THE CHEMISTRY OF INSECT HEMOLYMPH

III. GLYCEROL*

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(Received for publication, December 8, 1958)

ABSTRACT

Free glycerol has been identified as a major solute in plasma of pupae of the silk moth, *Hyalophora cecropia*. Glycerol is not found in the blood of larvae; it appears about the time of pupation, and then gradually accumulates during diapause to reach after 6 months a level of about 0.3 M. When diapause is broken and the adult develops, glycerol rapidly disappears. In diapausing pupae of the related species, *Telea polyphemus*, about 0.05 M glycerol was found; in 3 other saturniid species, there was little or none. It is suggested that glycerol may be a product of a modified glycolytic pathway.

During the course of some analyses, by paper chromatography, of the carbohydrates in insect hemolymph (Wyatt and Kalf, 1957), our attention was attracted by a spot which was revealed after oxidation by periodate and which moved much more rapidly on the chromatograms than any of the usual sugars. The substance responsible for this spot was so abundant in the blood of *cecropia* silkworm pupae during diapause that we decided to investigate its nature. It proved to be free glycerol, as already reported in preliminary form (Wyatt and Kalf, 1958; Wyatt, Meyer, and Kropf, 1958; Wyatt, 1958). This finding has been extended to other species by Salt (1957, 1958). Independently, Chino (1957, 1958) has described the presence of glycerol as well as sorbitol in diapausing embryos of the silkworm, *Bombyx mori*. We now report in detail on the occurrence of glycerol in the blood of *Hyalophora cecropia* and some related saturniid moths.

Materials and Methods

Hemolymph was collected from punctures in the insects' integuments, centrifuged, and used fresh or after storage at −20°. Glycerol was also obtained from lyophilized plasma, but this was avoided in quantitative work because of possible loss by evapora-

* This work was supported by research grants from the United States Public Health Service.

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tion. Some samples were deproteinized and deionized as described previously (Wyatt and Kalf, 1957) prior to chromatography, but later it was found satisfactory simply to mix insect plasma with an equal volume of water, heat at 100° for 3 minutes, centrifuge, and apply the supernatant fluid to the paper.

Chromatograms were run on Whatman No. 1 or No. 3 paper by the descending method, generally with *N*-butanol–acetic acid–water (5:1:2; Hough, 1950) as the solvent. Sec-butanol (80 per cent; Wyatt and Kalf, 1957) also gave good isolation of glycerol, but sometimes contained an impurity which reduced periodate and obscured the spots. Glycerol was at first revealed on chromatograms with the reagent of Lemieux and Bauer (1954). Later, it was found more satisfactory to spray with 0.5 per cent sodium metaperiodate which was allowed to dry and followed by a freshly prepared mixture of 5 per cent $AgNO_3$ and concentrated NH_4OH (5:1 by volume; *cf.* Hough, 1950; Evans and Dethier, 1957). Glycerol was not resolved from dihydroxyacetone, but could be distinguished by its lack of reaction with triphenyltetrazolium chloride (Wallenfels, 1950).

Glycerol and related substances were determined quantitatively by the procedure of Lambert and Neish (1950) scaled to a final reaction volume of 3.6 ml. This was applied both to the aqueous extracts from paper chromatograms and directly to samples of deproteinized plasma. Chromatograms for this purpose were marked into lanes as described previously (Wyatt and Kalf, 1957), the marginal lanes being used to locate the spots. The unsprayed portions for quantitative analysis were stored in a humid box and for minimal time before elution, in order to avoid loss of glycerol by evaporation. Glycerol standards were recovered from paper chromatograms 98 to 100 per cent.

RESULTS

Identification of Glycerol in the Blood of cecropia Silkmoth Pupae.—The major periodate-reducing substance in deproteinized plasma of diapausing *cecropia* silkmoth pupae was found to migrate on paper chromatograms identically with known glycerol. This was shown with 6 different solvent systems, including the two mentioned above. The same result was obtained whether protein was removed with $HClO_4$, $Zn(OH)_2$, ethanol, or heat, suggesting that the glycerol did occur free in the original plasma. The substance did not reduce triphenyltetrazolium chloride. A sample of the substance obtained by eluting a band from a paper chromatogram gave a strongly positive acrolein test (Segur, 1953).

The identity of the substance from *cecropia* plasma with glycerol was established by preparation of the tribenzoate. The deproteinized, deionized extract from 1 gm. of lyophilized pupal plasma (corresponding to 6.2 ml. fresh plasma) was streaked on two sheets of Whatman No. 3 paper, and subjected to chromatography. The extracts of the glycerol bands were evaporated to dryness (182 mg.) and benzoylated (Segur, 1953). The product was recrystallized from 96 per cent ethanol; yield, 264 mg., m.p. 71 to 72. Tribenzoate prepared similarly from known glycerol had m.p. 72 to 73; mixed m.p. 72.

Quantitative Changes in Glycerol during Development of the cecropia Silkmoth. —We first observed the presence of glycerol in blood from *cecropia* pupae in diapause. When blood from mature larvae was examined, glycerol was not found. It was then of interest to know whether this substance made its appearance relatively suddenly during the metabolic reorganization which occurs

TABLE I

Glycerol and Related Substances in Blood of Hyalophora cecropia during Development

Stage	Sex	No. of Animals	Mean analysis	
			Glycerol	Related substances*
			mM	mM
Larvae commencing to spin	?	3	2	2
Pupal molt (10 days‡)	?	4	15	6
Pupae, 20 days at 25°	?	4	24	5
" 58 " " "	♀	2	126	—
Pupa, 58 " " "	♂	1	100	—
Pupae, 71 " " "	♀	2	172	—
Pupa, 71 " " "	♂	1	121	—
Pupae, 4 mo. at 25°	♀	3	172	—
" 3 " " " then 1 mo. at 6°	♀	4	208	19
" 3 mos. at 25°, then 3 mos. at 6°	♀	3	323	17
" 7 mo. at 25°	♀	3	306	18
" 2 " " ", then 5 mo. at 6°	♂	3	325	23
" brains removed, 16 mos. at 25°	♀	4	151	18
Developing adults, day 2§	♀	3	141	14
" " " 5	♀	3	98	11
" " " 10	♀	2	28	7
" " " 13	♀	3	12	8
" " " 20	♀	2	2	8

* Difference between glycerol isolated on chromatograms and total substances reacting in the method of Lambert and Neish (1950), calculated as glycerol.

‡ Pupal age reckoned from the commencement of spinning.

§ Days of adult development correspond to the timetable of Schneiderman and Williams (1954); the adult emerges about day 21 at 25°C.

in the prepupal period, or whether it accumulated gradually during the ensuing pupal diapause. Accordingly, glycerol was determined in the blood of a series of individual animals taken at intervals from the beginning of cocoon spinning by the mature larva through diapause and subsequent development to the adult. The results (Table I; Fig. 1) show that glycerol first appears in the blood in small amounts about the time of the pupal molt, and that it accumulates slowly thereafter at a roughly constant rate during several months of diapause. Several pupae which had been kept in diapause for more than a

year by surgical removal of the brains (Williams, 1946) contained considerably less glycerol than those taken after only 6 or 7 months of diapause; after such long storage, however, these animals were in poor condition, and the significance of this result is doubtful. Upon re-initiation of development by returning chilled pupae to 25°C., the glycerol content of the blood falls rapidly, approaching 0 at the time of emergence of the adult.

The results of similar analyses on diapausing pupae of four other species of saturniid moths are shown in Table II. Of these, only *Telea polyphemus* contained any substantial amounts of glycerol, and here the concentration was only some 15 per cent of that found in *cecropia* pupae at a comparable stage.

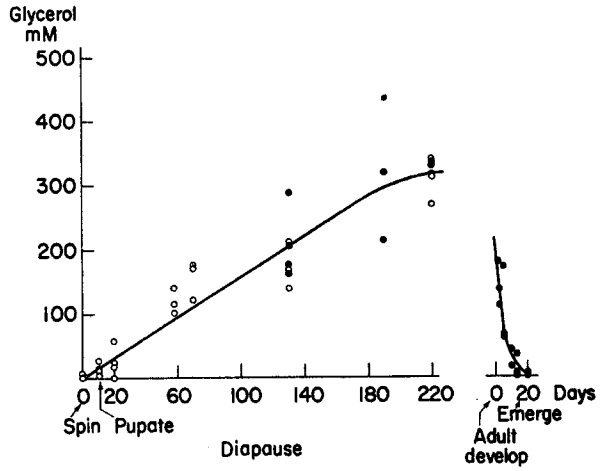

FIG. 1. The glycerol content of *cecropia* blood during diapause and development. Each point represents one animal analyzed individually; ●, animals chilled, ○, animals not chilled. Conditions of storage as in Table I.

The values for "related substances" in Tables I and II refer to compounds other than glycerol which yield formaldehyde upon being oxidized by periodate. These would include α-glycerophosphate. We find that 1 mole of α-glycerophosphate yields only 0.6 mole of formaldehyde during the 5 minute oxidation period used, whereas glycerol yields 2 moles. Thus, the 6 to 20 mM α-glycerophosphate found in *cecropia* plasma at various stages (Wyatt and Kropf, 1959) would correspond to 2 to 6 mM apparent glycerol in the procedure of Lambert and Neish. This would fully account for the "related substances" found in mature *cecropia* larvae, but during diapause the presence of other substances is indicated. These may include sorbitol, which Chino (1957) has found in large amounts along with glycerol in embryos of *Bombyx mori*. The values show, however, that in *cecropia* and the other species which we

have examined, any sorbitol must be small in amount compared with the levels found in *Bombyx*. We did not observe sorbitol on the paper chromatograms.

TABLE II

*Glycerol and Related Substances in Blood of Diapausing Pupae of other Saturniid Species**

Species and conditions of storage	Sex	No. of animals	Mean analysis	
			Glycerol	Related substances‡
			mM	mM
Samia walkeri, 2 mos. room temp.	?	2	0	8
" " 6 " 25°, then	♂	1	0	6
12 " 6–8°	♀	2	0	7
Antheraea mylitta, 5 mos. 25°, then	♂	1	1	13
1 mo. 8°	♀	1	0	1
Telea polyphemus, 1 mo. 25°, then	♂	1	47	15
5 mos. 6°	♀	1	46	12
Rothschildia orizaba, 6 mos. 25°, then	♂	1	2	4
12 mos. 6°	♀	2	3	5

* We are grateful to Professor C. M. Williams for the gift of most of these animals.

‡ Defined as in Table I.

DISCUSSION

Although glycerol is a normal intermediary in metabolism, its accumulation in substantial amount is exceptional in the animal kingdom. Among vertebrate animals, blood glycerol is generally less than 1 mM (Tangl and Weiser, 1906), but it is now evident that in certain insects it can become the most abundant plasma solute. Its presence has been demonstrated in several different stages of species belonging to several orders—embryos of the silkworm, *Bombyx mori* (Chino, 1957), larvae of the webworm, *Loxostege sticticalis*, the gallfly, *Eurosta solidaginis*, and certain parasitic wasps (Salt, 1957, 1958), as well as pupae of certain saturniid moths. In each case, the stage in which glycerol has been found is the stage in which the species undergoes diapause and passes the winter.

When added to various tissues, glycerol is known to protect against injury by freezing (Lovelock, 1954). The suggestion therefore naturally arises that the accumulation of this substance in certain insects may be a physiological adaptation conferring resistance to cold. This possibility has been explored by Salt (1957, 1958). In the larvae of certain parasitic wasps, he has found phenomenally high levels of glycerol (up to 25 per cent) which do have great

effects upon both the supercooling points and the likelihood of tissue injury when freezing occurs. The lower levels of glycerol (less than 4 per cent) found in *cecropia* and several other species, however, would be expected to have little influence upon the effects of low temperatures, and indeed both Salt's results and ours indicate a lack of general correlation between glycerol content and cold hardiness among various species.

In both *Bombyx mori* and *H. cecropia* it has been shown that the appearance and disappearance of glycerol coincide with the initiation and breaking, respectively, of diapause, and that production of glycerol is not dependent upon exposure to low temperature. Although other species pass through diapause without accumulating glycerol, it appears that the production of glycerol, when it does occur, may be related to the metabolic peculiarities of the diapausing state.

In *Bombyx*, Chino has shown the appearance of glycerol and sorbitol to coincide quantitatively with the disappearance of glycogen, and in *cecropia*, although we have not analyzed for glycogen, the amount of glycerol formed is too large to be readily accounted for by fat metabolism, and carbohydrate is presumably its chief source. It is known that anaerobic glycolysis in insects often yields less than the expected amount of lactate, and that instead the products may be equimolar amounts of pyruvate and α-glycerophosphate (Kubista, 1957; Chefurka, 1958). This apparently results from the presence of exceedingly little lactic dehydrogenase together with very active α-glycerophosphate dehydrogenase (Zebe and McShan, 1957). In some preliminary assays of these two enzymes in extracts of tissues of *H. cecropia* and *T. polyphemus*, we have found a similar relationship, glycerophosphate dehydrogenase being many times more active than that of lactate. In addition, the presence of α-glycerophosphate in the blood plasma of these and other insect species has now been demonstrated (Wyatt, Meyer, and Kropf, 1958; Wyatt and Kropf, 1959). Glycerol could arise by enzymic hydrolysis of this ester.

It is also possible that glycerol might be derived by direct reduction of glyceraldehyde, as suggested by Faulkner (1958), by means of the triphosphopyridine nucleotide-linked dehydrogenase studied by him.

The question remains why glycerol should accumulate only during diapause, and then only in certain species. In fly flight muscle, Estabrook and Sacktor (1958) have presented evidence that α-glycerophosphate may be formed by the action of a soluble DPN-linked dehydrogenase and reoxidized by a particle-bound cytochrome-linked enzyme. Our preliminary assays indicate that the soluble DPN-linked dehydrogenase remains active in *cecropia* tissues during diapause as well as in developing stages. This is in contrast to the behavior of certain enzymes of electron transport, notably cytochromes b and c, which virtually disappear during diapause (Shappirio and Williams, 1957). The decline in the cytochromes could lead to increased ratio of DPNH to DPN and to reduced effective activity of the particulate α-glycerophosphate

oxidase. Both of these effects would favor accumulation of α-glycerophosphate, which could presumably by hydrolyzed to glycerol. It may appear inconsistent with this view of the cause of glycerol accumulation that blood α-glycerophosphate is as abundant in stages and species which do not accumulate glycerol as in those that do (Wyatt and Kropf, 1959). However, concentrations in the plasma may not reflect those at the site of glycerol production. Clearly, further knowledge of the balance of enzyme activities, coenzyme levels, and the distribution of substrates between tissues and plasma will be necessary for understanding this situation. The actual rate of glycerol production by *cecropia* pupae in early diapause is rather small—about 1.5 μmole/ml. blood/day—and it is evident that a slight shift in relative rates could bring about or prevent a net accumulation of this magnitude. Yet, the accumulation of glycerol in *cecropia* appears to be a characteristic feature of diapause as opposed to development, and in view of the interest attached to understanding regulatory steps in metabolism, it will warrant further analysis.

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