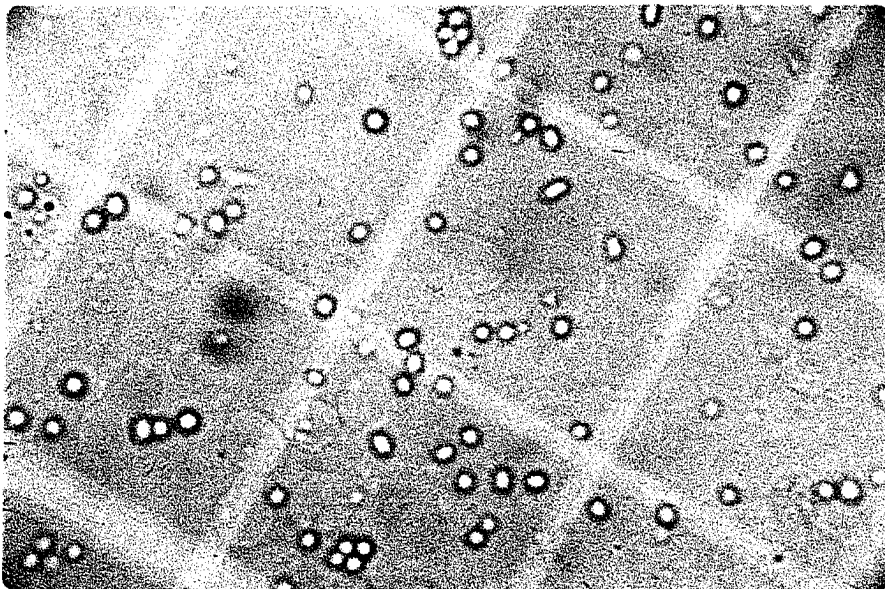


Figure 5. Photographs of Artemia Yolk Platelets.
 (b) Crude 700 g pellet, nuclei and yolk platelets, unstained, (700 X).

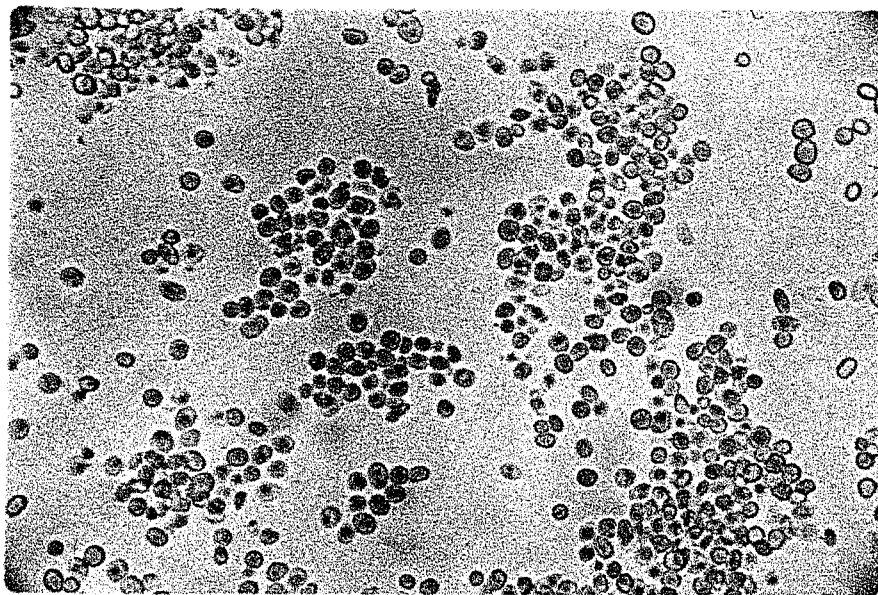


Figure 5. Photographs of Artemia Yolk Platelet Preparations.
(c) Final preparation, yolk platelets, unstained, (400 X).

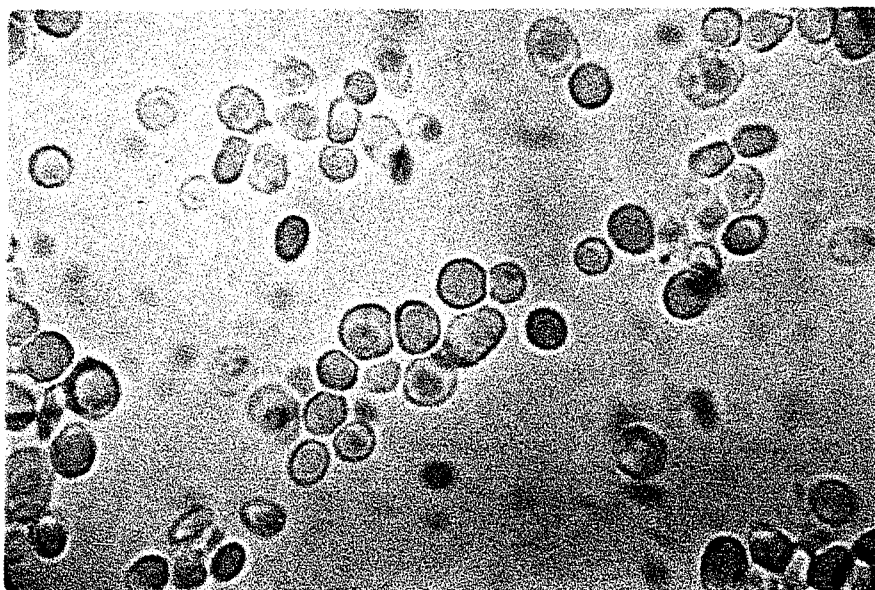


Figure 5. Photographs of Artemia Yolk Platelet Preparations.
(d) Final preparation, yolk platelets, unstained, (1000 X).

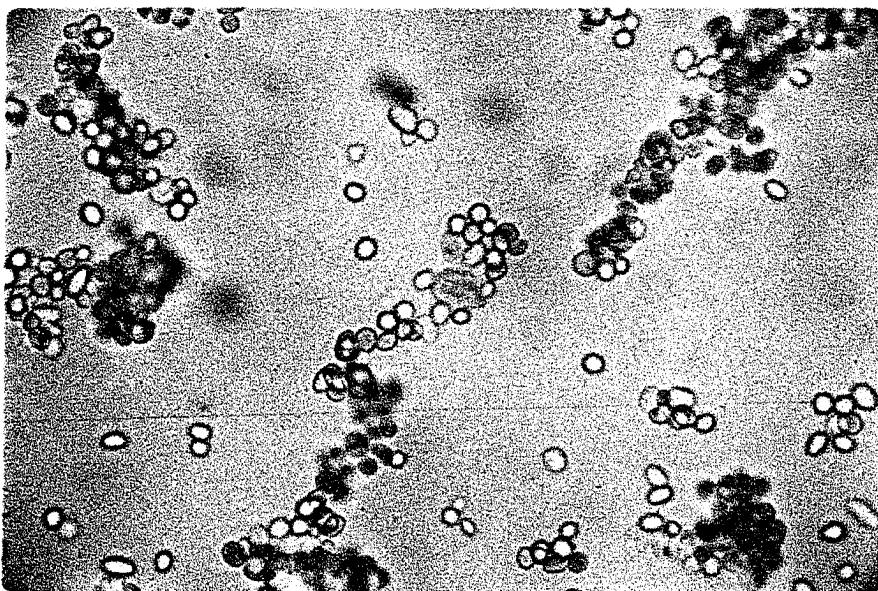


Figure 5. Photographs of Artemia Yolk Platelet Preparations.
(e) Yolk platelets with nucleus, methyl green stain, (400 X).

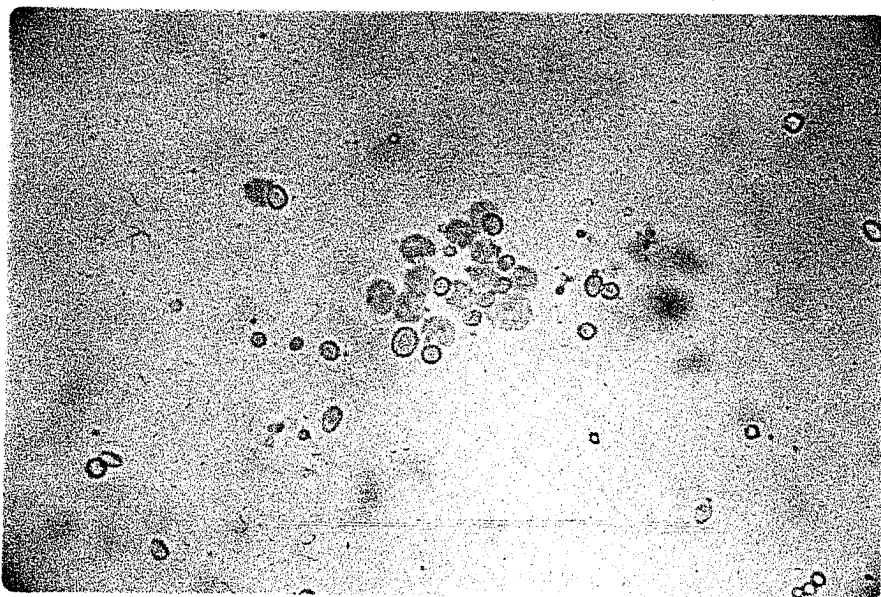


Figure 5. Photographs of Artemia Yolk Platelet Preparations.
(f) Crude 700 g pellet, nuclei and yolk platelets, methyl green stain, (400 X).

Chemical Composition of Purified Artemia Yolk Platelets

Purified yolk platelets were fractionated chemically according to Procedures 1, 2, 3 and 4 and found to be composed of the following components: 74.1 % protein, 8.1 % lipid, 4.0 % free carbohydrate, 3.3 % nucleotide, 0.11% nucleic acid and 3.5 % insoluble material (residue). These data are shown in Table II. It should be noted that Procedures 1 and 2 yield slightly different values for total lipid and protein content, but the reasons for the differences are, as yet, unknown. The nucleotide content was determined using Procedure 3 and the nucleic acid by Procedure 4.

A spectrophotometric analysis of the ethanol-ether soluble fraction is presented in Figure 6. The absorption peak is at 478 nm. and the solution gives a deep orange color. The visible spectrum of this extract corresponds closely to the spectrum for carotenoids isolated from intact Artemia embryos (Dutrieu, 1960).

The free carbohydrate components, analysed by ascending paper chromatography, were found to exhibit two spots with Rf values of 0.138 and 0.279. Standards of trehalose and glucose gave Rf values of 0.185 and 0.285, respectively. The quantity of trehalose, as indicated by the degree of reduction of the silver nitrate, was considerably greater than that of the glucose.

Localization of Diguanosine Nucleotides in Artemia Yolk Platelets

Cell fractionation experiments of Artemia cysts have indicated that about 93 % of the total diguanosine nucleotide fraction (GP₄G and GP₃G) is found associated with the 700 g sediment and that the particle-bound diguanosine nucleotides are associated with the purified yolk platelets. In the crude preparation, the 700 g sediment contains cyst debris, nuclei and yolk platelets, however, the nucleotide composition

TABLE II

Analysis of the Major Chemical Components of Artemia Yolk Platelets^a

Component	Per Cent of Dry Weight			Amphibian Yolk Platelets ^b
	Experiment 1	Experiment 2	Average	
Protein	78.0	70.2	74.1	78
Lipid	8.5	7.7	8.1	14
Carbohydrate	4.0	4.0	4.0	2.0
Nucleotide	3.3	3.3	3.3	---
Nucleic Acid				
DNA	0.1	0.1	0.1	0.10
RNA	0.01	0.01	0.01	0.11
Residue	4.4	2.0	3.2	---
Total	98.2	87.8	92.8	94.2

^aProcedure 1 was used in Experiment 1 for determination of protein, lipid and carbohydrate content. Procedure 2 was used in Experiment 2 to measure the protein and lipid content, whereas the carbohydrate content in Experiment 2 was determined using Procedure 1. In both experiment the nucleotide content was determined using Procedure 3, and the nucleic acid content was determined using Procedure 4.

^bIsolated from Rana pipiens egg (Wallace, 1963).

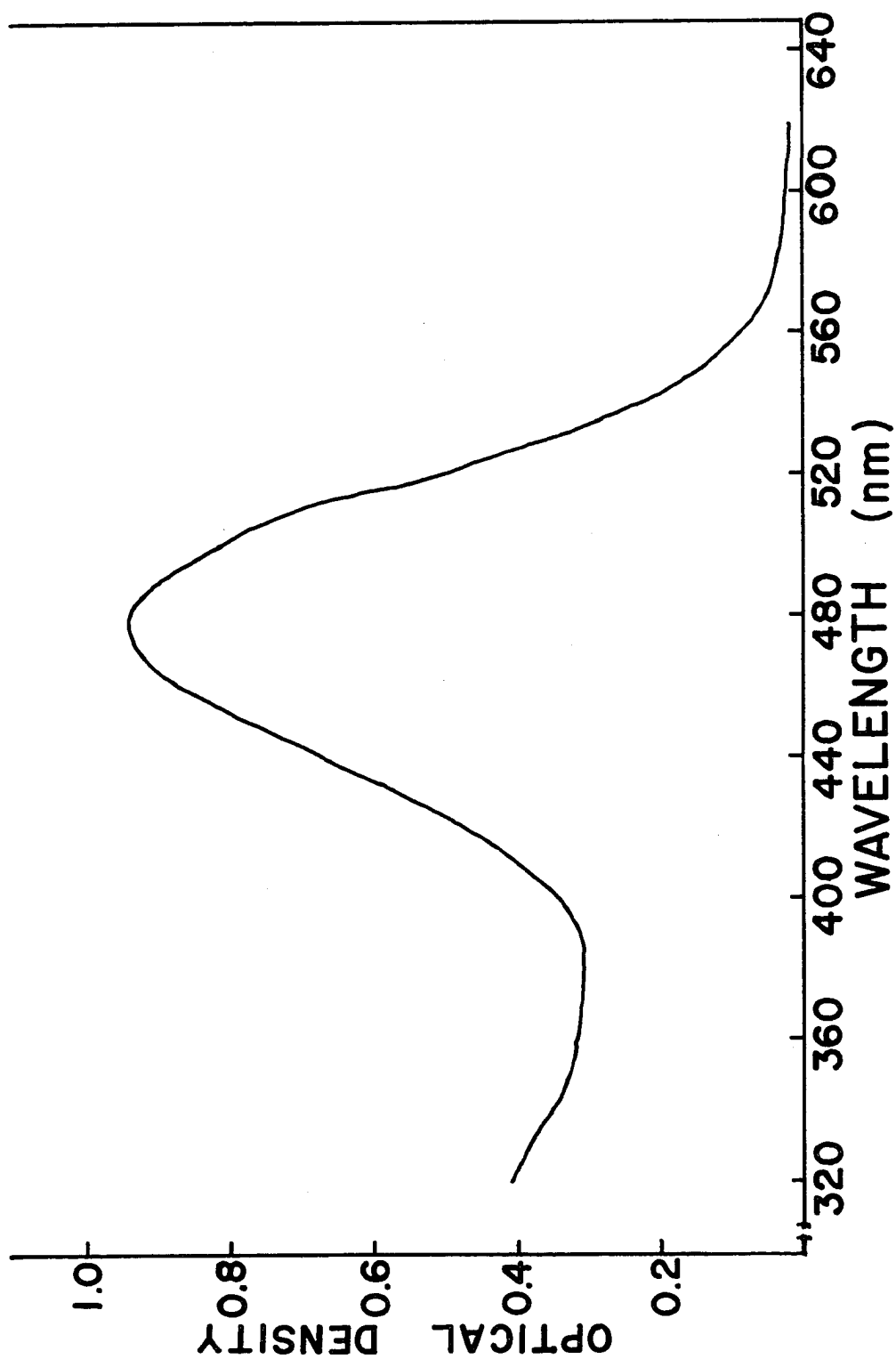


Figure 6. Absorption Spectrum of Artemia Yolk Platelet Ethanol-Ether Soluble Fraction.

of this fraction is very similiar to highly purified yolk platelets. This is apparent when the data in Tables III and IV are compared.

Isolation and Characterization of Artemia Yolk Platelet Protein

When yolk platelets from Artemia cysts are extracted with 1.0 M NaCl containing 0.025 M EDTA, pH 5.0, then percolated through a column of Sepharose 6B, three distinct fractions are obtained. The results of this experiment appear in Figure 7. From the light absorption characteristics of each fraction, it appears that the second peak is a lipoprotein species, whereas peak 1 contains the nucleic acids and peak 3 the nucleotide components. The material absorbing at 480 nm. in peak 2 is extractable with ether and has a spectrum similiar to the ethanol-ether extract of whole yolk platelets. Molecular weight determinations were performed on peak 2 and on the 66 % $(\text{NH}_4)_2\text{SO}_4$ insoluble material prepared from the NaCl-EDTA extracts and both fractions indicated a molecular weight of 220,000 for the lipoprotein. In addition, the phosphoprotein phosphorous content of isolated yolk platelets was found to be only 0.003 % of the total extractable yolk platelet protein.

Stability of Artemia Yolk Platelets in Various Media

The stability of Artemia yolk platelets in various media was monitored by measuring the quantity of ultraviolet-absorbing material at 260 nm in the dialysate. The results of this experiment are described in Figure 8. In the presence of high NaCl concentrations (0.1 M to 1.0 M) a considerable amount of UV-absorbing material is lost from the dialysis bag within 3 days. In the H_2O , 0.25 M sucrose and 20 % glycerol media, some material is lost during the first day, but the rate of loss thereafter is low and appears to reflect a decrease in the particle concentration with ageing. The amount of UV-absorbing material lost from the dialysis bags in the NaCl treated samples is similiar to the total

TABLE III

Effect of Ageing Artemia Yolk Platelets on Nucleotide Composition^a

Compound	Per Cent of Total Nucleotide Content		
	Fresh Preparation	Day 1	Day 12
GMP	8.9	4.4	4.6
GDP	9.2	4.4	4.9
GTP	6.3	2.4	7.7
GP ₃ G	4.4	3.5	3.8
GP ₄ G	71.0	85.1	78.8
Total	99.8	99.8	99.8

^a Purified yolk platelet preparations were aged in distilled water at 0-5° C. and the acid soluble nucleotides were fractionated on columns of DEAE-cellulose as described in **Methods and Materials**.

TABLE IV

Nucleotide Content in Artemia Cell Fractions

Compound	Per Cent Composition	
	700 g Sediment	700 g Supernatant
AMP	0	11.0
GMP	7.7	23.0
AMP-X	0	9.3
GDP	6.2	5.6
ADP	0	2.8
ATP	0	1.0
GTP	2.1	1.8
GP ₃ G	7.1	2.0
GP ₄ G	70.0	4.2
Wash	6.9	44.0
Total	100.0	100.0

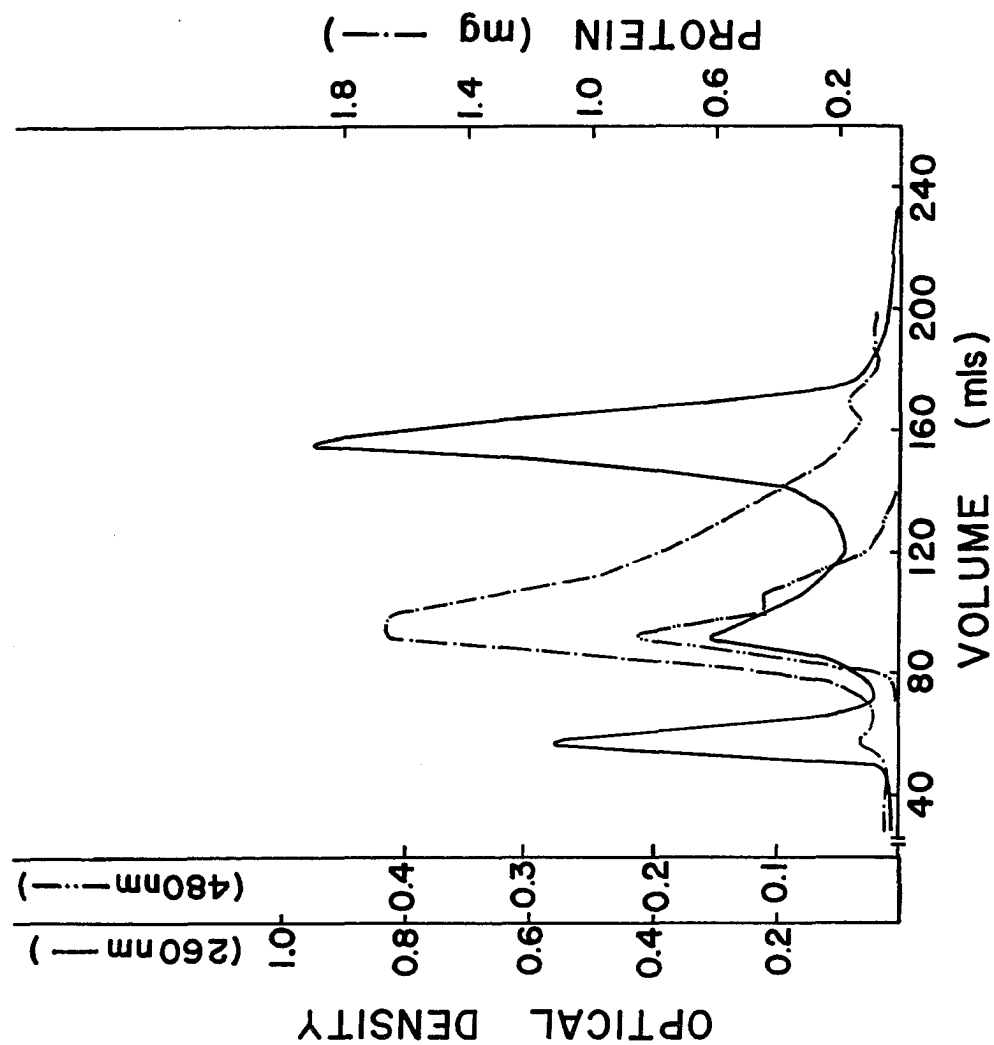


Figure 7. Fractionation on Sepharose 6B column of 1.0 M NaCl-0.025 M EDTA, pH 5.0, Extract of Artemia Yolk Platelets.

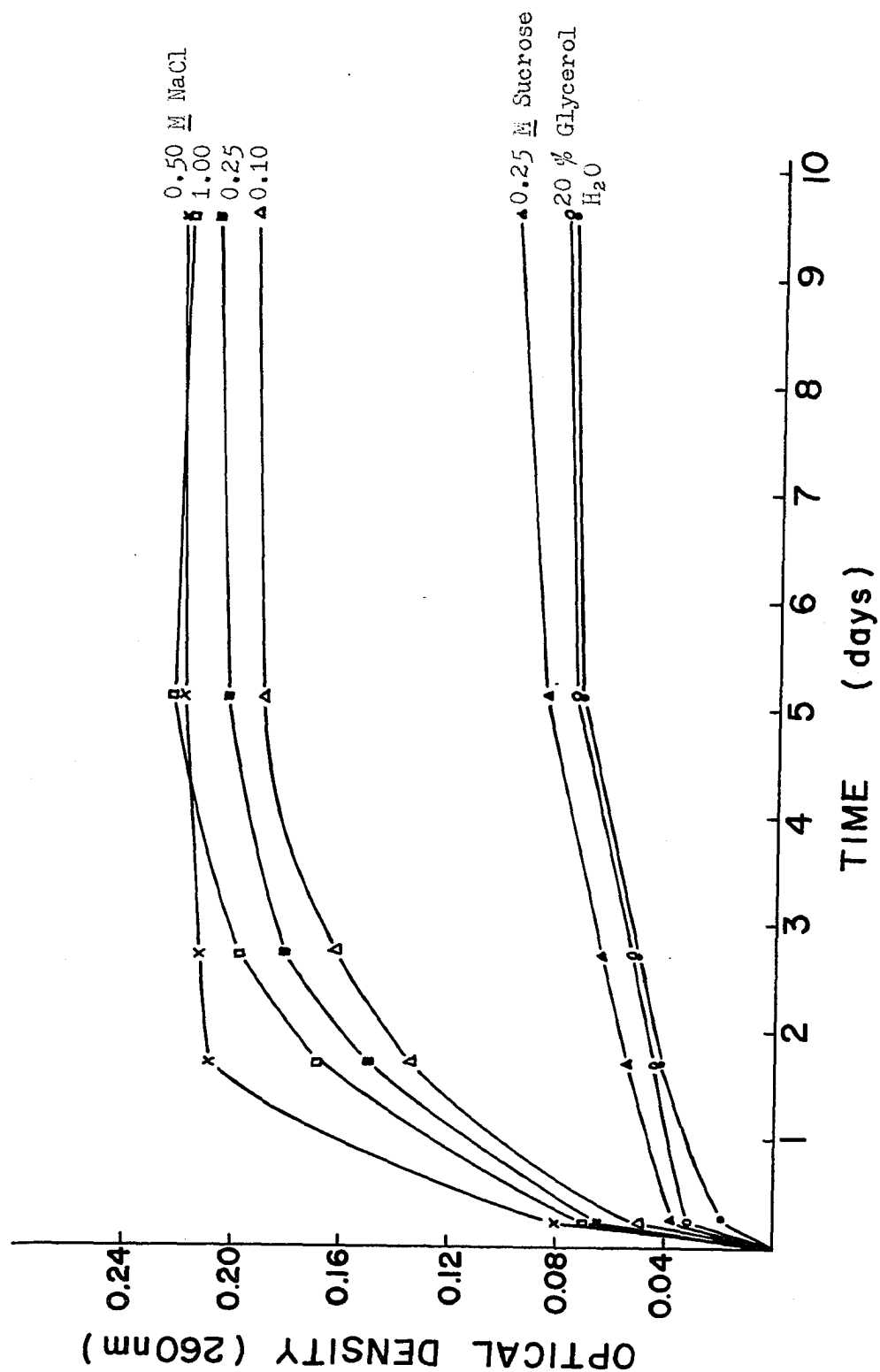


Figure 8. Stability of Artemia Yolk Platelets in Various Media.

acid-soluble nucleotide content present in the yolk platelets. However, a test for protein on day 3 of the ageing experiment revealed an increase of protein material in the dialysate related to the increased salt concentration.

Microscopic examination of the yolk platelets during the ageing process in various media, revealed a decrease in size and an increase in tendency to clump and lyse rapidly. When the nucleotide composition was determined on yolk platelets aged in H₂O for various periods of time, the data in Table IV were obtained. Although the nucleotide content decreases upon ageing, the per cent GP₄G increases slightly, whereas the other nucleotides decrease. Also, it was noted that aged preparations contain less non-identifiable UV-absorbing material than the unaged preparation.

DISCUSSION

A prerequisite for the study of any subcellular particle is a method of isolation that provides appreciable quantities of structurally intact and physiologically active particles free of undesirable contamination. In an earlier study on amphibian (Rana pipiens) eggs, Ringle and Gross (1962) isolated yolk platelets using 0.1 M NaCl, however, Wallace and Karasaki (1963) noted that the procedure failed to preserve the peripheral layers of the yolk platelets. In a similar study using R. pipiens eggs, Wallace and Karasaki (1963) were successful in isolating intact yolk platelets using a mixture of 0.25 M sucrose and 5 % polyvinylpyrrolidinone (PVP).

In the present study it was determined that yolk platelets from encysted embryos of the brine shrimp, Artemia salina, could easily be isolated using a 0.25 M sucrose solution containing a detergent, Tween 80 (0.1 %). Stirring the cysts in the sucrose-detergent solution with a magnetic stirring device permitted gentle rupture of the cysts and the cyst debris could easily be removed by filtering through a cheesecloth-glass wool-cheesecloth filter pad. Large intact nuclei are clearly visible in the crude filtered preparation.

The photographs of isolated yolk platelets and nuclei indicate that a range of sizes exist within both species of particles. Upon aging the yolk platelets become spherical, decrease in size and tend to clump. In addition, the nuclei tend to clump upon application of the methyl green stain.

Yolk platelets were also isolated by a non-aqueous procedure using mixtures of cyclohexane and carbon-tetrachloride to vary the specific gravity. The yolk platelets were isolated at specific gravity 1.26.

However, morphologically the platelets appeared to be broken, due possibly to the extraction of lipid from the yolk platelet membranes. This approach was abandoned because of the problems involved with preparing intact particles.

In 1963 Wallace and Karasaki analysed purified, intact yolk platelets from R. pipiens for their major components and their results are included in Table II for comparison with the Artemia data. It is apparent that although the protein, carbohydrate, and nucleic acid contents in amphibian yolk platelets are similar to that for Artemia, the amphibian lipid content is higher and they did not measure any free nucleotides. It is known, however, that R. pipiens eggs contain no measurable amounts of diguanosine nucleotides (Warner and Finamore, 1963) and therefore, it would be unreasonable to expect any in their yolk platelets.

The major component of Artemia yolk platelets is protein, however the exact amount measured depends upon the method of chemical fractionation. When compared to the protein content for amphibian eggs, the results are comparable although the methods of isolation and estimation were different.

In 1960 Dutrieu reported that carotenoids are present in the egg of Artemia. Krinsky (1965) identified the carotenoids in Artemia to be canthaxanthin (4,4'-diketo- β -carotene) and echinenone (4-keto- β -carotene) and present in the ratio of 19:1. Since the visible absorption spectrum of the ethanol-ether extract from Artemia yolk platelets is similar to the spectra of these pigments, it appears that Artemia yolk platelets contain canthaxanthin and possibly echinenone and may be the primary sites of storage for these compounds. The function of these carotenoids in Artemia development and the role of the yolk platelets in carotenoid metabolism remain to be investigated.

In most studies on yolk platelets little attention beyond quantitative examination is usually paid to the nature of the carbohydrate(s) in the yolk platelets. Clegg (1962, 1964) reported that dormant blastulae of Artemia contain 15 % by weight trehalose and very little glucose and glycogen. Trehalose is a non-reducing disaccharide of glucose and appears to be the major carbohydrate component of Artemia yolk platelets. However, only a small portion of the total cyst trehalose is sequestered in the yolk platelet. Trehalose, which is synthesized in the embryo and not in the maternal tissue, accumulates only in those Artemia embryos destined to enter dormancy. Therefore, it may be that yolk platelets of embryos not undergoing encystment would differ in both trehalose and total carbohydrate content. Glucose was also found in the Artemia platelets but since it may be a byproduct of acid hydrolysis of trehalose, its existence in vivo remains questionable.

In the preliminary cell fractionation experiments it was consistently observed that most of the diguanosine nucleotides were present in the pellet (93 %) with only a small portion in the supernatant (7 %). Throughout the purification procedure used it was observed that the nucleotide composition did not change appreciably. It was concluded that GP₄G is an intrinsic component of Artemia yolk platelets and not merely adsorbed to the surface of the platelets. Although these data may appear equivocal in some respects, the stability experiments also support these conclusions. The large quantity of diguanosine nucleotides in the yolk platelets compared to the amount found in the 700 g supernatant fraction suggests that the yolk platelets are the site of storage or compartmentalization of these unique nucleotide anhydrides. Finamore and Clegg (1969) reported that although the acid-soluble guanine-containing

nucleotides appear to be the source of adenine-containing nucleotides and the precursors of RNA, GP₄G is the sole source of DNA adenine. Their data clearly indicate that separate nucleotide pools exist for RNA and DNA synthesis and suggest that some kind of compartmentalization exists within the egg. The observation that diguancosine tetraphosphate is localized primarily in the yolk platelets supports this hypothesis. If the GP₄G is converted to dATP for DNA synthesis, questions arise as to the mode of transportation of GP₄G from the yolk platelets to the nucleus and their subsequent conversion to dATP in the nucleus. Such questions warrant further investigation of the role played by the yolk platelet in metabolism of GP₄G during the development of Artemia embryos.

Some differences exist between lipoprotein isolated from decapod egg (Wallace et al., 1967) and the Artemia lipoprotein observed in this thesis. In the decapods, the lipoprotein may be purple, blue or green, whereas in the branchiopod, Artemia salina, it is orange. Similiar to other crustacea, Artemia lipoprotein is soluble in 0.5 M NaCl containing 0.005 M EDTA, pH 5.0, but a higher ionic strength solution (about 1.0 u) was used with Artemia because more protein was found soluble at the higher strength. The EDTA concentration was also found to be important. At a low concentration (0.005 M) considerable UV-absorbing material binds tightly to Sepharose 6B columns, whereas this problem is circumvented by using higher concentrations of EDTA (0.25 M). The reason(s) for this difference is unknown at the present time. Using the most favorable extraction and filtration medium, it was observed that the molecular weight of the lipoprotein is 220,000 for Artemia compared to 330,000 - 370,000 for other crustacea (Wallace et al., 1967). In either case, the carotenoids are not covalently bound to the protein and could be easily removed by ether extraction. The lipid moiety is believed to reside in

hydrophobic pockets of the native protein(Wallace et al., 1967).

When the phosphoprotein phosphorous content of Artemia platelets was determined, it was found to be very low (0.003 % by weight of the extractable protein) compared to the levels found in amphibian yolk platelets (8.4 %). The function of the lipoprotein in Artemia is uncertain, but in the amphibia, it binds with two phosphovitin molecules to form a complex which is stored in the yolk platelet.

The role played by the yolk platelets in the developing embryo is usually one of storage. In Artemia the yolk platelets are unique in that they contain large quantities of nucleotides, particularly GP₄G, in addition to protein, lipid and carbohydrate. The role of Artemia yolk platelets in development remains to be elucidated but it appears that they may play an active role in diguanosine nucleotide development. It is hoped that this thesis has carried out some of the preliminary ground work necessary for future investigations into the role of the yolk platelet in Artemia development.

SUMMARY

1. Yolk platelets from encysted embryos of the brine shrimp, Artemia salina, have been isolated and purified extensively. In the most homogeneous preparations nuclear contamination is about 0.003 %.
2. The purified yolk platelets have been found to have the following composition: protein(74.1 %), lipid (8.1 %), free carbohydrate (4.0 %), nucleotides (3.3%), DNA (0.11 %) and RNA (0.01 %).
3. The ethanol-ether soluble fraction contains carotenoids similar in nature to canthaxanthin and echinenone.
4. The carbohydrate fraction appears to be mainly trehalose, however, small amounts of glucose have been detected.
5. The nucleotide composition of yolk platelets is virtually all guanine-containing compounds and GP₄G comprises about 70 % of the total nucleotide fraction in purified preparations. In addition, most of the embryo GP₄G is found in the yolk platelets (98 %) compared to the non-particulate fraction (2 %).
6. The major protein appears to be a lipoprotein and has a molecular weight of 2.2×10^5 .
7. The yolk platelets are most stable in 0.25 M sucrose, 20 % glycerol and H₂O and least stable in dilute (0.1 M NaCl) salt solution and ageing in H₂O produces little change in the overall nucleotide composition although the quantity of nucleotides decreases.

APPENDIX A

Deacidification of acid-soluble extracts of yolk platelet preparations was performed by a method similiar to that previously described (Warner and Finamore, 1966). To the perchloric acid extract was added an equal volume of N alamine in chloroform (Alamine 336 s, General Mills Inc., Kankakee, Illinois) and the mixture inverted slowly several times until the aqueous phase tested neutral or slightly basic with universal indicator paper. The phases were separated by mild centrifugation and the aqueous layer retained. The organic layer was washed once with H₂O and the wash was added to the deacidified aqueous layer. The combined aqueous fractions were stored at -20° C. until needed.

APPENDIX B

Sephacrose 6B gel, previously equilibrated with 1.0 M NaCl containing 0.025 M EDTA, pH 5.0, was poured into a column (2.0 X 56 cm) whose inner surface had been rendered hydrophobic by treatment with dimethyldichlorosilane solution. The gravity packed column was then washed with additional 1.0 M NaCl containing 0.025 M EDTA, pH 5.0. The sample was applied to the column and the material eluted at the rate of 30 mls per hour with the same NaCl-EDTA solution.

Once the column was prepared, molecular weight determination of yolk lipoprotein was carried out in the following manner. The void volume of the column was determined by eluting a freshly prepared solution of Blue Dextran 2000 (1.0 mg per ml) and monitoring it at 280 nm. The column was calibrated by eluting aldolase and chymotrypsinogen A in one run and ovalbumin and ribonuclease in a second run. Finally, the NaCl-EDTA extract of purified platelets was run under the same conditions

The elution volumes (V_e) for the standards were determined using the following equation.

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

V_e : elution volume for the protein
 V_o : elution volume for Blue Dextran
 V_t : total bed volume of the column

The selectivity curve was prepared by plotting the K_{av} values for each protein on the linear scale and the molecular weight on the logarithmic scale of semi-logarithmic graph paper. From the best fitting straight line the molecular weight of

the unknown was calculated by plotting its K_{av} and extrapolating a perpendicular line to the molecular weight scale. These procedures are similiar to those suggested by Pharmacia for determining molecular weights using Sephadex gels.

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