FREE GLYCEROL IN DORMANT CYSTS OF THE BRINE SHRIMP ARTEMIA SALINA, AND ITS DISAPPEARANCE DURING DEVELOPMENT [1]

JAMES S. CLEGG [2]

Department of Biology, The Johns Hopkins University, Baltimore 18, Maryland

During part of a previous study on the stored carbohydrates of various dormant organisms (Clegg and Filosa, 1961), large amounts of a carbohydrate-like substance were observed in extracts of *Artemia salina* cysts. On the basis of mobility and reactivity on paper chromatograms, this substance appeared to be glycerol. Since large amounts of free glycerol have been shown to accumulate during diapause, and in other hypometabolic stages of certain insects (*cf.* Salt, 1961), a more thorough analysis was undertaken. In addition, although several studies have been made of the chemical components of these cysts, no mention has been made of glycerol (the extensive literature on *Artemia* has recently been cited by Dutrieu, 1960). The present report deals chiefly with the identification of free glycerol in *Artemia* cysts and the changes in its concentration during development. A preliminary report on the presence of glycerol in *Artemia* cysts has been published (Clegg and Evans, 1962).

Materials and Methods

Dried cysts of *Artemia,* which are embryos in the early stages of development covered by a chitinous shell (Dutrieu, 1960), were obtained as a gift from the Brine Shrimp Sales Co., Inc., Hayward, California. Unless designated otherwise, the cysts used were collected in the fall of 1960 from the evaporating ponds near Hayward, and analyses of these cysts were begun in the summer of 1961. They were washed briefly with distilled water to remove any empty shells, and were then dried at room temperature for at least twenty days before use. Over 70% of these cysts produced active nauplii when incubated in sea water at 24–26° C.

For the isolation of glycerol, about 5 g. of cysts were homogenized in a Ten Broeck homogenizer with 30 ml. of 80% ethanol. The homogenate was filtered and the filtrate decolorized with Norit (1% w/v). After removal of the Norit, the clear filtrate was concentrated under reduced pressure and then extracted with benzene. The organic phase was discarded and the remaining solution was purified by paper chromatography (Evans and Dethier, 1957). The combined eluates from the chromatographic separation were concentrated by evaporation at 50° C., and then dried over $CaCl_2$ to a viscous syrup (about 300 mg.) which then was used for the identification studies.

For quantification of glycerol, 40–80 mg. of cysts were homogenized in 1.0 ml. of 80% ethanol; the homogenate was transferred to a graduated centrifuge tube

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[2] Present address: Department of Zoology, University of Miami, Coral Gables 46, Florida.

with four 1-ml. washings, and the volume was made up to 5.0 ml. with distilled water. After centrifuging for 15 minutes at 3000 rpm., aliquots were taken for determination of glycerol in the supernatant by the colorimetric method of Lambert and Neish (1950) as modified by Burton (1957). In preliminary experiments, the glycerol was first isolated and identified by paper chromatography and then eluted for quantification. It was found later that results obtained by this method differed from direct determinations on the supernatant by not more than 3%. As a result, direct colorimetric determinations were carried out on the supernatant.

Trehalose was determined by the anthrone method of Dimler *et al.* (1952) after its isolation from the supernatant by paper chromatography (Clegg and Evans, 1961). The pellet from the ethanol extraction was analyzed for polysaccharide by re-homogenizing in 5.0 ml. of 5% trichloroacetic acid, centrifuging, and using an aliquot of the supernatant for the anthrone method of Dimler *et al.* (1952). Results obtained by this procedure were similar to those obtained by conventional alkali extraction and alcohol precipitation methods. This material, when hydrolyzed by acid, yielded only glucose, as judged by paper chromatography, and will be referred to as glycogen in the present study.

To obtain the nauplii, a known amount of cysts (40–80 mg.) was incubated in a Petri dish containing filtered sea water at 24–26° C. In all cases, the nauplii were collected within three hours of emergence from the cysts and were separated from the mixture of developing cysts and shells by virtue of the fact that the nauplii were positively phototactic while the cysts and shells floated on the surface. The nauplii were pipetted from the medium, filtered, and washed with distilled water. They were then dried to constant weight and analyzed by the same methods given for the cysts. The empty shells were collected and analyzed after 96 hours of incubation. Average weights of the cysts, shells, and nauplii were obtained by placing 50 to 100 individuals on a pre-weighed coverslip, drying to constant weight, and re-weighing on a Mettler Micro Balance (sensitive to about 1 μg).

Results and Discussion

Identification of glycerol

The substance in question migrated on paper chromatograms with authentic glycerol in the following solvent systems (v/v): (1) water-saturated ethyl acetate; (2) n-butanol, ethanol, acetone, water (5:4:3:2); (3) chloroform, ethanol (8:2); (4) ethyl acetate, ethanol, water (12:2:1); and (5) n-propanol, ethyl acetate, water (7:1:2). When mixtures of the substance and authentic glycerol were chromatographed, no separation was observed in these solvent systems. Positive identification of the substance as glycerol was established by preparation of the tribenzoate derivative (Segur, 1953). The product, recrystallized from 90% ethanol, had a melting point of 71–72.5° C. The tribenzoate prepared from authentic glycerol had a m.p. of 71.5–72.5° C., and the mixed m.p. was 72–73° C.

Levels of glycerol and glycogen in the cysts and nauplii

Dutrieu (1960) has shown that net glycogen synthesis occurs in *Artemia* during the transition from the dormant cyst to the active nauplius. Net glycogen synthesis also occurs after diapause is broken in the eggs of the silkworm, *Bombyx mori* (Chino, 1957), and glycerol and sorbitol were shown to be its precursors (Chino,

TABLE I

Glycerol and glycogen content of the cysts, and nauplii of newly emerged Artemia

Stage	Per cent of the dry weight			
	Glycerol		Glycogen	
	Mean ± S.E.	No.	Mean ± S.E.	No.
Cyst	4.91 ± 0.42	(9)	1.13 ± 0.09	(8)
Shell	0.19	(3)	0.04	(3)
Embryo	6.30* ± 0.48	(9)	1.86* ± 0.14	(8)
Newly emerged nauplius	4.85 ± 0.21	(13)	15.1 ± 0.2	(6)

* 1 mg. cysts = 0.78 mg. embryo (Table II); 4.91 ÷ 0.78 = 6.30% glycerol of the embryo dry weight.

1958). Therefore, studies were undertaken to determine whether or not glycerol was converted to glycogen following the termination of dormancy in *Artemia*. Sorbitol, incidentally, was not found in these cysts (limit of detection = 0.2% of the dry weight).

Table I summarizes the results obtained by incubating cysts in 2% NaCl. Glycerol was present in the dried cysts before incubation to the extent of about 5% of the dry weight and, on the basis of cyst dry weight, no decrease was measured during the transition from cyst to nauplius. Similar values were obtained by homogenizing the cysts in distilled water at 0–4° C. This indicated rather strongly that the amount of glycerol found was present as free glycerol in the cyst. The small values given for the shells are maximal since a few undeveloped cysts might also be present in the shell fraction. In addition, it should be pointed out that the assay system used is not wholly specific for glycerol, so these low values may not be glycerol at all. In any event, it was clear that most, if not all, of the glycerol was confined to the embryo. In order to compare the glycerol levels in the embryo with those in the nauplius it was first necessary to estimate the weight of the embryo. This was so because the shell, constituting a large percentage of the cyst weight, was shed when the nauplius emerged, and would not be used as a basis for estimating glycerol levels in the nauplius. This information, given in Table II, indicated that the embryo constituted about 78% of the cyst dry weight. This figure was then used to calculate the concentration of glycerol in the embryo, shown in Table I as over 6% of the dry

TABLE II

Dry weights of the cyst, shell, and nauplius

Stage	Mean weight		Per cent of the cyst weight
	μg. ± S.E.	No.	
Cyst	2.55 ± 0.03	(7)	100
Shell	0.57 ± 0.01	(7)	22
Embryo	1.98 ± 0.03	(7)	78
Newly emerged nauplius	1.93 ± 0.16	(8)	—

weight. Even on this basis the glycerol content decreased by only about 1.5% of the dry weight during the formation of the nauplius. At the same time, glycogen levels increased by about 14% of the weight, on the basis of cyst and embryo dry weight, as shown in Table I. Clearly, the small decrease observed in the glycerol content could not account for the amount of glycogen synthesized. Therefore, the source of most of this glycogen, unlike *Bombyx* eggs, was not glycerol. It would appear from the study of Dutrieu (1960) that trehalose, a non-reducing disaccharide of glucose,

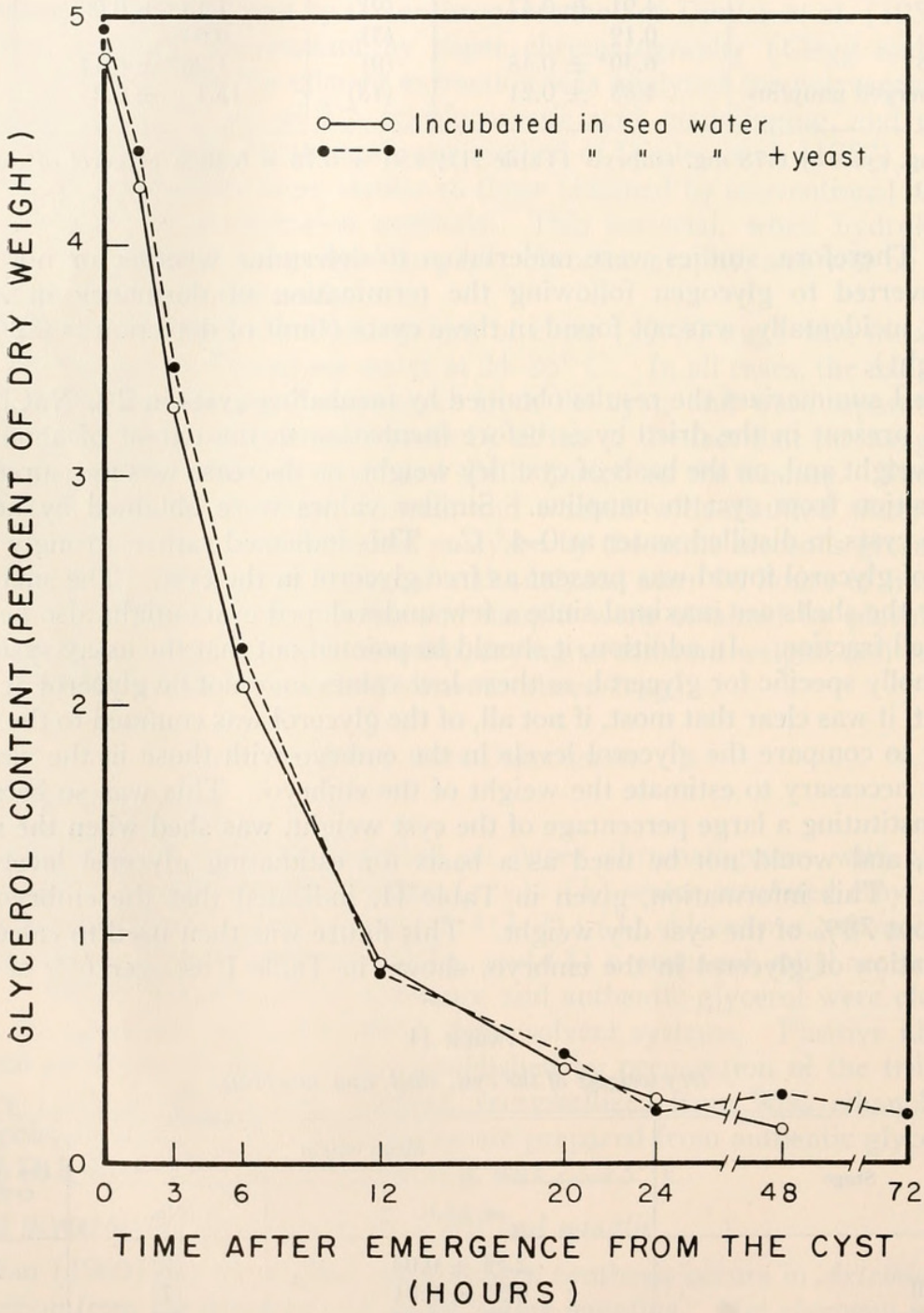

FIGURE 1. Glycerol content of fed (•) and unfed (o) nauplii as a function of time after emergence from the cyst.

might be the chief substrate for the glycogen synthesis observed during the development of *Artemia*. This aspect will be considered in a future publication. The values given in Table I for glycogen concentrations in the nauplius are more than twice those reported by Dutrieu (1960). There are several obvious possible explanations for this difference.

The results given above indicated that glycerol was not being used either as an important source of energy during development or as a major substrate for glycogen synthesis. Accordingly, the fate of glycerol in the nauplius was examined.

Glycerol levels as a function of nauplius age

A large number of newly emerged nauplii was collected as described and divided into two groups. One group was incubated in filtered sea water and the other in sea water containing 1 mg. of dried yeast per ml. as a source of food. After various periods of incubation the nauplii were analyzed for glycerol content. The averaged results of three separate experiments are given in Figure 1. The amount of glycerol in the nauplii decreased sharply during the first 24 hours of incubation and then remained at a very low, constant level. These latter values are probably due to the presence of small amounts of non-glycerol substances that produce color with the reagents, since glycerol could not be detected in 72-hour-old nauplii when these extracts were analyzed by paper chromatography (limit of detection = 0.2% of the dry weight). Since the rapid decrease in glycerol content was observed in fed and unfed nauplii it seems that glycerol disappearance is not influenced by nutrition. Comparisons of the glycogen content of these two groups of nauplii were not made since, in the case of those incubated with yeast, the amount of glycogen present in the gut lumen, due to the presence of ingested yeast, was uncontrollable. Consequently, it is not known whether the decrease in glycerol is accompanied by an increase in glycogen. Because the nauplii are so small, attempts have not yet been made to follow the metabolic fate of injected radioactive glycerol. The present results do show, however, that glycerol essentially disappears during the first day following emergence from the cyst.

Glycerol, trehalose, and glycogen contents of aged cysts

Next, the effect of source, age, and storage condition on the carbohydrate composition of the cyst was examined. These aged cysts, and a brief resumé of their history, were generously supplied by Mr. Maurice Rakowicz of Brine Shrimp Sales Co., Inc., Hayward, California. At least 200 mg. of cysts from each group were analyzed for trehalose, glycerol, and glycogen content by the methods described above. Dutrieu (1960) has shown that trehalose and small amounts of glucose are the main alcohol-soluble sugars present in *Artemia* cysts and this has been confirmed in the present study. The per cent hatch was obtained by incubating at least 500 cysts from each of the groups for 72 hours in sea water at 24–26° C., and then counting the number of viable nauplii produced. The results, summarized in Table III, showed that the trehalose content of these several groups was quite constant, whereas the glycogen and glycerol contents showed considerable variation. The most striking difference between these groups was the per cent hatch, none of the cysts producing viable nauplii in the 1938 group. Noteworthy was the increased viability of those cysts stored *in vacuo* compared with those stored in air since 1951.

TABLE III

Glycerol, glycogen, and trehalose contents of aged cysts

Origin of cysts and date collected	Storage	Per cent of the dry weight			Average % hatch
		Glycerol	Trehalose	Glycogen	
San Francisco, 1961	air	4.91	14.27	1.13	73
San Francisco, 1951	air	2.48	16.49	1.18	5
San Francisco, 1951	vacuum	2.49	17.29	1.65	62
San Francisco, 1938	air	2.45	14.68	1.05	0
Great Salt Lake (Utah), 1951	air	4.73	15.09	2.67	4

The fact that cyst viability greatly decreased with aging in air, while the trehalose and glycogen content did not appear to diminish appreciably, suggests that a source of energy is not the limiting factor determining viability during aging for long periods. These findings also emphasize the "metabolic dormancy" of these cysts, at least with respect to carbohydrate metabolism. For the present, however, the main conclusion derived from these results was that trehalose, glycogen, and glycerol are the normal and principal carbohydrates of dormant *Artemia* embryos. A detailed study is presently being made to determine the origin of glycerol in the embryo, the metabolic fate of glycerol in the nauplius, and the role of glycerol and trehalose in the dormancy of *Artemia* cysts.

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SUMMARY

1. Free glycerol was identified as a major carbohydrate component of the dormant cysts of *Artemia salina.*

2. The amount of glycerol present in cysts aged for a year in the dry state was found to be about 5% of the cyst weight, and was shown to be restricted to the embryonic part of the cyst.

3. Glycerol content decreased slightly during the formation of the nauplius and then rapidly decreased to a very low level after the nauplius emerged from the cyst. The decrease in glycerol content could not account for the synthesis of glycogen during formation of the nauplius.

4. The glycerol, trehalose, and glycogen contents, and the viability of cysts aged up to 28 years were determined.

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