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Studies of carbohydrates in eggs of Aulocara elliotti (Thomas) (Orthoptera, Acrididae) in relation to development, temperature, maternal age and crowding
by Kenneth Leon Quickenden

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Entomology
Montana State University
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Abstract:
The qualitative and quantitative carbohydrate changes occurring in the egg of the grasshopper species Aulocara elliotti (Thomas) at various stages of development were investigated= Carbohydrate distribution, interconversions and temperature effects were considered. Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified by chromatographic means in 70% ethanolic extracts of eggs = Mannose was detected in early prediapause eggs but was not detected in diapause or post-diapause eggs. As mannose levels declined, mannitol accumulated but the quantity of free mannose was not sufficient to serve as the only precursor for mannitol synthesis. Neither trehalose nor "glycogen" levels declined as mannitol accumulated. Mannitol was temporally associated with diapause. Although mannitol synthesis does not depend on cold exposure, cold might have an elevating effect. Glycerol appeared to be an extremely variable moiety, was not associated with diapause and would not appear to be important to cold-hardiness. There appeared to be slight early and late utilization of glycogen but levels were higher in the definitive egg than in the newly laid egg. Trehalose was generally 91 + 5% of the free neutral sugars in eggs, increased by two to three-fold during diapause and then declined following blastokinesis. Glycogen, trehalose and mannitol distributions in the egg are discussed in relation to membrane permeability and changes occurring at blastokinesis. Radioactive tracing indicated that glucose could be converted to trehalose and mannitol and that, as mannitol disappeared from the eggs, conversion of mannitol to trehalose and "glycogen" was possible even though these two compounds did not accumulate at that time.

The effects of maternal age and crowding on egg weight and trehalose and "glycogen" levels, at a time when embryonic development had not progressed beyond the blastoderm stage, were measured in eggs collected from adults reared at three densities throughout the fecund period. Glycogen levels in these eggs increased with maternal age as did egg weight. Parental density had no noticeable effect on glycogen content. The greater the -density, the greater the amount of trehalose that was partitioned to eggs during the first two-thirds of the reproductive period. During the last half of the fecund period, trehalose decreased from 57.6 to 20.2yμg/egg in eggs obtained from adults reared at the highest density. This is probably due to the combined effect of crowding and maternal age. Maternal age and density effects on trehalose levels partitioned to eggs are discussed in relation to rate of development and a density-stress response mechanism which may be likened to that of vertebrates.

STUDIES OF CARBOHYDRATES IN EGGS OF AULOCARA ELLIOTTI
(THOMAS) (ORTHOPTERA, ACRIDIDAE) IN RELATION TO
DEVELOPMENT, TEMPERATURE, MATERNAL AGE AND CROWDING

by

KENNETH LEON QUICKENDEN

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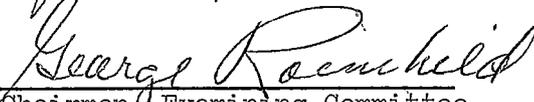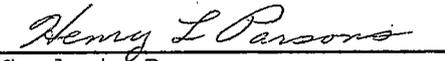fulfillment of the requirements for the degree

of

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ABSTRACT

The qualitative and quantitative carbohydrate changes occurring in the egg of the grasshopper species <u>Aulocara elliotti</u> (Thomas) at various stages of development were investigated. Carbohydrate distribution, interconversions and temperature effects were considered. Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified by chromatographic means in 70 % ethanolic extracts of eggs. Mannose was detected in early prediapause eggs but was not detected in diapause or post-diapause eggs. As mannose levels declined, mannitol accumulated but the quantity of free mannose was not sufficient to serve as the only precursor for mannitol synthesis. Neither trehalose nor "glycogen" levels declined as mannitol accumulated. Mannitol was temporally associated with diapause. Although mannitol synthesis does not depend on cold exposure, cold might have an elevating effect. Glycerol appeared to be an extremely variable moiety, was not associated with diapause and would not appear to be important to cold-hardiness. There appeared to be slight early and late utilization of glycogen but levels were higher in the definitive egg than in the newly laid egg. Trehalose was generally 91 $\pm$ 5 % of the free neutral sugars in eggs, increased by two to three-fold during diapause and then declined following blastokinesis. Glycogen, trehalose and mannitol distributions in the egg are discussed in relation to membrane permeability and changes occurring at blastokinesis. Radioactive tracing indicated that glucose could be converted to trehalose and mannitol and that, as mannitol disappeared from the eggs, conversion of mannitol to trehalose and "glycogen" was possible even though these two compounds did not accumulate at that time.

The effects of maternal age and crowding on egg weight and trehalose and "glycogen" levels, at a time when embryonic development had not progressed beyond the blastoderm stage, were measured in eggs collected from adults reared at three densities throughout the fecund period. Glycogen levels in these eggs increased with maternal age as did egg weight. Parental density had no noticeable effect on glycogen content. The greater the density, the greater the amount of trehalose that was partitioned to eggs during the first two-thirds of the reproductive period. During the last half of the fecund period, trehalose decreased from 57.6 to 20.2 μg/egg in eggs obtained from adults reared at the highest density. This is probably due to the combined effect of crowding and maternal age. Maternal age and density effects on trehalose levels partitioned to eggs are discussed in relation to rate of development and a density-stress response mechanism which may be likened to that of vertebrates.

INTRODUCTION

Biologists are often called upon to explain population fluctuations
in animals. Dempster (1963) indicates that a combination of factors,
external and internal, account for these fluctuations.

Richards (1961) states that the four most likely causes of insect
mortality are external features. These are weather, parasites, predators
and disease. While these external features are important, a reliance
solely upon the external environment has failed to adequately explain
many sudden changes in numbers.

Internal population regulation is a widespread phenomenon in the
animal kingdom. Among the vertebrates it is theorized to operate through
the pituitary-adrenal and/or the pituitary-gonadal axis in response to
stress. Research involving mammals indicates that such a feature occurs
in this class (e.g. Christian, 1950; Christian and Davis, 1964). Hane
et al. (1966) have demonstrated that salmon show an increase in the
levels of plasma 17-hydroxycorticosteroids, adrenal cortex hormones, in
response to stresses of handling and migration. There is also decreased
responsiveness to ACTH, a pituitary hormone, when injected at the end of
migration. Flickenger (1966) recorded changes in gonad size in response
to greater social conflict resulting from larger group size.

The mechanisms by which the internal factors integrate into the
problem of population fluctuations are largely unknown in insects. Many
responses to density and other stresses, however, have been observed.
There are reports that crowding retards fecundity in some Acrididae
(Norris, 1952) and induces changes in ovariole numbers in progeny

(Albrecht, et al. 1958). Maternal stress thus extended to the developing
embryos. O'Brien and Wolfe (1964) state, without giving reference, that
overcrowding is known to transform locusts from the solitary to gregarious
form and influences the rate of yolk deposition in eggs.

Field studies of the grasshopper Aulocara elliotti (Thos.), which is
an economic pest of rangelands (Pfadt, 1949; Anderson and Wright, 1952;
Anderson, 1964), indicate that wide fluctuations in density occur in this
species. Hastings and Pepper (1964) have shown that nymphs of different
populations of A. elliotti vary in their ability to withstand the stresses
of temperature extremes and starvation. One of their suggestions to
explain such population dependent variation is that stresses imposed on
the adult may be transmitted through the developing embryo to the nymph.

In an effort to understand the internal environment of developing
embryos of A. elliotti and to study density or other stress effects, a
multifacited approach was begun at Montana State University. Van Horn
(1963, 1966a, 1966b) studied the embryonic development noting wide varia-
tion with respect to rate of development and numbers of retarded embryos
in different populations. She also found that density may change the
developmental pattern of these embryos. In a population studied in 1966,
Van Horn (1968) found that the density effect was modified by photoperiod
such that the rate of embryonic development became successively greater
for long day-one pair per cage, long day-two pairs per cage, short day-one
pair per cage and short day-two pairs per cage.

There have been two attempts to establish the existence of biochem-

ical differences in eggs of A. elliotti from different populations and differences resulting from density effects. Svoboda (1964) and Svoboda et al. (1966) could detect no differences in lipids nor could Bunde (1965) or Bunde and Pepper (1968) find differences in amino acids of eggs from different populations of A. elliotti which could be attributed to density. Still, changes in the vigor of a population might be reflected in the biochemical constitution of the eggs. Vuillaume (1955) showed that crowded nymphs of the acridid Zonocerus variegatus had a higher fat content than isolated nymphs and Matthée (1945) found that the gregarious phase of several locusts and noctuids contained a higher percentage of fat than did the solitary phase.

There have been a number of studies directed at the embryonic developmental physiology of A. elliotti. Roemhild (1961, 1965a, 1965b, 1967, 1968b) has directed his attention principally toward egg compartmentation and the blastokinesis occurring at diapause termination. He has found that membrane structures divide the egg into compartments containing different physical and chemical properties. He believes that the integrity of these membranes is important to the maintenance of the diapause state and has found that their rupture at blastokinesis exposes the embryo to a very different pH (7.4 to 6.6). Laine (1966) investigated temperature effects on the embryonic oxygen consumption and Leopold (1967) conducted a histochemical study of ovarian development and vitellogenesis.

With the previously noted investigations and with the many hormonal

effects on carbohydrate metabolism observed in insects recently sum-
marized in a review by Wyatt (1967), it is reasonable to expect that a
density or other stress effect might be reflected in the carbohydrates
found in eggs and embryos of A. elliotti. A study of the principal car-
bohydrates would contribute some needed basic information concerning the
internal environment and developmental physiology of this species.

The disaccharide trehalose, which is the main blood sugar of insects,
has been identified in an impressive list of lower plants and inverte-
brates. The importance of this sugar in such physiological events as
molting, reproduction and flight has been well noted. Its occurrence in
a number of life stages is tabulated in a recent biochemical review of
sugars and polysaccharides in insects by Wyatt (1967). Reports of tre-
halose in insect eggs are more restricted but it probably occurs univer-
sally. Clegg and Filosa (1961) simply state that it occurs in eggs of
Aedes aegypti (Diptera). In eggs of the oak silkworm, Antheraea pernyi,
Egorova and Smolin (1962) found 0.026 % of the dry weight is trehalose.
According to Dutrieu (1961b), newly laid eggs of Carausius morosus
(Phasmida) lack trehalose but it then appears and increases from 2.5 to
3 % during the last month of embryonic life.

Glycogen has been reported to be present in oocytes of the orth-
opteran Tachycines (Radecka, 1962) and the dermapteran Anisolabis
(Bonhag, 1956) but absent in oocytes of the cockroach Periplaneta (Kugler
et al., 1956; Bonhag, 1959) and the hemipteran Oncopeltus (Bonhag, 1955).
Leopold (1967) came to the conclusion that glycogen was absent in oocytes

of A. elliotti on the basis of several histochemical techniques. He
decided that a major portion of the yolk was in the form of protein-
carbohydrate complexes with significant amounts of acid and neutral muco-
polysaccharides. However, his case may remain to be proven as it rested
in part on the assumption that treatment with cereal amylase would digest
glycogen and yield a negative Schiff's reaction. Whatever the nature of
polysaccharides in eggs of A. elliotti, glycogen-like material was assayed
at various developmental stages to determine its contribution to embryo-
genesis and will be referred to henceforth as "glycogen."

There are many factors which may influence the identity or amount of
various carbohydrates found in insects. These include development, temp-
erature and age and will be discussed in turn where they may have some
application to this paper.

Changes of trehalose during embryogenesis have been followed in eggs
of Bombyx mori (Dutrieu, 1961 a, b; Yamashita, 1965) and Melanoplus dif-
ferentialis (Randall and Derr, 1965). Dutrieu reports that in the non-
diapause eggs of bivoltine and tetravoltine strains of B. mori trehalose
increases from a low level (1.5 % dry weight) to a maximum of 15 % of the
dry weight at blastokinesis. She states that hibernating eggs are
initially about the same in trehalose level. Yamashita found that the
initial levels of about 4 mg/g wet weight in hibernated eggs were greater
than the 0.5 mg/g occurring in non-diapause eggs. Randall and Derr noted
infertile eggs of M. differentialis held 88 mg/100g fresh weight as treha-
lose and diapause samples reflected an initial level of 174 mg/100g.

After 4 and 14 days at 5°C they found levels of 196 and 166 mg/100g

respectively. The trehalose levels had quadrupled by the time eggs were

about to hatch. It was accordingly expected that this carbohydrate would

occur in eggs of A. elliotti and that by tracing levels throughout their

embryonic development and correlating results with previous studies some

insight might be gained of its contribution as an energy and/or as a

carbon source.

Although, as Agrell (1964) pointed out, fat metabolism predominates

in most insect eggs, the importance of polysaccharides as an energy source

has long been known. Rothstein (1952) cites at least six workers dating

back to 1885 who observed a decrease in fat, glycogen and protein in post-

diapause eggs of B. mori. Glycogen decreases to approximately 87 % of

initial levels. An early, more rapid decrease was noted by Moulinier

(1957) and Chino (1957). Yamashita (1965) notes that a slightly higher

level of glycogen was found in hibernated eggs than in non-diapause eggs

and that glycogen in both decreased throughout development. Agrell cited

four authors who noted that both fats and polysaccharides were steadily

consumed during "embryogenesis of the silkworm and grasshopper."

According to a theory of sequence of metabolites used as an energy

source during development, the order of utilization is carbohydrate,

protein and fat (Needham, 1931, 1942). Along with those studies just

noted, other studies of insect eggs seem to bear out an early reliance

upon carbohydrate as an energy source. Rothstein (1952) reports that

glycogen (when expressed as a percentage of the constant nitrogen content)

decreases from 58.4 % to 25.9 % in developing eggs of the Japanese

beetle, Popillia japonica. He states that the loss occurs in two phases

and that it furnishes the energy for early embryogenesis. Ludwig and

Ramazzotto (1965) point out that fat furnishes the main source of energy

for embryogenesis in most insects but in the yellow mealworm, Tenebrio

molitor, glycogen is the main energy source. They state that the 51.5 %

loss of glycogen (4.7 to 2.2 mg/100 eggs) occurred throughout the embry-

onic period and fat was not utilized until the last day. No evidence was

found of protein utilization for energy in either the mealworm or the

Japanese beetle.

As the diapause state is entered by eggs of Bombyx mori, there is an

increase in cyanide resistance (Wolsky, 1949) and a depression of respira-

tion rate concurrent with a rapid conversion of glycogen to sorbitol and

glycerol (Chino, 1957, 1958, 1960). These polyols were then reconverted

to glycogen at diapause termination. He advanced the explanation that

accumulation of polyols in B. mori occurred as a result of the inactiva-

tion of the electron transport system, the hydrogen being transferred to

unusual intermediates in this anaerobic condition. Harvey (1962) states,

however, that this hypothesis "faces serious difficulties" and points out

that Chino gives no evidence that such a block occurs. Wyatt (1962) came

to the same conclusion as Harvey.

Melanoplus differentialis also shows increased cyanide resistance

during embryonic diapause (Bodine and Boell, 1934) and a low rate of

oxygen consumption (Bodine, 1929). Randall and Derr (1965), however,

report that the accumulation of sorbitol and glycerol does not occur in this species. The absence of glycerol is also a feature of hibernating eggs of Melanoplus bivittatus (Salt, 1957).

As pointed out by Roemhild (1965a), diapausing eggs of A. elliotti have a number of characteristics different from those of M. differentialis, including a requirement for low temperature in breaking diapause. Laine (1966) discovered that when A. elliotti entered diapause the respiration rate did not drop but continued at a fairly high level. Roemhild (1965b) noted that reducing materials were highest during the diapause period. Roemhild (1966) also found an increase in osmotic pressure coincident with diapause initiation. This month-long period of elevated levels occurred without any low temperature exposure. On the basis of thin layer chromatography in one solvent system, Roemhild tentatively attributed this temporary increase to glycerol production by the embryo. Unfortunately the levels declined before this could be confirmed in other solvent systems. In view of the unlike results obtained with B. mori and M. differentialis with respect to carbohydrate metabolism in diapause and the different character of diapause in A. elliotti, it is of interest to see if any polyhydric alcohols accumulate in this species.

Diapause occupies a major time interval in the embryonic development of A. elliotti. Van Horn (1963) reported that, during a prolonged cold storage of eggs, there was marked increase in the size of the embryonic fat body and a deposition of lipid. Svoboda (1964) and Svoboda et al. (1966) were unable to confirm this deposition by separately analyzed

lipid extractions of embryo and yolk.. They state, however, that small.... ...

changes in lipid location may have gone undetected. The fat body, a

tissue noted for glycogen accumulation, has been shown to be the major

site of trehalose synthesis in the adult locust, Schistocerca gregaria

(Candy and Kilby, 1961). In view of the above findings, the present

study investigates the effect of the lengthy embryonic diapause of A.

elliotti on carbohydrate levels in the egg.

Howe (1967) comments that many biochemical and physiological studies

are conducted at uncontrolled temperatures even though temperature has

been shown to influence the amounts of unsaturated fatty acids in the

mosquito, Culex tarsalis and the proteolytic activity in Tenebrio and

Tribolium beetles. Temperature changes are also known to influence some

carbohydrate levels in insects. Somme (1966) showed that in larvae of

the Mediterranean flour moth, Anagasta kuehniella, hemolymph levels of

glycerol and glucose rose at low temperatures and trehalose increased

at $0^{o}C$ but decreased at $-6^{o}C$. During prolonged storage of Hyalophora

cecropia pupae at $5^{o}C$, there is an increase in trehalose (Wyatt and Kalf,

1957).

Temperature effects on carbohydrates have also been observed in

insect eggs. Somme (1964) found that in eggs of the black willow aphid,

Pterocomma smithia, glycerol was 5.5 % at $20^{o}C$ but only 1.2 % if the eggs

were stored at $5^{o}C$ for one week. Yamashita (1965) states that, while

there is little trehalose in newly laid diapause eggs of Bombyx mori,

cold exposure favors its appearance. Dutrieu (1961b) reported that

trehalose levels in the "directly developing" eggs of B. mori were aug-
mented by the action of cold. She also says that cold does not change
trehalose levels in diapause eggs of B. mori which are newly laid, in
prediapause or in early estivation. She also notes that cold has no
action on trehalose levels in eggs of Carausus morosus (Phasmida).
The possibility then remained that any change observed during prolonged
cold storage of diapausing eggs of A. elliotti could be due to either the
temperature or the developmental state. The present study makes a brief
attempt to distinguish between any temperature or developmentally induced
changes in carbohydrate levels which might occur during this period.

Clark and Rockstein (1964) review papers that indicate that the time
of oviposition may be important to the rate of development or viability
of a number of insect eggs. For example, Richards and Kolderie (1957)
noted that eggs of the milkweed bug, Oncopeltus fasciatus, weighed less
and took longer to develop when laid very early or very late in the
fecund period. Wellington (1965) suggests that an unequal partitioning
of maternal food reserves in eggs of the tent caterpillar might account
for the greater vigor in larvae from early egg masses as opposed to late
laid eggs. Since carbohydrate is utilized first in the embryonic devel-
opment, it is possible to hypothesize that there was more carbohydrate in
the early laid eggs and that this may partially account for their more
rapid development or the greater vigor of resultant larvae. Senescence
in some insects is known to influence the amount of trehalose and glycogen
available to adults for flight or in the fat body (Clark and Rockstein,

1964; Rockstein and Srivastava, 1967).

Van Horn (1966b) has observed a different phenomenon in eggs from a population of A. elliotti. Eggs laid the last two weeks of the laying season developed to a 30 day mean stage of 16.8, according to criteria established by Van Horn (1963, 1966a), while those laid during the first two weeks only reached a mean stage of 12.3 in 30 days. She stated that, although it seemed unlikely that the quantity of yolk alone could be responsible for more rapid development in late laid eggs, there was no data relating egg weight to maternal age. The present investigation seeks to discover if later laid eggs are indeed heavier and what effect aging has upon the maternally donated carbohydrate substrate in eggs collected from three different parental densities.

The study of the principal soluble carbohydrates found in Aulocara elliotti eggs presented here is intended to compliment previous studies of eggs of this species. The study was conducted in two phases. The first dealt with the qualitative and quantitative changes in carbohydrates during the development of the eggs and considers temperature effects; the second with the effects of maternal age and density as reflected in newly laid eggs.

METHODS AND MATERIALS

Experimental Design

During the first phase of this study, conducted in 1966-1967, whole eggs in a number of stages of development were analyzed qualitatively and quantitatively for glycogen and mono- and oligosaccharides. Since the distribution of substrates within the egg is important to development, yolk and embryo were also separately analyzed at each stage marked (*) below. The changes in carbohydrate constitution and distribution were traced under the times and conditions schematically represented as:

	Prediapause State	Diapause State	Post-diapause State
Days of development in state when sample was taken:	1 3 7 15 25 45	25 40 175	1 2 7 14 20

Morphological stage (Van Horn, 1966a):	1 5 10 18
	      * *
		19 19 19 20-22
		        * *
			23-24 25 26 27
			   *

Temperature at which eggs were maintained:	$25^{\circ}C$	$8-12^{\circ}C$	Group I: $8-12^{\circ}C$ Group II: $25^{\circ}C$ $25^{\circ}C$

The effect of temperature was further evaluated by analysis of a stage 19 sample after 139 days at $25^{\circ}C$. In an attempt to verify possible synthesis of trehalose and establish any possible interconversions, samples in each state were treated with radioactive glucose and late diapause samples with the appropriate radioactive polyol. Late diapause and post-diapause field-collected eggs were compared with known age eggs of the same stage.

During the second phase of this investigation, eggs were collected weekly throughout the reproductive period from grasshoppers maintained in the greenhouse at three different densities and the effects of maternal

age and density on egg weight and carbohydrate partitioned to the eggs were studied.

Biological Samples

Third and fourth instar nymphs were collected from a population of A. elliotti near Decker, Montana early in the summers of 1966 and 1967. Nymphs were reared under greenhouse conditions in cylindrical clear plastic-walled cages, 20.5 cm in diameter and 27.5 cm high, set on dirt-filled pans as described by Anderson and Hastings (1966). This population markedly decreased in abundance from 1966 to 1968. In 1966 the nymphs were reared and maintained in 47 cages at a density of 3 pairs per cage. On alternate days, the grasshoppers were provided with fresh cuttings of western wheatgrass, Agropyron smithii, which is one of their preferred food-plants (Pfadt, 1949; Anderson, 1964).

Egg pods were collected daily in 1966 by sifting dirt in the early evening hours when oviposition activity had ceased. The egg pods were then stored in plaster of paris blocks as described by Van Horn (1966a), at 25°C until needed. After a period of from 63 to 84 days, when the remaining embryos had entered diapause, the blocks were transferred to a refrigerator maintained at 8 to 12°C. Eggs were also field collected in the fall of 1966 and stored at this temperature. After at least 153 days of cold exposure, eggs that were not yet selected nor destined for longer cold exposure were replaced in the 25°C cabinet to initiate post diapause development. A number of embryos, however, began blastokinesis at the lower temperature. Egg pods and blocks were watered lightly on each

third day while at 25°C and about weekly while at 8-12°C.

Only eggs which had developed to the desired degree and appeared to be viable when removed from their pods were included in samples. In the samples which were 15 or 25 days old, a representative egg was selected from the center of each pod for staging. The staging criterion used in this study was that established by Van Horn (1966a). At all other ages investigated, the embryo either had not formed or was visible from the exterior and could be staged while immersed in water. Generally, each sample size was 100 eggs. Each whole egg sample was rinsed, surface dried, weighed, triturated for five minutes in distilled water at 5°C and freeze-dried to preserve it in that state. This latter procedure was made necessary due to the number of samples involved and is a recommended procedure for storing biological material (Hais and Macek, 1963; Burchfield and Storrs, 1962). Thirty-six whole egg samples were treated in the above fashion. The distribution of samples in each previously designated stage may be observed in the included tables.

Eight 100-egg samples were taken at stages previously indicated for separate analysis of yolk and embryo. The embryos were dissected from the remainder of the eggs under distilled water at 5°C. Three such operations were performed in a volume of 0.4-0.5 ml water. As little water as possible was removed with removal of embryos from the distilled water. The remaining egg parts and water constituted the yolk fraction. Both fractions were freeze-dried.

Five radioactive egg samples were prepared with the view of demon-

strating enzyme systems capable of converting glucose to other carbohy-
drates during prediapause, diapause and post-diapause but incubations are
of such duration that results do not yield information on specific enzy-
mic pathways followed. The method of introducing radioactive material
was that used by Bunde (1965). Two 75-egg prediapause samples were col-
lected from a parental density of three pairs per cage in 1967 and each
was exposed to $0.75 \mu c$ of glucose-U-C14 in 0.75 ml water for 42 hours,
following three days of desiccation over silica gel and calcium sulfate
crystals. The prediapause samples were 25 and 45 days old at the initia-
tion of this procedure and had developed to mean stages of 11.9 and 18.9
respectively. Two post-diapause samples were treated the same except one
field collected sample of 100 eggs was exposed to $10 \mu c$ of radioactive
glucose in 1.0 ml and one greenhouse collected sample of 80 eggs was ex-
posed to $8 \mu c$ of radioactive glucose in 0.8 ml. The post-diapause samples
were in stages 20 - 22 when incubated and ranged from 23 to 25 when
processed. A 50-egg diapause sample was desiccated for six days, incuba-
ted for a like period in $2.5 \mu c$ of glucose-U-C14 in 0.5 ml water and
processed the day following incubation. Carbohydrates were extracted
from all other samples that had been exposed to radioactive glucose im-
mediately after incubation and rinsing with distilled deionized water.
The extraction technique is described in the next section of this paper.

After a desiccation period of three days, four samples, each com-
posed of 34 field collected eggs that had received sufficient cold to
break diapause, were exposed to $2.5 \mu c$ of D-mannitol-1-C14 in $280 \mu l$ of

water for 70 hours at 10°C. Carbohydrates were immediately extracted

from one sample and the other samples were placed at 25°C for 1, 3 and 5

days prior to extraction. Nothing was known about the changing pattern

of carbohydrates in postdiapause development of A. elliotti at the time

this procedure was initiated. It was reasoned that if the polyhydric

alcohols were reconverted to glycogen at diapause termination (as is the

case in Bombyx mori) an accumulation of radioactivity would be observed.

Since a similiar feature did not exist, exposure of A. elliotti eggs to

the appropriate polyol at diapause termination only serves to demonstrate

the existence of enzyme systems capable of converting mannitol to other

carbohydrates.

Egg samples for the 1967-68 study of density and age effects were

collected and treated in the same fashion as the whole egg samples taken

for the study of carbohydrate changes occurring during development.

Nymphs were maintained to adulthood at one, three and six pairs per cage,

with 18 pairs at each density contributing eggs for study. High parental

mortality and lowered egg production at the highest density made contin-

uation of this experiment impossible after six weeks. Each of the 18

samples taken during this phase of the project was composed of 100 eggs

except for those laid by the parental density of six pairs per cage during

weeks 1, 5 and 6. Here the sample sizes were 70, 85 and 80 eggs respect-

ively. Since eggs were less than seven days old when freeze-dried, de-

velopment was thought not to have progressed beyond the blastoderm stage

(Van Horn, 1966a).

Two hemolymph samples were obtained from females that had been used for the study of maternal age effects. These females had been reared at densities of one and six pairs per cage and had survived the sixth week of oviposition. The hemolymph quantities were approximately 40 and 35 μl respectively. They were obtained by severing one leg near the thorax and, with gentle squeezing, drawing up the hemolymph that exuded from the wound with a 100 μl syringe. These samples were also freeze-dried.

Carbohydrate Extraction

Two extracts were obtained from each freeze-dried sample in a fashion similiar to that described by Kemp and Van Heijningen (1954) for separate analysis of glucose and glycogen. Each sample was extracted 3 times in 4 ml of 70 % aquaeous ethanol. Following the initial 10 minute trituration, the homogenate was centrifuged for 8 minutes and the supernate removed with a pipette. The residue was twice resuspended in 70 % ethanol, centrifuged as above, and the supernatant liquid added to the first extract. The ethanol was removed in vacuo at 36°C on a Rinco rotary evaporator and the composite extract was deproteinated with Somogyi's reagent (2 volumes each of 0.3 N barium hydroxide and 5 % zinc sulfate) as recommended by Hais and Macek (1963). After filtration through sintered glass and rinsing the residue with 20 ml of distilled, deionized water, the volume was reduced in vacuo at 36°C to about 5 ml. The extract was clarified by successively passing it through a column containing 3 grams of Dowex 50 (H+) and a column containing 2 grams of

Dowex 1 ($CO_3^=$) prepared after Burchfield and Storrs (1962). The columns

were fitted with glass joints and drip tips in order that the eluate from

the Dowex 50 column might drip directly on the Dowex 1. The neutral

sugars in the percolate were displaced through the columns with 100 ml of

distilled, deionized water and the extract was again reduced in vacuo to

a volume of about 2 ml. Following three rinses of the flask, the resul-

tant volume of 6 to 7 ml was reduced under a stream of nitrogen to less

than 0.5 ml and then brought up to a final volume of $1^{\pm}.01$ ml. In the

case of those samples that had been exposed to radioactive mannitol, amino

acids were eluted from the Dowex 50 column and organic acids from the

Dowex 1 column after Burchfield and Storrs (1962).

The residue which remained after the 70 % alcohol extraction was de-

fatted by suspending it in 5 ml of diethyl ether, centrifuging and de-

canting. The ether was evaporated from the residue at room temperature.

Next, "glycogen" was extracted from the residue with 5 ml of 5 % tri-

chloroacetic acid (TCA) in a boiling water bath for 30 minutes, centri-

fuged 8 minutes and the supernatant liquid placed on a column con-

taining 1.0 g of Dowex 50 (H+). The remaining residue was twice re-

suspended in 5 ml of 5 % TCA, centrifuged and the supernatant liquid

placed with the hot TCA extraction. The composite extract was not run

through a Dowex 1 ($CO_3^=$) column since it was experimentally determined

that loss of oyster glycogen occurred in such a column. The glycogen ex-

tract was concentrated to a final volume of $10^{\pm}.03$ ml under reduced pres-

sure following a 75 ml rinse of the column with distilled, deionized H_2O.

Paper Chromatography

The concentrated 70 % ethanolic extract was subjected to qualitative analysis by chromatography on Whatman No. 1 filter paper. Resolution of components was performed by the descending technique at room temperature. The chromatographic cabinets were fashioned of wood and sealed with wax. Identification of the carbohydrates in A. elliotti eggs was begun by co-chromatography of the extract and standards in at least four solvent systems and noting similiar migration distances. In all, seven solvent systems were employed in the identification of sugars and polyhydric alcohols in the egg. These solvent systems were: ethyl acetate:pyridine: water (8:2:1 v/v), iso-propanol:pyridine:acetic acid:water (8:8:1:4 v/v), phenol:water (4:1 w/v), n-butanol:ethanol:water (4:1:5 v/v, upper phase) (all listed by Block et al., 1958), n-butanol:ethanol:water (4:1:1 v/v) (Hough and Jones, 1962), 88 % phenol:water (4:1 v/v) and n-butanol:acetic acid:water (4:1:1 v/v).

The positions of the sugars and polyols on chromatograms were detected by the alkaline silver nitrate method of Trevelyan et al. (1950). Selective visualization of different moieties and further characterization of them was performed by using more specific indicator sprays. The modified Fleury's reagent, for the detection of polyols, may be followed with p-anisidine and results in the formation of multi-colored spots when observed in daylight and under ultra-violet illumination (Lambou, 1956). The p-anisidine phosphate reagent, prepared as modified by Mukherjee & Srivastava (1952), gave differently colored spots for aldohexoses and

ketohexoses, aiding in identification. The ketohexose was also detected
with 0.2 % w/v naphthoresorsinol in acetone:3 N phosphoric acid (5:1 v/v)
(Block et al., 1958). This spray did not detect the aldohexoses or the
polyhydric alcohols. The alpha-naphthol:phosphoric acid reagent de-
scribed by Block et al. (1958) is more sensitive for ketose although
aldose will react. When used on chromatographic separations in this
study only the standard and egg ketoses were detected.

Gas-liquid Chromatography

The identity of neutral free moieties soluble in aqueous ethanol was
further confirmed by gas-liquid chromatography of their respective O-tri-
methylsilyl ethers. Biological material used for preparation of these
derivatives was that which remained after paper chromatographic identifi-
cation and quantitative estimation. This material was freeze-dried and,
as were the carbohydrate standards, stored over desiccating silica gel
crystals and phosphorous pentoxide prior to preparation of the O-tri-
methylsilyl ethers. These derivatives were prepared by the method of
Perry (1964) using pyridine which had been redistilled over potassium
hydroxide and trimethylchlorosilane and hexamethyl disilizane (K & K Lab-
oratories, Plainview, N.Y.) which were used as received.

The F and M Biomedical Gas Chromatograph model 400 equipped with a
flame ionization detector was used for analysis. The trimethylsilation
reaction mixtures (0.5 - 1.0 μl) were injected on U-shaped glass columns
(1/8 in. I.D. x 4 ft.) which had been packed with either 3.8 % silicone
gum rubber on Gas Chrom Q (100 x 120 mesh) or 5 % silicone gum rubber on

Gas Chrom Z (100 x 120 mesh). Helium was used as the carrier gas at a flow rate of 40 ml/min. The column was maintained at a temperature of 150°C to note retention times of hexoses and the hexitol and 210°C for trehalose characterization.

Quantitative Analysis

Colorimetric quantitative estimation of sugars and polyols in the 70 % ethanolic extract were made in conjunction with paper partition chromatography. The solvent system iso-propanol:pyridine:acetic acid: water (8:8:1:4 v/v) was chosen for use in developing chromatograms since there was good resolution of all compounds of interest and none was washed from the Whatman #1 paper empolyed in the 18 hour run required. Previous to development, the egg extract and standards were spotted parallel to the aliquot to be analyzed. Following development, the resolved spots were visualized on the guide strips by the alkaline silver nitrate method of Trevelyan et al. (1950). The compounds thus located were eluted with distilled, deionized water from strips parallel to the detected spots and filtered through sintered glass in preparation for colorimetric assay. Measurements of glycogen and the total free sugars (unfractionated 70 % ethanol soluble neutral carbohydrates with reducing or potentially reducing groups) were made without individual chromatographic resolution.

The above determinations were made using the phenol-sulfuric acid method of Dubois et al. (1956). The instrument used in taking readings was the Bausch and Lomb Spectronic 20 Colorimeter. Absorbancy readings,

which were made at a wavelength of 490 mµ, were preferably read in the range of 0.250 to 0.450 where the third decimal place could be estimated. Standard curves were prepared for both glycogen and trehalose by plotting micrograms of known vs. absorbance at 490 mµ. The estimates of total free sugars were made by reference to the standard trehalose curve since greater than 90 % of the absorbancy values were found to be attributable to this moiety. Blanks were prepared for standard curves, glycogen and trehalose determinations by substituting purified water for the sugar solution. Since trehalose in eggs or egg parts was measured following chromatographic separation, its blank was prepared by eluting a parallel strip of the chromatogram representing the same area. Measured aliquots of chromatographically isolated trehalose and samples of glycogen or total free sugars were analyzed in triplicate to minimize the danger of accidental contamination of cellulose lint. Further, each ethanolic extract was chromatographed three times to avoid errors resulting from missing a section of paper containing trehalose. This resulted in 9 absorbance readings for trehalose and 3 each for glycogen and total free sugar in each sample. The error observed with standards was less than 5%.

Where the hexitol existed in amounts greater than 5 µg/egg, it was estimated after elution from the same chromatograms used to resolve the trehalose. The method used depends on a short periodate oxidation and assay of formaldehyde produced as described by Lambert and Neish (1950). The Spectronic 20 Colorimeter was used at a wavelength of 570λ. Blanks and the standard curve were prepared similarly to those previously

described. Restricted amounts and sensitivity allowed only duplicate
readings of absorbances for each of the replicated spots. Six observa-
tions of the amount of this polyol in each sample resulted. Observed
error was again less than 5 %. Where less than 5 μg/egg existed, esti-
mates of amount were derived by comparing extract spot size and intensity
on chromatograms with spotted standards of known quantity.

Glycerol was estimated in the same fashion as hexitol. Reference
was made to the standard hexitol curve and multiplication of the μg of
polyol so obtained by a conversion factor of 0.5055 to get μg glycerol
present per egg. Where no other polyol was present, glycerol was deter-
mined directly on the extract. Lambert and Neish (1950) stress that
error resulting from glucose present in equal concentration to glycerol
amounts to only 2.5 to 5.0 % and no such concentration existed here. It
was verified that trehalose in concentrations greater than are found in
the egg did not interfere with polyol measurement since synthetic mix-
tures of polyol and trehalose were determined by this method of accura-
cies of 101 and 104 %.

Radio-assay

Sugars and polyols in the radioactive samples were subjected to
paper chromatographic resolution in preparation for counting of activity
and blanks for background counting were prepared in the same way that
trehalose was quantitatively measured. Here, however, the phenol:water.
(4:1 v/v) solvent system was chosen for use since it completely separated
the ketose, aldose, polyol and trehalose occurring in eggs of A. elliotti.

Following elution of radioactive compounds from chromatograms, aliquots were reduced under a stream of nitrogen to a volume of about one ml and 15 ml of scintillation fluid were added to the counting vials. The liquid scintillation medium was made up of 6.0 grams of PPO and 120 grams of naphthalene per liter of dioxane after Bush and Hansen (1965) modified as recommended by Howald (1966).

Aliquots were counted for a ten minute period with a model 6804 Nuclear Chicago liquid scintillation counter. In order to correct for different counting efficiencies, the observed counts per minute were adjusted to disintigrations per minute with the aid of a quenching curve prepared by Dr. G. Strobel, Botany and Microbiology Department, Montana State University.

RESULTS AND DISCUSSION

Qualitative Analysis

Mannose, glucose, fructose, trehalose, mannitol and glycerol were identified in the neutral 70 % ethanolic extracts taken from eggs of A. elliotti. Corresponding Rgl values (range relative to glucose) were obtained by co-chromatography on paper of standards and egg extracts in at least four solvent systems (Table I). Five solvent systems were used in mannose characterization, six for glucose and fructose and seven for mannitol and trehalose.

Selective sprays were used in conjunction with paper chromatography to further aid in identification of the sugars and polyols. As indicated by Lambou (1956), standard and egg mannitol were both viewed as gray spots on heated chromatograms which had been sprayed with the modified Fleury reagent and, after overspraying with p-anisidine phosphate, they appeared as dull yellow spots in daylight and yellow under ultraviolet light. Under the above conditions, glycerol appeared as gray, white and lavender spots respectively; fructose spots were observed as tan, golden brown and gold; glucose as black, dull yellow and dull yellow; and mannose spots appeared as pale tan, pale tan and yellow. Trehalose was not detected following treatment of chromatograms with the Fleury reagent but made a fleeting pale pink appearence under daylight conditions when the p-anisidine was oversprayed. When chromatograms were only sprayed with the improved p-anisidine phosphate reagent of Mukherjee and Srivastava (1952), egg and standard mannose and glucose were viewed as light brown spots and fructose spots were yellow. Polyols were not detected with this

Table I

Rgl values from chromatograms of extracts of <u>Aulocara elliotti</u> eggs and of various sugars and polyhydric alcohols.

Extract or Standard	Solvent System*						
	BAW	EPW	IPAW	PW$_1$	PW$_2$	BEW$_1$	BEW$_2$
A. elliotti eggs	100	99	100	100	100	101	---
	29	24	67	69	53	16	16
	122	95	98	131	129	113	111
	125	121	109	118	---	---	---
	142	136	---	154	156	143	---
	378	266	129	307	---	---	---
	26**	23**	39	39		**	
Glucose	100	100	100	100	100	100	100
Trehalose	29	24	67	69	53	16	16
Mannitol	122	94	98	132	129	113	111
Mannose	125	121	109	119	---	---	---
Fructose	141	136	109	153	156	142	---
Glycerol	378	266	129	307	---	---	---
Sorbitol	117	92	---	141	133	105	---
Sorbose	121	131	109	---	---	---	102
Ribose	197	239	---	---	---	---	---
Xylose	---	---	119	---	---	---	---
Galactose	---	90	---	---	---	---	---

* Solvent systems used were: BAW, n-Butanol:Acetic Acid:Water, 4:1:1 v/v; EPW, Ethyl Acetate:Pyridine:Water, 8:2:1 v/v; iso-Propanol:Pyridine: Acetic Acid:Water, 8:8:1:4 v/v; PW$_1$, Phenol:Water, 4:1 w/v; PW$_2$, 70 % Phenol; n-Butanol:Ethanol:Water, 4:1:5 v/v (upper phase), BEW$_1$; BEW$_2$, n-Butanol:Ethanol:Water, 4:1:1 v/v.
** Not completely resolved from trehalose in egg extract.

reagent when used alone. Fructose was visualized as a red spot when the

naphthoresorcinol spray reagent outlined by Block et al. (1958) was used

and as a blue-violet spot when the alpha-naphthol:phosphoric acid reagent

described by Block et al. was used.

The identities of trehalose, mannitol and mannose were further con-

firmed by gas-liquid chromatography and comparison of retention times of

the trimethylsilyl ether derivatives that had been prepared from neutral

ethanolic egg extracts and carbohydrate standards (Table II).

Trehalose characterization was concluded by hydrolysis in sulfuric

acid and paper chromatography of the neutralized hydrolyzate. Material

in the egg, which had migrated on paper the same distance as standard

trehalose in iso-propanol:pyridine:acetic acid:water (8:8:1:4 v/v),

yielded only glucose when hydrolyzed in this acid. A one normal solution

of sulfuric acid released only small amounts of glucose in 30 hours at

room temperature (as judged by co-chromatography with standard glucose and

trehalose in phenol:water (4:1 v/v), ethyl acetate:pyridine:water (8:2:1

v/v) and iso-propanol:pyridine:acetic acid:water (8:8:1:4 v/v)). The

trehalose spot was much reduced in intensity and a prominent glucose spot

was observed when the material was chromatographed in the above solvent

systems following hydrolysis in boiling 3 $\underline{N}$ sulfuric acid for one hour.

Due to deproteination by Somogyi's reagent, which includes the use of

barium hydroxide, production of fructose by the Lobry de Bruyn - van

Ekenstein transformation is possible. Since it was a minor component in

eggs, (always less than about 0.5 μg/egg, as judged by spot size and

Table II

Retention times of trimethyl silyl ethers of standard and egg carbohydrates.

a) Column of 3.8 % SE-30 on Gas Chrom Q.

TMS ethers prepared from:	Retention times (minutes)				
	at 210°C		at 205°C		at 150°C
Diapause egg carbohydrates	0.65	8.7	0.65	9.2	7.6
Trehalose		8.6		9.2	
Mannitol	0.65		0.65		7.6
β-D-Glucose	0.70				9.6
β-D-Fructose					4.3

b) Column of 5 % SE-30 on Gas Chrom Z.

TMS ethers prepared from:	Retention times (minutes)				at 160°C
	at 150°C				
Prediapause egg carbohydrates	3.8	5.8	8.8	10.2	
Diapause egg carbohydrates			6.8	10.2	5.5
Mannose	3.4 & 3.8				
Mannitol			6.7		5.5
Undetermined		X	X	X	

detection limits), no effort was made to confirm its true occurrence in eggs of A. elliotti. However, fructose was detected in hemolymph samples where Somogyi's reagent was not used. Therefore, it is believed that fructose naturally occurs in eggs of this species.

There was one unidentified spot, detected with $AgNO_3$, that migrated less than trehalose in most solvent systems employed (Table I). This moiety could not be detected with the more selective sprays previously noted nor with the Morgan-Elson reagent for the detection of amino sugars (Waldi, 1965) and did not form a color complex when the phenol-sulfuric acid method of Dubois et al. (1956) was used. It was therefore judged not to contain an aldo or keto sugar, nor be a polyol or acetylated amino sugar and further characterization was not attempted.

In two hemolymph samples from females that had survived the sixth week of oviposition, only glucose, trehalose and a slight amount of fructose were identified. In a recent review, Wyatt (1967) notes that about 20 sugars were found in Locusta migratoria hemolymph and their occurrence was diet dependent. Mannose was not detected in the hemolymph of A. elliotti even though it was found in newly laid eggs in greater amounts than glucose.

Most of the carbohydrates identified in A. elliotti eggs have been found in eggs of other insects. The occurrence of trehalose and glucose in insect eggs is probably universal. No previous report was found of mannose in eggs. Somme (1964) has previously reported the presence of a polyhydric alcohol that was "probably mannitol" in eggs of the fall

cankerworm, Alsophila pometaria, and the black willow aphid, Pterocomma smithia, but no other reports of its occurrence in insect eggs could be located. Figure 1 and Table I distinguish between mannitol and sorbitol, which has been observed in a number of insects. Roemhild (1966) tentatively identified glycerol in A. elliotti eggs. Glycerol has been reported in eggs of a number of other insect species (Somme, 1964, 1965; Chino, 1958; Hanec, 1966) but has not been detected previously in any other acridid egg studied (Salt, 1957; Randall and Derr, 1965).

Quantitative Analyses of Developing Eggs

Changes in mannose and mannitol during development

During the development of A. elliotti eggs, the carbohydrates that were detected changed in quantity and identity. Mannose was present in week old eggs in quantities of up to about 7 μg/egg. The quantity of mannose present in eggs was markedly reduced between 15 and 25 days of development (figure 2) and was not detected after 45 days of development.

The amount of mannitol present in each egg sample during development is tabulated in Table III. Figure 3 exhibits the means and extremes of mannitol during development. As mannose disappears from the eggs, mannitol makes its appearance. The quantity of free mannose is not sufficient to serve as the only precursor for mannitol synthesis. However, mannose may be an intermediate and/or a component of the protein-carbohydrate complexes that Leopold (1967) found to be a major portion of yolk material deposited in A. elliotti oocytes. This view is supported by the observations that there is a striking increase in the free amino acid