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II. Trehalose and Other Carbohydrates*

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A curious feature of insect blood, shown by many analyses, is the apparent absence of all but minute amounts of sugar (for reviews, see Beutler, 1939; Babers, 1941; Buck, 1953). Reducing substances are abundant, but (except in the case of a few species) the greater part of these are not fermentable by yeast and are therefore presumably not sugar. Typical are data on the silkworm, *Bombyx mori*, in which the reported levels of blood sugar range from zero up to about 30 mg. per 100 ml. (Florkin, 1937; Kuwana, 1937; and others) —remarkably small amounts for a metabolically active animal with a high dietary intake of sugars. Twenty years ago, however, Kuwana (1937) made the significant discovery that acid hydrolysis of *Bombyx* hemolymph caused release of much reducing sugar from a substance which was not glycogen. Even earlier, Ronzoni and Bishop (1929) had reported an unidentified non-reducing "polysaccharide below glycogen" in the blood of honeybee larvae. These reports received little attention. Recently, unaware of them, we made essentially the same finding. The blood of *Bombyx mori* and of two other insect species was found to contain large amounts of anthrone-reactive material which was neither a reducing sugar, sucrose, nor glycogen (Wyatt, Loughheed, and Wyatt, 1956).

As already reported in preliminary form (Wyatt and Kalf, 1956), we have now identified as a major sugar of insect plasma the non-reducing disaccharide α,α-trehalose. In the present paper, we give details of the isolation and characterization of this sugar together with some quantitative analyses of trehalose and certain other carbohydrates in insect plasma. Their distribution and significance will be considered.

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Materials

Larvae of the silk moths, *Telea polyphemus* and *Platysamia cecropia*, were reared in outdoor cages on maple and wild cherry respectively; cocoons containing diapausing pupae were obtained from dealers. Larvae of *Drosophila repleta* were provided by Professor D. F. Poulson. Specimens of the milkweed bug, *Oncopeltus fasciatus*, the flour beetle, *Tribolium confusum*, the mosquito, *Aedes aegypti*, and the tobacco worm, *Protoparce sexta*, were obtained through the courtesy of Mr. Neely Turner and other

TABLE I

Properties of Hemolymph Plasma Samples

Sample No.*	No. of insects	Sex	Stage	Dry matter
				gm./100 ml.
Tp 11	15	Mixed	Mature larvae	7.6
Tp 13a	3	♂	Pupae, kept 7 mos. at 5°C.	12.4
Pc 15	66	Mixed	Mature larvae	7.9
Pc 7a	3	♂	Pupae kept 5 mos. at 5°C.	16.0
Pc 7b	3	♀	Pupae kept 5 mos. at 5°C.	15.7
Pc 10a	3	♂	Pupae kept 4 mos. at 5°C., then 5 days at 25°C.	12.7
Pc 10b	3	♀	Pupae kept 4 mos. at 5°C., then 5 days at 25°C.	17.0
Pc 21a	6	♀	Pupae, from the same collection as Pc 7 and Pc 10, kept 12 mos. at 5°C.	15.5
Pc 21b	6	♀	Developing adults (after 12 mos. diapause), 10th day‡	12.6
Pc 21c	4	♀	Developing adults (as above), 15th day	17.0
Pc 21d	6	♀	Developing adults (as above), 20th day	12.6

* Tp, *Telea polyphemus*; Pc, *Platysamia cecropia*.

‡ Stages in adult development according to the time-table of Schneiderman and Williams (1954).

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Lyophilized plasma was prepared as previously described (Wyatt, Loughheed, and Wyatt, 1956). Samples used for quantitative analysis are listed in Table I. Analyses were also carried out on some of the samples described in the earlier paper (Bm 7, 11, 12, and 14, are from larvae of the oriental silkworm, *Bombyx mori*; Bm 15, from pupae of *B. mori*; Gm 1, from larvae of the wax moth, *Galleria mellonella*; Dh 1, from larvae of the spruce sawfly, *Diprion hercyniae*).

Methods

Preparation of Deproteinized and Deionized Extracts

Large samples of hemolymph for isolation of trehalose were deproteinized by adding an equal volume of 0.6 N $HClO_4$ and centrifuging. The precipitates were reextracted with 0.3 N $HClO_4$, then the combined extracts were neutralized with KOH, chilled,

and freed of $KClO_4$ on the centrifuge. Solutions were then deionized by passage first through a bed of Dowex 1 (× 10, formate) and then a bed of Dowex 50 (× 12, acid). Neutral carbohydrates were washed from the columns with water, and their distribution in the effluents was followed by analysis with anthrone.

Smaller volumes of insect blood or of tissue extracts for paper chromatography and electrophoresis were usually freed of protein and glycogen with hot ethanol (final concentration 60 to 70 per cent) and then deionized with a small column containing a layer of each of the above resins. Effluent solutions were dried in a stream of air; the residues were rinsed with petroleum ether to remove lipides and then redissolved in small volumes of water. In a few cases, protein was coagulated in aqueous extracts of tissues by heating at 100° C.; such solutions, however, tended to clog the resin bed.

Extracts for quantitative chromatography were prepared from about 10 mg. of lyophilized plasma. This was extracted 3 times with 0.4 ml. portions of 70 per cent ethanol, the mixture being heated each time to 75° C. for 5 minutes. The extracts were applied successively to a layered deionizing column (4 mm. × 50 mm. containing Dowex 1 and Dowex 50) and elution was completed with a further 0.4 ml. of 70 per cent ethanol. After evaporation of the effluent solution, the residue was carefully rinsed with 0.5 ml. of petroleum ether and redissolved in 0.05 ml. of water. 10 μl. portions were used for chromatography.

To prepare samples for quantitative determination of trehalose by chemical isolation, 5 to 10 mg. of lyophilized plasma was suspended in a measured volume (usually 1 to 2 ml.) of 60 per cent ethanol. The tube was stoppered, heated at 75° C. for 5 to 20 minutes, and then centrifuged. Portions of 0.1 ml. were pipetted into pyrex test tubes and evaporated to dryness before analysis. Removal of ethanol was necessary since its presence was found to enhance the color from sugars in the anthrone reaction. In a few experiments, portions of such extracts were deionized by passing them through layered resin columns (as above), the effluent being evaporated and redissolved in water before taking samples for analysis.

Paper Chromatography

Whatman No. 1 paper was used, generally in the descending technique, the solvent being allowed to drip from the serrated lower end of the paper. A solvent system which we found satisfactory for qualitative and quantitative work is described in Table II. Good distribution of R_G values, but slower mobility, was given by *n*-butanol–acetic acid (Partridge, 1948). We also occasionally used ethyl acetate–pyridine–water (Jermyn and Isherwood, 1949) and butanol–ethanol–acetone–water (Gray and Fraenkel, 1954), in the ascending technique, but these fast-flowing systems gave, in our experience, rather diffuse spots.

For detection of carbohydrates including trehalose and glycogen the permanganate-periodate reagent of Lemieux and Bauer (1954) was used. To prevent migration of the spots it was found advantageous to spray the papers while they rested horizontally on a rack. For detection of sugars other than trehalose, benzidine trichloroacetate (Bacon and Edelman, 1951) was found useful. After chromatography, 1 μg. of maltose was detectable by this reagent, whereas as much as 300 μg. of trehalose gave no visible spot.

In the earlier stages of the study, trehalose and glucose were determined by quanti-

tative chromatography. For this purpose, the paper was marked in lanes 3 cm. wide. Each sample to be analyzed was spotted on each of three lanes, one of which was next to the margin of the paper. Two lanes were always left blank. After running the chromatogram, the marginal lanes were cut off, treated with periodate-permanganate reagent, and then used to locate the sugars in the unsprayed lanes. The appropriate areas were cut out, usually as 3 cm. squares, and the sugars eluted with about 0.5 ml. of water (Consden, Gordon, and Martin, 1947). The eluates were evaporated to dryness in a desiccator and analyzed with anthrone. Blanks from 9 sq. cm. of paper gave color equivalent to 4 to 6 μg. of glucose; this value was not materially lowered by prior washing of the paper. The procedure was tested with a solution containing

TABLE II

Chromatographic Separation of Sugars

Solvent system: 80 per cent (by volume) *sec.*-butanol in water, run at 37°C. for about 44 hours. Movement of glucose: 10 to 14 cm. R_G = movement of sugar/movement of glucose

Sugar	R_G	Sugar	R_G
Raffinose	0.13	Galactose	0.90
Melezitose	0.20	Glucose	1.00
Trehalose	0.38	Mannose	1.24
Maltose	0.39	Fructose	1.28
Cellobiose	0.39	Arabinose	1.35
Sucrose	0.56	Ribose	1.91
		N-Acetylglucosamine	1.56

2.68 mg. glucose and 15.41 mg. trehalose per ml. Four analyses gave: glucose, mean 2.54, range 2.25 to 2.73 mg. per ml.; trehalose, mean, 16.0, range 15.1 to 17.4 mg. per ml.

Electrophoretic Separation of Sugars

Since paper chromatography failed to resolve trehalose from maltose and cellobiose, we used electrophoretic mobility in borate (Consden and Stanier, 1952) as a further means of separating sugars. Solutions were applied to a strip of Whatman No. 1 paper, which was moistened with 0.02 M $Na_2B_4O_7$ and then immersed in CCl_4 in the apparatus of Markham and Smith (1952). A potential gradient of 40 v. per cm. was applied for 2 hours. The paper was dried, sprayed with dilute ethanolic acetic acid to neutralize the borate, and again dried before spraying with permanganate-periodate reagent. Trehalose and sucrose, which would not be expected to form borate complexes, moved about 4 cm. toward the cathode, presumably because of migration of the buffer. Relative to the position of trehalose, cellobiose moved 3 cm., maltose 5.5 cm., and glucose 29 cm. toward the anode.

Chemical Isolation of Trehalose for Quantitative Determination

In later quantitative analyses, we supplanted the rather tedious chromatographic isolation of trehalose by a procedure which takes advantage of the exceptional sta-

bility of this sugar to both acid and alkali. Samples containing 20 to 75 μg. of trehalose were evaporated to dryness in pyrex tubes and redissolved in 0.2 ml. of 0.1 N H_2SO_4. Each tube was capped with aluminum foil and heated at 100° C. for 10 minutes to hydrolyze any sucrose or glucose-1-phosphate. The solution was made alkaline by addition of 0.15 ml. of 6 N NaOH and again heated at 100° C. for 10 minutes to destroy reducing sugars. The sample was then chilled, 2.0 ml. of anthrone reagent was added, and the analysis completed as described below. Trehalose standards and blank tubes were carried through the acid and alkaline treatments. Loss of trehalose during this treatment is small (about 5 per cent). A number of carbohydrates were tested for interference in amounts of 100 μg. and 500 μg. Color yields per 100 μg. of carbohydrate, expressed as micrograms of trehalose giving equal color, were: glucose, galactose, sorbose, lactose, sucrose, melezitose, glucosamine, *N*-acetylglucosamine, glucose-1-phosphate, glucose-6-phosphate, arabinose, ribose, rhamnose, less than 1; mannose, fructose, maltose, cellobiose, fructose-1,6-diphosphate, 1 to 2; raffinose, 5. Glycerol, which gives an orange color with the anthrone reagent (Schutz, 1938), does not interfere at 630 mμ.

Determination of Glycogen

The alcohol-insoluble residues after extraction of sugars from plasma were suspended in 0.1 ml. of 30 per cent KOH and heated at 100° C. for 20 minutes. 0.4 ml. of 80 per cent ethanol was added, and the mixture was warmed to flocculate the precipitate, and centrifuged. The precipitate was washed once with absolute ethanol, dried, and its glycogen content determined with anthrone. It is possible that the fraction thus isolated may contain anthrone-reactive substances other than true glycogen (Balmain, Biggers, and Claringbold, 1956).

Anthrone Reaction

Anthrone reagent was prepared according to Mokrasch (1954) and used by mixing 2.0 ml. of reagent with 0.35 ml. of aqueous sample in a 13 mm. pyrex tube. For use with dried samples, the reagent was diluted with one-sixth its volume of water. Aldose and ketose could be distinguished by reading the color after different periods of heating (Mokrasch, 1954); when this was not necessary, the tubes were heated at 100° C. for 15 minutes, then cooled to room temperature. With solutions which had contained NaOH, excessive chilling led to crystallization of sodium sulfate. Color was read in the Beckman spectrophotometer at 630 mμ or in the Klett-Summerson colorimeter (adapted for 13 mm. tubes) with No. 66 filter.

Isolation and Determination of Glucose-6-Phosphate

In order to account as completely as possible for the carbohydrate content of some samples of insect blood, and to confirm the earlier inference that larval *Bombyx* blood contains glucose-6-phosphate (Wyatt, Loughheed, and Wyatt, 1956) we performed some analyses by ion exhcange chromatography. Plasma was deproteinized with $HClO_4$ and the extract was applied to a column of Dowex 1 (× 10, formate) and eluted with a concentration gradient of formic acid (Hurlbert, Schmitz, Brumm, and Potter, 1954). Fractions were collected and analyzed for carbohydrate with anthrone

and for phosphorus (method of Lowry, Roberts, Leiner, Wu, and Farr, 1954, scaled to 2 ml. volume).

Glucose-6-phosphate was determined enzymically in fractions from the columns and in unfractionated $HClO_4$ extracts of insect plasma by spectrophotometric measurement of reduction of triphosphopyridine nucleotide coupled with the action of glucose-6-phosphate dehydrogenase. When results were negative, the absence of inhibitory substances was demonstrated by addition of known glucose-6-phosphate. Enzyme and coenzyme were obtained from the Sigma Chemical Co., St. Louis, Missouri, and the reaction was carried out as recommended in Sigma Bulletin No. 201.

RESULTS

Isolation and Characterization of Trehalose from Hemolymph

From 100 diapausing pupae of *Telea polyphemus*, 63.4 gm. of plasma was obtained. After removing protein and ionic substances, the solution was lyophilized, yielding 958 mg. of crude carbohydrate. The chief anthrone-reactive substance was shown to be freely dialyzable, and therefore not glycogen. It did not reduce Benedict's reagent. After chromatography on paper it could not be detected by spraying with benzidine trichloroacetate or with aniline phthalate (Partridge, 1949), but with permanganate-periodate reagent a spot appeared having a mobility similar to that of maltose. After hydrolysis (N H_2SO_4, 105°C., 1½ hours) glucose was the sole product detected by chromatography. These properties suggested that the substance might be trehalose.

For purification, 400 mg. of the crude carbohydrate was dissolved in 3.0 ml. of water. Since analysis of the effluent from the columns had shown incomplete separation of carbohydrates from phosphorus-containing compounds, the latter were precipitated by addition of 1.6 ml. of saturated $Ba(OH)_2$ followed by ethanol to a concentration of 70 per cent and heating. A flocculent precipitate was centrifuged off, and the excess of barium was removed as the carbonate. The alcoholic solution was stored at 3°C. After 24 hours a small amorphous precipitate was removed and the solution was evaporated to dryness. The residue was dissolved in 0.5 ml. of water and absolute ethanol was slowly added. At 66 per cent concentration, a fine amorphous precipitate formed and was removed on the centrifuge. Alcohol was added to a concentration of 80 per cent and the clear solution was left in the cold. After 24 hours, large colorless rhombic crystals had formed. These were collected, recrystallized twice from 80 per cent ethanol, and dried; yield, 121 mg. *Anal.* Loss on drying at 105°, 9.07. Calcd. for $C_{12}H_{22}O_{11} \cdot 2H_2O$: H_2O, 9.52. The anhydrous material gave C, 41.44; H, 6.40. Calcd. for $C_{12}H_{22}O_{11}$: C, 42.10; H, 6.48. M.P. 96.8–97.2, corr. Optical rotation (for the dihydrate) $[\alpha]_{22}^{D}$ + 184° (H_2O, *c* 1). Commercial trehalose dihydrate, twice recrystallized, gave M.P. 96.5–97.5, $[\alpha]_{22}^{D}$ + 174°. The sugar isolated from hemolymph migrated identically with commercial trehalose when subjected to paper chromatography in four solvent systems.

Trehalose Octaacetate.—This derivative was prepared from 20 mg. of the isolated trehalose with acetic anhydride in pyridine, by an adaptation of the procedure of Pangborn and Anderson (1933). The product was twice recrystallized from ethanol, yielding 11 mg. of colorless needles, M.P. 78–79°. An acetate prepared by the same procedure from commercial trehalose had identical

TABLE III

Presence of Trehalose in Various Organisms

Organism	Material examined	Trehalose found	Remarks
Insects			
Oncopeltus fasciatus (Hemiptera)	Whole adults	+	
Tribolium confusum (Coleoptera)	Whole adults	+	
Aedes aegypti (Diptera)	Whole larvae	+	
Drosophila repleta (Diptera)	Whole larvae	+	
Diprion hercyniae (Hymenoptera)	Larval plasma	+	
Galleria mellonella (Lepidoptera)	Larval plasma	+	
Bombyx mori (Lepidoptera)	Larval and pupal plasma	+	
Protoparce sexta (Lepidoptera)	Larval plasma	+	
Telea polyphemus (Lepidoptera)	Larval and pupal plasma	+	
Platysamia cecropia (Lepidoptera)	Pupal plasma	+	
Other			
Lobster (*Homarus americanus*)	Plasma	−	Limit of detectability: 10 mg. per 100 ml.
Human	Plasma	−	Limit of detectability: 5 mg. per 100 ml.
Tobacco	Fresh leaves	−	

crystal form and melting point, which correspond to those of trehalose octaacetate (Pangborn and Anderson, 1936).

Recognition of Trehalose in Other Species of Insects

Recognition of trehalose in other species rests on the following criteria: (1) by paper chromatography of a deproteinized, deionized extract, a substance was demonstrated having the mobility of trehalose and reacting with the periodate-permanganate reagent, but not reacting with benzidine trichloroacetate; (2) the substance eluted from a paper chromatogram and examined by electrophoresis in borate exhibited the immobility characteristic of trehalose; (3) the substance when eluted from a paper chromatogram, hydrolyzed with

H_2SO_4, and rechromatographed, gave glucose as the sole product. As shown in Table III, trehalose was found in all the insects examined, but not in preparations from a crustacean, a mammal, nor a flowering plant.

Characterization of Glucose-6-Phosphate from Hemolymph

Anion exchange fractionation has been applied as yet only to plasma from *T. polyphemus* pupae and from *Bombyx mori* larvae. With the former, all of the carbohydrate passed through the columns with the effluent front, indicating the absence of phosphorylated sugars. In the *Bombyx* extracts, however, a peak containing both carbohydrate and phosphorus came off the columns at the position in the acid gradient expected of a hexose monophosphate. This

TABLE IV

Analysis of Fraction from Bombyx Hemolymph Containing Glucose-6-Phosphate

	Total P	10 min. labile P*	Aldohexose (anthrone)	Ketohexose (anthrone)	Glucose-6-phosphate (enzymic)
	μmol. per ml. solution				
Fraction from hemolymph	13	0	7.6	2.2	6.2
Known glucose-6-phosphate	7	0	6.8	0.1	6.1

* 1 N H_2SO_4, 100°C.

fraction, after concentration, gave the analyses shown in Table IV. These indicate the presence of glucose-6-phosphate together with other stable phosphate compounds, apparently including a ketose derivative. Paper chromatography (Wyatt, Loughheed, and Wyatt, 1956) also showed a major component migrating identically with glucose-6-phosphate. Because of lack of material, further fractionation was not attempted.

Quantitative Content of Carbohydrates in Hemolymph

The results of various quantitative determinations are summarized in Table V.

The values for total sugars were obtained by anthrone analysis, before chromatographic or chemical isolation of trehalose, on samples of the substances extracted from lyophilized plasma by 60 to 70 per cent ethanol. For the chromatographic method, these solutions had been deionized; for the chemical method, they were deionized in only a few cases, as indicated. The close agreement between total carbohydrate and trehalose content in the extracts from *Bombyx* plasma (even without deionization) must signify that glucose-6-phosphate was not extracted from the protein.

It will be noted that when both methods of trehalose analysis were used on the same sample, results from the chemical method are higher by 12 to 40

per cent. That the two methods give similar ratios of trehalose to total sugars (in all samples except Dh-1) suggests that both afford satisfactory isolation of trehalose, and that the differences arose in preparation of the extracts. One difference in procedure is that the dried extracts for chromatography were

TABLE V

Quantitative Analyses of Carbohydrates of Hemolymph

0, none detectable; —, not tested. Except when indicated, values are averages of two or more replicate analyses.

Sample No.	Chromatographic method			Chemical method		Glycogen	Glucose-6-phosphate‡
	Total sugars*	Trehalose	Glucose	Total sugars	Trehalose		
	Mg./100 ml. plasma						
Bm 7a	—	—	—	305	306	—	88
Bm 7b	—	—	—	397	407	—	121
Bm 12	427	419	3	530	523	12	39
Bm 14a	—	—	—	453	419	—	—
Bm 11	451	442	2	—	—	15	—
Bm 15	200	202	0	—	—	38	—
Gm 1	1356	1312	21	1766	1703	41	0
Dh 1	894‡	664‡	28‡	1089	926	32‡	0
Tp 11	—	—	—	1100, 1017*	1036	—	0
Tp 13a	—	—	—	1408, 1304*	1398, 1324*	—	0
Pc 15	—	—	—	1255	1194	—	—
Pc 7a	439	406	—	587	581	25	14
Pc 7b	536‡	530‡	—	619, 559*	596, 561*	31‡	—
Pc 10a	502‡	475‡	0	620	539	31‡	0
Pc 10b	436‡	416‡	8	598	545	55‡	0
Pc 21a	—	—	—	987	949	—	—
Pc 21b	—	—	—	709	652	—	—
Pc 21c	—	—	—	1046	966	—	—
Pc 21d	—	—	—	1199	1159	—	—

* Deionized.

‡ Single analysis only.

washed with petroleum ether; although sugars are insoluble, some fragments of the residue may have been dislodged and lost. Another factor may be deionization, for deionization of a few extracts before analysis by the chemical method lowered the results by 5 to 10 per cent, suggesting removal of interfering substances, perhaps organic acids. Sugar standards passed through similar columns were recovered quantitatively. It seems likely that the true trehalose levels may be intermediate between the values obtained by the two procedures.

It is also apparent that the present results are lower than those reported

earlier for total carbohydrate in some of the same hemolymph samples (Wyatt, Loughheed, and Wyatt, 1956). Three sources of difference are evident. The earlier extracts were made with acid instead of alcohol and thus included glycogen and glucose-6-phosphate. We have also subsequently found that the trichloroacetic–perchloric acid mixture interferes slightly in the anthrone reaction, enhancing color yield by about 5 per cent. A further difference (5 per cent, for water eliminated in the glycosidic link) is due to the earlier data being expressed as glucose rather than trehalose.

In all the blood samples analyzed from lepidopterous insects (*B. mori*, *G. mellonella*, *T. polyphemus*, and *P. cecropia*) trehalose accounted for more than 90 per cent of the total blood sugar (except Pc 10a by the chemical method, for which the value is 87 per cent). In several samples, the fraction represents 100 per cent, within experimental error. In the sawfly *Diprion hercyniae* the proportion of blood sugar represented by trehalose is less (74 per cent by the chromatographic method, 85 per cent by the chemical method). Chromatograms from this species showed two unidentified periodate-reactive spots, one migrating more slowly than trehalose (possibly a trisaccharide) and one moving rather faster than glucose; it is possible that these substances include a carbohydrate which resists acid and alkaline degradation.

DISCUSSION

Trehalose is know to occur in many fungi and is commonly regarded as a product characteristic of lower plants (Myrbäck, 1949). Nearly one hundred years ago, this sugar was isolated from cocoons (collected in Syria and known as "*trehala*") of the weevil *Larinus nidificans* (Berthelot, 1859), and more recently it has been identified in another "desert manna" believed to be the excretion of a scale insect (Leibowitz, 1944). Nevertheless, the possibility of its occurring in insect blood and having a normal role in insect metabolism has apparently not been considered before the present investigation. It is now clear that trehalose is widely distributed among insects. Since our preliminary publication, trehalose has been reported in the locust, *Schistocerca gregaria* Howden and Kilby, 1956), the blowfly *Phormia regina*, and the honeybee (Evans and Dethier, 1957). In the species that we examined quantitatively, it is the major blood sugar. This is not true of all insects, however, for the principal blood sugar of *Gastrophilus* is fructose (Levenbook, 1950) and the blood of adult bees contains very large amounts of glucose together with some fructose (Beutler, 1936; von Czarnowski, 1954).

Trehalose has also recently been found in invertebrates other than insects. Fairbairn and Passey (1957) have isolated crystalline trehalose from *Ascaris* and have demonstrated this sugar chromatographically in several other parasitic worms. On the other hand, we were unable to detect trehalose in the blood of a lobster.

Of the insect species which we examined, only one (*Drosophila*, feeding on yeast) is known to have a source of trehalose in its diet. We have been unable to detect trehalose in tobacco leaves, the food of the tobacco hornworm. Insects must therefore synthesize this sugar. Synthesis of trehalose from glucose undoubtedly accounts for the observations of Kuwana (1937), who fed glucose to silkworms and followed the subsequent changes in blood sugar. Reducing sugar reached a maximum 4 hours after feeding and then fell sharply, but the fall in reducing sugar was accompanied by a rise in the level of a substance which gave reducing sugar upon hydrolysis: this reached a maximum some 10 hours after feeding, and was still elevated after 24 hours. The rapid conversion of other sugars to trehalose is also signalled by the fact that *Galleria* larvae, on a diet containing honey, have 1.5 per cent of trehalose in their blood and only 0.02 per cent of glucose.

That various insects are capable of utilizing trehalose is known. Bee larvae and adults can metabolize ingested trehalose efficiently, as indicated by measurements of survival time (Bertholf, 1927; Phillips, 1927; Vogel, 1931). It will also maintain adult blowflies (Fraenkel, 1940; Hassett, Dethier, and Gans, 1950) and *Drosophila* (Hassett, 1948); in *Drosophila* it is one of the few sugars which will support continuous flight (Wigglesworth, 1949). Moreover, aphids contain an enzyme which digests trehalose (Duspiva, 1954). These results could be attributed to the presence of non-specific digestive α-glucosidases in insects. However, trehalose is at least as good a substrate as glucose for respiration of housefly flight muscle homogenates (Sacktor, 1955), and Frèrejacque (1941) found that enzyme preparations from a number of insect species split trehalose much more rapidly than sucrose or maltose, and that the rate is increased by the presence of phosphate. This last finding, which we have confirmed, might be explained by phosphorolytic cleavage; such a mechanism would be of evident physiological value, permitting conservation of the energy of the glycosidic linkage. A study of the metabolism of trehalose by insect tissues will be reported in a later communication.

It is of interest to compute what fraction of an insect's carbohydrate reserve is in the form of trehalose, and how this changes during metamorphosis. If one assumes (1) that the total carbohydrate in the mature larva of the Cecropia silkworm consists of tissue glycogen and blood trehalose, (2) that glycogen comprises 1 per cent of the fresh weight, as in *Prodenia* (Babers, 1941), and (3) that the blood, making up 30 per cent of the body weight, contains 1 per cent of trehalose, then trehalose would account for some 23 per cent of the total carbohydrate of the insect. In the pupa, if glycogen has risen to 2 per cent of the body weight, as in *Prodenia* and in *Antheraea pernyi* (Drilhon, 1935), and the blood contains 0.5 to 1.0 per cent of trehalose, then the fraction of total carbohydrate accounted for by trehalose becomes 7 to 13 per cent. These approximations are probably representative of the changes occurring at the

time of pupation in many lepidopterous insects. They suggest conversion of trehalose into glycogen. This possibility is significant in relation to a long-standing problem; namely, the source of the net increase in glycogen that occurs in various insects after the cessation of larval feeding. Increase in glycogen at this time was noted, for example, in *Bombyx* (Bataillon and Couvreur, 1892), in *Prodenia* (Babers, 1941), and in *Popillia* (Ludwig and Rothstein, 1949). An interpretation which originates with Couvreur (1895) and recurs in the entomological literature postulates that glycogen is synthesized from fat. Whether net conversion of fat to carbohydrate does in fact take place in insects is of great interest. Mammals are apparently incapable of this process (Deuel and Morehouse, 1946), but it does occur in plants (Murlin, 1933; Christiansen and Thimann, 1950), and evidence has recently been adduced in support of its occurrence in *Ascaris* (Passey and Fairbairn, 1956). In the analyses which suggest the conversion of fat to glycogen in insects, trehalose was overlooked. A decision as to whether utilization of trehalose can account quantitatively for the glycogen synthesis during pupation must await a thorough analytical study.

In the Cecropia pupa, our analyses (Pc 7a and 7b, 10a and 10b) fail to show any significant difference between the levels of trehalose in blood of male and female diapausing animals. An increase in blood trehalose during prolonged storage at 5°C., presumably reflecting conversion from glycogen, is indicated by the analysis of sample Pc 21a. Samples Pc 21b to 21d, taken at intervals during adult development, show a fall, then a rise, in blood trehalose concentration. To obtain total blood sugar one must, of course, take into account the blood volume, which decreases during adult development. The change in blood volume has not been accurately measured in Cecropia, but it is indicative that the average yield from bleeding fell progressively from 28 per cent of body weight in diapausing pupae to 10 per cent on the 20th day of development. Multiplying the sugar concentrations by these values indicates that the total trehalose may drop by adult emergence to about half of its amount in the pupa. The proportional decline in trehalose during adult development is thus less than that of glycogen, which falls by the time of emergence to one-fourth or less of its level in the pupae (*Bombyx*, Needham, 1929; *Antheraea pernyi*, Drilhon, 1935). Evidently, trehalose plays a relatively minor role as an energy reserve during metamorphosis. It may be more significant in carbohydrate transport, perhaps conveying glucose units from fat body glycogen to sites of metabolism in other tissues.

Since various mycobacteria contain lipides which are esters of fatty acids with trehalose (see Myrbäck, 1949), the question arose whether such lipids might be found also in insects. In unpublished experiments by G. R. Wyatt and W. L. Meyer, lipides were extracted from *T. polyphemus* pupae with ethanol ether and saponified. Chromatography of the products revealed large amounts of glycerol but failed to detect trehalose.

Whereas trehalose accounts for the greatest part of the carbohydrate in the plasma samples which we examined, several other carbohydrates are present. Glucose occurs in small amounts. The trace amounts of fructose and sucrose previously reported in *Bombyx* (Wyatt, Loughheed, and Wyatt, 1956) are below the limits detectable by our present methods. Apparent glycogen occurs in small amount in larval blood, increasing after pupation (Bm 15) presumably owing to histolysis. Glucose-6-phosphate, the presence of which in insect plasma has previously been indicated (Smolin, 1952; Wyatt, Loughheed, and Wyatt, 1956), has now been characterized by a specific enzymic method, and evidence has been obtained for smaller amounts of a ketose phosphate. In view of Faulkner's (1956) demonstration of phosphohexose isomerase in *Bombyx* blood, the latter is likely to be fructose-6-phosphate. In larval *Bombyx* hemolymph, glucose-6-phosphate can occur at quite high levels; it undoubtedly serves as a substrate for the triphosphopyridine nucleotide–linked sugar phosphate reductase of *Bombyx* plasma recently described by Faulkner (1956).

SUMMARY

α,α-Trehalose, a sugar previously regarded as a product characteristic of certain lower plants, has been identified as a major blood sugar of insects. Trehalose has been isolated in pure form from the blood of pupae of the silk moth, *Telea polyphemus*, and has been recognized chromatographically in all the insects examined, which comprise 10 species belonging to 5 different orders. Trehalose has been determined quantitatively with anthrone after either chromatographic separation or chemical degradation of other sugars. In larvae and pupae of 4 species of Lepidoptera it ranges from 0.2 to 1.5 gm. per 100 ml. of blood and makes up over 90 per cent of the blood sugar; in larvae of a sawfly, about 80 per cent of the blood sugar is trehalose. In *Bombyx mori* and *Platysamia cecropia*, the pupal blood trehalose level is about half that in the mature larva, suggesting utilization of trehalose for glycogen synthesis during pupation. Small amounts of glucose and apparent glycogen are also present in the plasma of these insects. In *Bombyx* larval plasma there is also 0.04 to 0.12 gm. per 100 ml. of glucose-6-phosphate and smaller amounts of an apparent ketose phosphate.

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