THE CHEMISTRY OF INSECT HEMOLYMPH

ORGANIC COMPONENTS OF THE HEMOLYMPH OF THE SILKWORM, BOMBYX MORI, AND TWO OTHER SPECIES*

BY G. R. WYATT, T. C. LOUGHHEED, AND S. S. WYATT

(From the Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada, and the Department of Biochemistry, Yale University, New Haven)

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The composition of insect blood, or hemolymph, has been extensively investigated, a recent review on the subject being that of Buck (1953). Most of this work has been concerned with the chemical differences between phylogenetic groups or with the changes in major groups of metabolites during metamorphosis. Only one study of which we are aware (Levenbook, 1950 *c*) had as its object the formulation of a physiological solution for use in studies on an insect's tissues. The majority of "insect Ringer solutions" in use (see Buck, 1953, p. 161) bear no relation to the composition of insect body fluids, and the lack of a balanced physiological solution hindered experiments by one of us (S. S. W.) on culturing tissues of the silkworm, *Bombyx mori* L. To assist in formulation of such a solution, it was decided to obtain more detailed information on the composition of silkworm hemolymph, making use of some recently developed techniques, notably chromatography on filter paper.

Our principal object was the estimation of individual compounds, such as free amino acids and sugars, on which there is little published information. We also measured hydrogen ion concentration and nitrogen and phosphorus distribution. To give the results more physiological significance, we examined hemolymph from a series of developmental stages of the silkworm, and also single samples from each of two other insect species.

Although it was necessary to conclude the study while it was very incomplete, the results were of value in the formulation of a tissue culture medium, as described in the preceding paper (S. S. Wyatt, 1956). The results also demonstrate that a substantial proportion of the organic components of insect hemolymph is yet unidentified, and suggest that further work on the composition and physiology of this fluid would be rewarding.

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TABLE I

Properties of Hemolymph Samples

Sample No.*	No. of insects	Stage	Approximate time from molt II	pH‡	Dry matter§	Protein‖	Non-protein N
			days		*gm./100 ml.*	*gm./100 ml.*	*mg./100 ml.*
Bm 16	42	Molt II	0	—	7.0	—	—
Bm 17	115	Early III	2	—	5.8	1.2	425
Bm 18	27	Later III	4	—	6.4	1.7	571
Bm 19	7	Molt III	6	—	6.0	—	—
Bm 20	26	Mid IV	9	—	5.4	—	264
Bm 21	23	Mid IV	—	—	5.6	1.0	520
Bm 9	30	Molt IV	13	6.54	7.8	1.7	522
Bm 8	49	Early V	17	6.39	9.4	1.3	630
Bm 10	31	Mid V	20	6.42	6.8	1.3	500
Bm 7a	15	Mid V, ♂	22	6.40	7.8	1.9	491
Bm 7b	6	Mid V, ♀	22	6.38	8.8	2.6	531
Bm 12	49	Late V	25	6.38	8.0	2.4	489
Bm 6	11	Late V	—	6.46	9.8	—	515
Bm 14a	45	Late V	26	6.47	10.6	5.3	512
Bm 14b¶	45	Late V	26	—	6.0	0.0	551
Bm 11	13	Start of spinning	27	6.50	9.3	4.8	460
Bm 15	*Ca.* 35	Early pupa	31	6.54	10.1	5.2	309
Bm 13	14	Late pupa	37	6.60	10.0	4.2	474
Gm 1	*Ca.* 50	Mature larva	—	—	15.9	8.5	580
Dh 1	*Ca.* 100	Mature larva	—	—	12.3	5.4	686

* Bm, *Bombyx mori*. Samples Bm 6 and Bm 21 are of a white strain, all others are of a yellow strain. Gm, *Galleria mellonella*; Dh, *Diprion hercyniae*.

‡ *In vivo* pH values are probably slightly lower: see text.

§ Based on weight of lyophilized solids, from which a further 6 to 10 per cent of water could be removed at 110°.

‖ TCA-$HClO_4$-precipitable N × 6.25.

¶ Ultrafiltrate of Bm 14a.

Materials, Methods, and Results

Source and Preparation of Hemolymph

The insects used for most of this study were silkworm larvae (*Bombyx mori* L.) of a yellow-blooded strain that has been maintained in the Laboratory of Insect Pathology for 4 years. They were kept at about 21°C. and 40 per cent relative humidity, and were fed several times daily on greenhouse-grown mulberry leaves. Their size (mean length of fully grown larvae, 5.2 cm.; mean weight, 1.4 gm.) was rather less than that achieved by commercial growers. Since there was some variation in rate of growth within each family, the insects were sorted at molting times in order to obtain groups as uniform as possible in development.

Larvae of the wax moth (*Galleria mellonella* (L.)) were raised at 37°C. following the procedure of Good, Morrison, and Mankiewicz (1953). European spruce sawfly larvae (*Diprion hercyniae* (Htg.)) were raised at 21°C. on spruce foliage.

The insects were bled by cutting a proleg and then collecting the hemolymph in a small test tube that was kept chilled and flushed with nitrogen to retard the action of tyrosinase. In the case of very small larvae, the fluid was collected in a capillary pipette and then delivered into a test tube. Each pooled sample was centrifuged in the cold to remove the cells, and the plasma was weighed and lyophilized. The dried blood solids were stored in a desiccator at 4°C. From one sample (Bm 14b) a protein-free ultrafiltrate was prepared by centrifuging through cellophane, using a low speed for 16 hours at 4°C.

For expressing results, the volume of hemolymph corresponding to each sample of solids was calculated using an assumed specific gravity of 1.035 (Ducceschi, 1902).

The samples of hemolymph that were used for analysis are listed in Table I.

Hydrogen Ion Concentration

Measurements of pH were made using a Beckman model G pH meter and "one-drop" glass electrode, calibrated with Beckman standard buffers at pH 4 and pH 7, and checked with buffers prepared in the laboratory. A drop of hemolymph was delivered directly from the insect to the electrode, and a reading was taken as quickly as possible (15 to 30 seconds after bleeding), followed by several readings during a period of 2 minutes or more. In almost all cases the pH reading rose at a rate which was rapid at first and decreased to give almost constant values after 2 minutes. The extent of the observed change from the first reading was 0.05 to 0.3 unit. This rise may be due to loss of CO_2. When the electrode assembly was held in an atmosphere of CO_2, pH values fell instead of rising during the first 2 minutes. Since we have no valid basis for correcting for this effect, we report the mean 60 second readings, which approached the final constant values. It is likely that the *in vivo* blood pH of most individuals may be lower by 0.1 to 0.2 unit.

The results of these pH determinations are given in Table I.

Nitrogen Distribution and Free Amino Acids

Methods.—

Free amino acids were separated by paper chromatography and estimated with ninhydrin. The procedure was as follows:—

Dried blood solids (6 mg.) were suspended in 0.08 ml. of a solution containing $HClO_4$ (1 N, to precipitate K), trichloroacetic acid (TCA, 7.5 per cent, to aid in the precipitation of proteins), and ethylene diamine tetraacetic acid (EDTA, 0.5 per cent, to form, after neutralization on the chromatogram, soluble complexes with the divalent metals). The value of each component of this mixture for producing clear chromatograms was established experimentally. After standing for 1 to 2 hours in the cold, the precipitated protein and $KClO_4$ were removed by centrifugation. From the supernatant solution two 10 μl. portions were taken for chromatography and two

2 μl. portions for N estimation. The remaining solution was heated in a sealed tube at 100°C. for 1½ hours to hydrolyze glutamine and asparagine, and a further two 5 μl. portions were taken for chromatography. To increase the sensitivity of detection of cystine and cysteine, a further 10 μl. portion was spotted on paper and oxidized by applying successively 2 μl. 0.4 per cent ammonium molybdate and 10 μl. 30 per cent H_2O_2 (Dent, 1948).

Non-protein N was estimated by a micro Nessler method (Weed and Courtenay, 1954) in the two portions taken from the acid extract before hydrolysis; results were corrected for the N content of the EDTA. To provide samples for estimation of protein N, the acid-insoluble residue was washed twice with 7.5 per cent TCA and dissolved in 1 N NaOH.

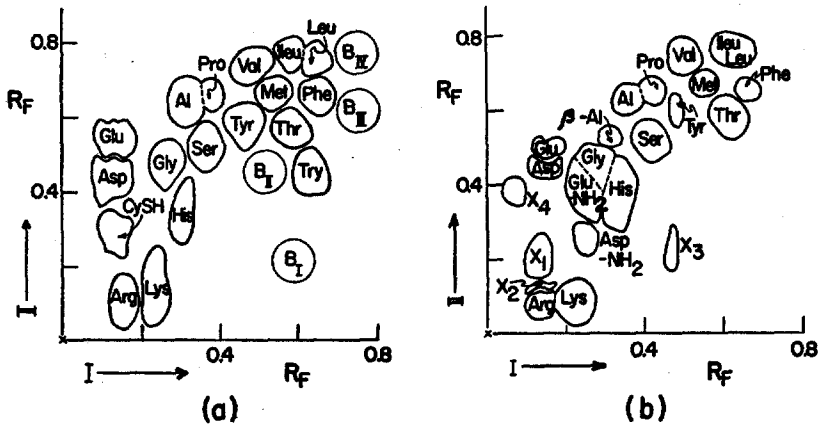

FIG. 1. Tracings of amino acid chromatograms, one-tenth original size. (*a*) Synthetic mixture. B_{I-IV} are the blank spots used in quantitative estimation. (*b*) Extract of silkworm hemolymph. Solvent I: *tert.*-butanol–methyl ethyl ketone-concentrated ammonia-water (10:10:3:5). Solvent II: methanol-pyridine-water (20:1:5).

For chromatography we used Whatman No. 1 filter paper sprayed before use with 0.02 per cent EDTA in 60 per cent ethanol, which was found to eliminate the pink "halos" that otherwise distorted the valine and leucine spots as revealed by ninhydrin. Chromatograms were run by the ascending method without prior vapor equilibration of the jars. The first solvent, modified from Redfield (1953), contained *tert.*-butanol 10, methyl ethyl ketone 10, concentrated ammonium hydroxide 3, and water 5 parts by volume. This mixture gives the same separations as Redfield's second solvent without the necessity for including diethylamine. It runs 30 cm. in about 16 hours. The second dimension was run in Redfield's solvent consisting of methanol 20, pyridine 1, and water 5 parts by volume. This runs 28 cm. in about 5 hours. Typical chromatograms are shown in Fig. 1.

The amino acids were located and estimated with ninhydrin as described by Wellington (1952, 1953). For each amino acid a calibration curve was prepared from chromatograms of standard mixtures. Blanks were cut at four levels in the paper and used for the amino acids at each level, since it was found that the blank absorption

varied in the direction of the second run. Corrections were made when the area of a spot differed greatly from that of the blank.

Since proline was not completely separated from alanine, it was estimated by the method of Chinard (1952), slightly simplified. After spraying with ninhydrin and development of color in the usual way, disks of paper containing proline together with any incompletely separated amino acids were cut from the chromatograms and placed in dry test tubes. To each was added 4 ml. of reagent containing 4.1 ml. concentrated H_3PO_4, (sp. gr. 1.7), 65 ml. glacial acetic acid, and 625 mg. ninhydrin per 100 ml. The tubes were heated at 100°C. for 1½ hours, cooled, and the optical densities read at 515 mμ. After subtraction of the paper blank, the results were read on a calibration curve.

Most of the amino acids were estimated from the chromatograms of unhydrolyzed extracts. Glutamine and asparagine were estimated from the increase in aspartic and glutamic acids following hydrolysis. The chromatograms of hydrolyzed extracts were used also for glycine (which overlaps glutamine), proline (which overlaps alanine), and often for arginine and lysine (which separate better in the smaller amounts used on these papers). Methionine was frequently found partially oxidized to the sulfoxide, and was estimated by combining the spots of the oxidized and unoxidized forms. In the samples oxidized with molybdate and H_2O_2 it was completely converted to the sulfone; as the latter overlaps tyrosine in our solvents, however, it cannot be estimated conveniently. Cystine and cysteine, when present, were estimated from oxidized samples as cysteic acid.

To check the accuracy of the method, mixtures of known composition, containing 8 to 20 μg. of various amino acids, were subjected to chromatography simultaneously with the unknowns. Out of a total of 43 such estimations, each being the average from duplicate chromatograms, 29 fell within 10 per cent of the true values, 38 within 15 per cent, and all but 2 within 20 per cent. With almost all the hemolymph samples two such analyses were carried out, on different days, and the results averaged.

Results.—The values for non-protein N and for protein (calculated approximately from protein N) are given in Table I.

A typical chromatogram of the free amino acids of silkworm blood is shown in Fig. 1 *b*. In addition to the known amino acids, the ninhydrin-reactive spots marked x_1 and x_2 were present in all samples from silkworm, and those marked x_3 and x_4 were occasionally present. Since x_1 occurred consistently in substantial amounts, an attempt was made to characterize a small sample eluted from paper. By hydrolysis (6 N HCl at 110°C. for 16 hours) it could be partially, but not completely, converted to several other ninhydrin-reactive substances. On oxidation with H_2O_2 and molybdate, it gave rise to two new spots, but part remained unchanged. By electrophoresis on filter paper in 1 M acetic acid, x_1 could be separated into two bands, both moving toward the cathode. There was not sufficient material for isolation and further study of these substances. Their molecular weights cannot be very large, since the amount of x_1 was not reduced in blood filtered through cellophane (sample Bm 14b). From these observations it appears that x_1 is a mixture and includes at least one peptide

and an easily oxidizable substance. The latter may contain sulfur, but is not glutathione, which does not migrate in our first solvent. The spot designated x_2 was not altered by hydrolysis, but being present in only small amount was not further studied. X_3 and x_4 were eliminated by hydrolysis and may therefore be peptides.

TABLE II

Free Amino Acids of Hemolymph

Mg./100 ml. plasma; 0, no visible spot, +, present but not estimated.

Sample No.	Bm 17	Bm 18	Bm 20	Bm 21	Bm 9	Bm 8	Bm 10, 7*	Bm 12, 14, 11*	Bm 6	Bm 15	Bm 13	Gm 1	Dh 1
Glycine	76	68	71	59	73	64	53	73	81	100	131	51	108
Alanine	16	15	11	10	32	74	53	50	33	21	20	225	99
β-Alanine	50	31	36	21	45	41	16	39	33	+	+	51	0
Valine	11	20	23	21	63	56	44	23	16	46	39	29	34
Leucine + isoleucine	11	19	16	18	66	60	54	29	16	48	53	42	22
Proline	6	12	16	20	49	20	18	36	135	67	61	520	262
Phenylalanine	2	5	1	4	12	8	13	11	6	17	16	11	10
Tyrosine	0	23	3	11	105	36	40	31	22	72	70	76	38
Serine	83	100	84	106	76	83	117	111	112	76	61	47	200
Threonine	12	19	23	20	50	49	57	36	35	31	38	62	34
Aspartic acid	14	10	8	10	11	13	8	10	13	13	14	38	12
Asparagine	43	41	52	52	64	119	60	59	17	12	36	13	63
Glutamic acid	16	14	27	12	13	15	11	10	11	19	21	22	17
Glutamine	211	262	234	203	190	300	198	143	116	104	126	369	146
Arginine	21	20	15	33	52	51	34	28	19	20	22	39	20
Lysine	83	198	81	99	192	201	158	164	68	106	128	68	43
Histidine	117	128	81	90	209	209	181	273	408	241	236	136	92
Methionine	11	7	2	5	23	11	15	14	14	75	71	27	68
Cystine + cysteine	0	0	0	0	0	13	0	0	0	0	0	0	43
X_1	59	71	39	63	67	74	29	87	44	189	159	15	0
Total	842	1063	823	857	1392	1497	1159	1227	1199	1297	1342	1841	1311
Total N of estimated amino acids	157	195	149	156	246	277	209	234	233	215	229	292	199

* Results for these samples averaged.

The quantitative results on free amino acids in the various blood samples are shown in Table II. The values for x_1 are based on the arbitrary assumption that its color yield is the same per unit weight as that of glycine; they are thus probably minimal estimates, and are included merely to indicate its relative amount in the different samples.

In view of our failure to find tryptophan, cystine, or cysteine on any but a very few chromatograms, a sample of hemolymph was analyzed after adding

tryptophan and cysteine (5 μg. and 8 μg. per chromatogram, respectively). Both amino acids were recovered almost quantitatively.

Carbohydrates

Methods.—

Chromatography of Free Sugars.—Free sugars were identified and semiquantitatively estimated by paper chromatography after a preliminary separation from charged substances by electrophoresis on filter paper. The procedure was as follows:—

A weighed sample (about 10 mg.) of hemolymph solids was dissolved in water and applied as a band across the center of a strip of Whatman No. 3 paper measuring 10 × 30 cm. At one end of the band was applied a marker spot consisting of another small portion of the same hemolymph sample. The paper strip was suspended in an enclosed chamber between two electrode vessels, and 0.1 N NH_4OH was allowed to soak into it from both ends to meet at the sample. A potential of 225 v. was applied for 5 hours, then the paper was removed and allowed to dry. The sugar spot in a guide strip cut from the edge of the paper was located with naphthorescorcinol reagent (Bryson and Mitchell, 1951), the corresponding zone of the quantitative sample was cut out, and the sugars were eluted chromatographically from it with water. The eluate was collected on a polyethylene disk, dried in a stream of warm air, and dissolved in a little water for chromatography on filter paper.

On preliminary chromatograms from silkworm hemolymph, the only sugars that were revealed by a number of spray reagents were glucose, fructose, and sucrose. Identification of these rests solely on R_f values and response to spray reagents. They were resolved satisfactorily on Whatman No. 1 paper developed at 37°C. with water-saturated butanol (Hough, Jones, and Wadman, 1950). The samples for quantitative analysis were therefore chromatographed in this system, together with standards containing the three sugars in amounts ranging from 0.5 to 10 μg. each. As none of the spray reagents that were tried gave satisfactory spots with all three of these sugars, the following procedure was employed. The paper was masked so as to expose only the 10 μg. standard, which was sprayed with naphthoresorcinol reagent and heated, revealing fructose and sucrose. A line was drawn across the paper just below the sucrose spot, and the remainder of the paper was sprayed, with appropriate masking, so that sucrose was revealed with naphthoresorcinol and the hexoses with triphenyl tetrazolium chloride (Trevelyan, Procter, and Harrison, 1950). Color from both reagents was developed by heating at 75° for 5 minutes. The amounts of the sugars in each unknown sample were estimated by visual comparison of the area and intensity of the spots with those of the standards. Such estimates were found to be reproducible between experiments and with different observers to about ±50 per cent.

Total Carbohydrate.—The anthrone reagent was used to obtain independent estimates of the carbohydrate contents of the hemolymph samples. The tests were conducted with portions of the same TCA–$HClO_4$ extracts used for determination of phosphorus, as described below. The technique was that of Fong, Schaffer, and Kirk (1953), slightly simplified, with the reagent prepared according to Fairbairn

(1953). Since glucose and fructose (and their derivatives) yield maximal colored products at different times, the amounts of the two sugars could be estimated by reading the optical densities of samples heated at 100°C. for 2 minutes and for 12 minutes, and calculating from equations derived from the color yields of standards at the two times. Similar procedures have since been published, and it has been shown that the phosphorylated derivatives behave in this reaction identically with their

TABLE III

Carbohydrate and Phosphorus Distribution of Hemolymph

Mg./100 ml. plasma; −, not tested, +, present but spot too distorted for estimation.

Sample No.	Free sugars*			Total carbohydrate‡		Phosphorus		
	Glucose	Fructose	Sucrose	Apparent glucose	Apparent fructose	Acid-insoluble	Acid-soluble inorganic	Acid-soluble total
Bm 16	−	−	−	310	39	2.7	10.5	116
Bm 17	−	−	−	166	6	2.5	14.6	160
Bm 19	−	−	−	381	21	1.9	10.1	164
Bm 20	−	−	−	223	0	2.0	12.5	127
Bm 21	−	−	−	210	2	1.4	12.3	135
Bm 9	>37	1.0	0.8	588	34	2.5	10.9	144
Bm 8	+	+	2.5	463	26	3.6	13.5	196
Bm 10	1.2	1.3	2.4	520	17	2.4	4.7	129
Bm 7a	−	−	−	550	33	2.1	4.8	154
Bm 7b	−	−	−	505	38	3.0	5.2	160
Bm 12	2.8	1.8	0.6	635	25	3.6	5.4	180
Bm 14a	−	−	−	529	14	6.8	5.4	167
Bm 14b	−	−	−	528	0	—	—	—
Bm 11	3.6	1.9	0.9	620	0	3.4	8.1	99
Bm 15	5.2	1.9	1.1	449	3	14.4	13.6	173
Bm 13	3.5	1.5	1.4	390	1	8.9	13.7	163
Gm 1	−	−	−	2120	37	4.9	30.4	240
Dh 1	−	−	−	1288	46	3.7	24.4	185

* Semiquantitative estimate by paper chromatography.

‡ By anthrone reaction.

parent sugars (Mokrasch, 1954; Bonting and Bonting, 1954). The method proved satisfactory, except that when there was extreme disparity between the amounts of the two sugars the estimate of that present in smaller amount was unreliable.

Amino Sugars.—To test for the possible presence of amino sugars, color reactions were performed on $HClO_4$ extracts of hemolymph. For hexosamines, we used the method of Dische and Borenfreund (1950), which depends on the reaction of indole with the deamination product of the amino sugars. For *N*-acetylhexosamines, we used the method of Aminoff, Morgan, and Watkins (1952), and in order to minimize the effect of interfering substances, based the calculations on the difference in optical densities at 585 mμ and 610 mμ.

Results.—The chromatographic estimates of free sugars and the results of the anthrone tests are shown in Table III. The free sugars in each sample of silkworm blood examined were glucose, fructose, and sucrose. The amounts, however, were very small, totalling less than 10 mg. per 100 ml. in every sample except Bm 9: in this sample the glucose spot was greater than any of the standards used. The results of the anthrone tests are in sharp contrast, indicating a level of material reacting as glucose equal to 15 to 100 times the total free sugars. The estimates of apparent fructose by this method are not reliable because of the presence of so much apparent glucose, but they do indicate a relatively low level of fructose and its derivatives.

The tests for amino sugars were largely negative or inconclusive. In the indole test for hexosamines, a colored product with the correct absorption maximum (490 mμ) was formed, but the density at this wave length was actually greater in control samples that had not been deaminated, so that no evidence was obtained for the presence of hexosamines. Also, since hexosamines react with ninhydrin, they should have been detected on the amino acid chromatograms if substantial amounts were present. In the test for *N*-acetylhexosamines, several of the blood extracts gave weak colors that had the characteristic double absorption maxima (545 and 585 mμ). The densities, referred to *N*-acetylglucosamine standards, indicate 2 to 6 mg. per 100 ml. in hemolymph from larval *B. mori*, *G. mellonella*, and *D. hercyniae*, and 9 to 13 mg. per 100 ml. in pupal *B. mori*. Because of the known interference of amino acids in the presence of sugars in this reaction, however, these values may be too high.

Phosphorus Distribution

Methods.—

For determination of the phosphorus distribution, about 8 mg. of hemolymph solids were suspended in 2 ml. of a chilled solution containing TCA (5 per cent) and $HClO_4$ (0.5 N). After standing for 1 hour, the mixture was centrifuged and the supernatant fluid was used for determination of orthophosphate (including any labile P) and total P by a micromodification of Allen's (1940) method. The acid-insoluble residue was used for determination of protein-bound P.

The acid-soluble phosphates were examined by paper chromatography, as follows. The hemolymph solids were extracted with $HClO_4$, the extract was neutralized with KOH, and the precipitated $KClO_4$ was removed by centrifugation. Cations were removed by passage through a small column of dowex 50 (H form), and the eluate was lyophilized, redissolved in a little water, and spotted on paper for chromatography. Whatman No. 1 paper, previously washed with 3 N acetic acid and water, was used. The most satisfactory solvents of many tested for resolution of known phosphate metabolites and of the compounds in silkworm blood were: (*a*) ethyl acetate 3, acetic acid 3, water 1 part by volume (Mortimer, 1952); (*b*) methanol 12, concentrated ammonium hydroxide 2, water 3 parts by volume (modified from Ban-

durski and Axelrod, 1951); and (*c*) as (*b*) but with the water replaced by 2 per cent aqueous H_3BO_3 and the paper previously sprayed with 1 per cent H_3BO_3 in 60 per cent ethanol. Borate, by forming complexes, retards the movement of glucose-6-phosphate and improves its separation from glucose-1-phosphate. Chromatograms were run at room temperature without prior vapor equilibration. The spots were detected with molybdate reagent and ultraviolet irradiation (Bandurski and Axelrod, 1951; Haney and Loughheed, 1955). For quantitative estimation, the spots were cut out and analyzed for total P. When standard solutions were subjected to the combined deionization and chromatographic procedures, 93 to 97 per cent of the P was recovered.

Results.—The distribution of phosphorus in the hemolymph samples is shown in Table III. It is evident that all the samples are characterized by high levels of acid-soluble organic phosphate. In most cases this amounts to 100 to 200 mg. per 100 ml., comprising more than 90 per cent of the total P of the hemolymph.

Chromatograms of the acid-soluble phosphates of silkworm hemolymph showed 5 to 7 spots. Small samples of these substances were prepared by elution of bands from large chromatograms. One was orthophosphate, as shown by its R_f values and by the fact that it gave a yellow color with the molybdate reagent before exposure to ultraviolet light. A second was tentatively identified by chromatography, paper electrophoresis, and the absorption spectrum of its anthrone complex as glucose-6-phosphate. The remaining P compounds of the hemolymph did not correspond chromatographically to glucose-1-phosphate, fructose-6-phosphate, fructose diphosphate, β-glycerophosphate, nor phosphoglyceric acid, and remain as yet unidentified. The P content of the chromatographically separated compounds from a number of the hemolymph samples accounted for only 20 to 50 per cent of the total acid-soluble P. One possible reason for this is that the samples may have contained positively charged compounds that would be held on the cation exchange resin.

Purine and Pyrimidine Derivatives

As a test for the presence of nucleic acid derivatives, a paper chromatogram (Wyatt, 1951) of $HClO_4$ extracts of two of the silkworm blood samples was inspected in ultraviolet light chiefly of wave length 254 mμ. The procedure was sensitive enough to detect any of the purines or pyrimidines of nucleic acids if more than about 10 mg. per 100 ml. were present in the hemolymph. No spot was visible.

DISCUSSION

The results show some quite large differences among pooled samples of hemolymph from silkworms at nearly the same stage of development. It is possible that more uniform rearing conditions, or selection of more homogeneous

samples, would have reduced this variation. However, previous work (see Buck, 1953) has indicated that insect hemolymph does vary more than mammalian blood. This must be borne in mind when assessing the significance of differences.

Hydrogen Ion Concentration

Some earlier measurements of the pH of silkworm hemolymph are listed by Buck (1953). These were obtained with indicators, the quinhydrone electrode, or the hydrogen electrode, methods which may be unreliable with this material because of interfering substances or gas exchange. Recently (since the present work was carried out) there have been two reports of the pH of silkworm blood measured with glass electrodes similar to the one which we used. Heimpel (1955) found a mean pH of 6.77 for blood of larvae in the last two instars, and Nunome and Horiba (1955) report values in the range 6.69 to 6.87 for groups of larvae of various ages. In both cases, the readings were taken within 15 or 30 seconds of bleeding and apparently did not change during the period of observation. Our results differ in two respects. First, we noted an initial rise of pH which we attribute to loss of CO_2. This seems reasonable in view of the high CO_2 content of silkworm blood (11 volumes per cent; Florkin, 1934). A similar effect is well known in mammalian blood (Peters and Van Slyke, 1932), and has been noted in other insects (Boche and Buck, 1942; Levenbook, 1950 *a*). Second, our values even when not corrected for CO_2 loss, are lower than those of the other authors by almost 0.4 unit. The reasons for these differences are not clear, although it is likely that the blood pH may vary somewhat with strain, food, and rearing conditions of the insects.

Our measurements also confirm the observation of Demjanowski *et al.* (1932) that there is a slight rise in blood pH at the time of spinning, which is continued in the pupal stage.

Nitrogenous Components

Protein.—The analyses of Florkin (1937) indicated a gradual rise in blood protein level during the development of *Bombyx mori* from about 1.2 per cent in instar IV to a maximum of over 5 per cent at the time of spinning. Our own results are in agreement, and also indicate a rise during each of instars III and IV with a fall at each following molt. This is probably due mainly to dilution by water taken up after molting. It may also include the effect of hydrolysis of protein to maintain blood amino acids during the non-feeding period, for Beadle and Shaw (1950) have shown that during starvation of *Sialis* blood proteins decrease while amino acids remain constant. The female, in instar V, appears to have somewhat higher blood protein than the male (samples Bm 7a and 7b).

Non-Protein Nitrogen.—Non-protein nitrogen is maintained at a high level

averaging 400 to 600 mg. per 100 ml., in agreement with earlier work (Florkin, 1937; Bialaszewicz and Landau, 1938), with some large variations between samples, but no obvious pattern of change during development. No sample was taken in the midst of spinning, at which stage Florkin (1937) noted a decrease in non-protein N. Comparing Tables I and II, it is evident that the free amino acids account for only 30 to 55 per cent of the non-protein N (except sample Bm 15, in which the fraction is 70 per cent). This is in agreement with the ratios of Florkin's values for amino N and total non-protein N, which range from 35 to 55 per cent. It leaves a substantial amount (100 to 350 mg. per 100 ml.) of unidentified acid-soluble N. Nitrogenous compounds that have been reported in insect blood include uric acid, urea, allantoin, and ammonia (Leifert, 1935), and pterins (Drilhon and Busnel, 1951), but the amounts of these were small in relation to the nitrogen mentioned above. Our tests indicated that no great amounts of amino sugars or nucleic acid derivatives are present. The nature of the residual nitrogen remains, therefore, as yet unknown.

Free Amino Acids.—Since the advent of paper chromatography, there have been numerous qualitative analyses of the free amino acids of insect blood. These include several reports on *Bombyx mori* (Drilhon and Busnel, 1949; Yoshitake and Aruga, 1950; Fukuda, 1951; Ishimori and Muto, 1951) and on *Galleria mellonella* (Auclair and Dubreuil, 1953; Boucly-Urison, 1953). The amino acids identified are in general the same as those that we have found. Several of these authors report cystine or cysteine, which we could detect in only one sample from the silkworm; this may be due to differences in strain of insects or of mulberry, or on the other hand the spot which we call x_1 could easily be mistaken for cysteine.

Quantitative analyses, by microbiological assay, of the free amino acids of fifth instar *Bombyx mori* hemolymph have recently been reported from two laboratories (Sarlet, Duchateau, and Florkin, 1952; Duchateau and Florkin, 1955; Fukuda, Kirimura, Matuda, and Suzuki, 1955). In these assays, separate values were obtained for leucine and isoleucine, but aspartic and glutamic acids were not distinguished from their amides. With minor differences, the proportions of the amino acids reported in three independent analyses are in good agreement.

This suggests some degree of constancy for the species, with glutamine, histidine, and lysine the preponderant amino acids of the blood. It is notable that *Galleria* and *Diprion* differ strikingly from *Bombyx* and from one another in the proportions of the amino acids in their hemolymph, although glutamine is in all three species a major component.

Tryptophan could not be detected on any of our chromatograms of insect hemolymph. Fukuda *et al.* (1955) report tryptophan in silkworm blood at a low level (3 to 5 mg. per 100 ml.), which would be at the sensitivity limit of our method. As cysteine and tryptophan are essential dietary constituents for

some insects (see Trager, 1953), it seems possible that they do not accumulate in the hemolymph because of a minimal supply from mulberry leaves.

β-Alanine, although not a constituent of proteins, seems to occur commonly in insect blood (Pratt, 1950; Auclair and Dubreuil, 1953).

The significance of changes during the development of the silkworm is difficult to assess because of the range of variation between samples. During growth from instar III to V, there is a general rise in total amino acid level, which is maintained in the pupa, although, as Florkin (1937) has shown, it may drop off temporarily during spinning. If the result from one sample (Bm 9) is significant, there is a marked increase in the tyrosine content of the blood during molting; this might be derived from the old cuticle by the proteolytic action of the molting fluid (Passonneau and Williams, 1953) and be stored for use in hardening of the new cuticle after molting. At pupation there is a decrease in methionine and, to a lesser extent, glycine and proline. These changes presumably reflect cessation of dietary amino acid intake.

Carbohydrates and Phosphorus Compounds

Fermentable reducing sugar in the hemolymph of larval *Bombyx mori* has been variously reported as 0 to 30 mg. per 100 ml., representing one-fourth or less of the total reducing power (see Buck, 1953, for references; also Florkin, 1937). All but one of our estimates of glucose plus fructose fall in the lower part of this range. It is possible that glucose-6-phosphate might appear as fermentable reducing power. There seem to be hitherto no analyses of specific carbohydrate components of silkworm blood. Our anthrone tests indicate a high level of carbohydrate apparently mainly derivatives of glucose or other aldohexose. While its nature has not been determined, it cannot consist of glycogen, since it was not removed by ultrafiltration (sample Bm 14b).[1] Blood of the wax moth and the sawfly contains even more anthrone-reacting material than that of the silkworm; these samples were not examined for free sugars, but, in *Galleria*, at least, whose diet contained honey, the free sugar levels may be higher than in the silkworm.

The only marked change in free sugars during development of the silkworm is the elevated level of glucose in the sample from molting insects (Bm 9). This may reflect altered carbohydrate metabolism during the non-feeding period. It would be desirable to have it confirmed by examination of other blood samples from feeding and non-feeding insects.

Substantial amounts of organic phosphate in the plasma of certain insects were reported many years ago (see Buck, 1953, p. 153, for references). There have been partial analyses of this fraction in blowfly larvae (Hopf, 1940) and in oak caterpillars (Smolin, 1952) by precipitation of barium salts, but these

[1] Since writing, the major component of this fraction has been identified as α,α-trehalose (Wyatt and Kalf, 1956).

did not lead to isolation and characterization of the components. Our chromatograms indicate that the fraction is complex, including at least one phosphorylated sugar derivative, which would account for part of the anthrone-reacting material. It seems likely that insect blood serves for storage of nutrients, and it is possible that phosphorylated derivatives may be better suited for this than free sugars because of less easy, or more selective, penetration into cells.

Surprisingly little attention has been paid to the phosphates of insect plasma, whose presence contrasts with the characteristically intracellular occurrence of such substances in vertebrates. Their contribution to the buffer capacity (Levenbook, 1950 *b*) and anion balance (Buck, 1953) of hemolymph seems also to have been generally underestimated. In our larval silkworm blood samples, the acid-soluble phosphate, which is mainly organic, is sufficient to balance 30 to 40 per cent of cations present, assuming monoesters with 50 per cent secondary phosphoryl dissociation. The nature and metabolism of these compounds are the subjects of further investigation.

CONCLUSION

The results of these analyses emphasize the complexity of insect hemolymph. Large proportions of the nitrogen, phosphorus, and carbohydrate of silkworm plasma occur in compounds not yet identified. It is clear that the conventional methods of human blood analysis are inadequate for this material. Further knowledge of its composition awaits continued application of chromatography and similar high resolution techniques.

SUMMARY

1. Hemolymph was collected for analysis from the silkworm, *Bombyx mori*, in a series of developmental stages ranging from the second molt to the late pupa. The mean pH of larval hemolymph after collection was found to be 6.45, that of pupal hemolymph, 6.57; *in vivo* values may be slightly lower. Total dry solids ranged from 5.4 to 10.6 per cent. Total protein ranged from 1.2 to 5.3 per cent, increasing rapidly during the fifth instar.

2. Free amino acids were separated chromatographically and estimated. Of 19 amino acids identified, amounting collectively to 823 to 1497 mg. per 100 ml., glutamine, histidine, and lysine generally occurred in greatest amount. Tryptophan was not detected, and cystine (or cysteine) was found in only one sample. The total free amino acids account for 35 to 55 per cent of the non-protein nitrogen of the plasma.

3. Free sugars, estimated semiquantitatively on chromatograms, comprise glucose, fructose, and sucrose in total amount ranging from about 5 to 40 mg. per 100 ml. Total acid-soluble, ultrafiltrable carbohydrate, estimated as glucose by the anthrone reaction, ranged from 166 to 635 mg. per 100 ml., indicating the presence of low molecular weight sugar derivatives.

4. Inorganic phosphate amounted to 5 to 15 mg. per 100 ml., and acid-soluble organic phosphate to 100 to 200 mg. per 100 ml. The latter fraction includes several substances, of which one was tentatively identified as glucose-6-phosphate and the remainder are as yet unidentified.

5. Single samples of hemolymph were also taken from larvae of the wax moth, *Galleria mellonella*, and the spruce sawfly, *Diprion hercyniae*. These contained even higher concentrations of solutes than the silkworm samples, but with a generally similar distribution. The proportions of the free amino acids were different in each species.

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