The Composition of Horse Bot Fly (*Gastrophilus intestinalis*) Larva Blood

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The extensive literature on the composition of insect blood or haemolymph has been reviewed by Maluf (1939), Needham (1942) and Timon-David (1945). Sufficient data have been accumulated to show that the composition of the blood varies widely not only from one insect to another, but also in the same insect under different physiological conditions. The present study on the blood of *Gastrophilus intestinalis* de Geer larvae was originally undertaken to devise an isotonic physiological solution for use in metabolic experiments with isolated tissues of this insect. But in view of the fact that the blood of a dipterous insect has not previously been systematically investigated, and that a parasitic form such as *Gastrophilus* might show certain characteristic features, the work was extended to cover a wider range of biologically interesting substances than required for the original problem.

The life history of *Gastrophilus* has been summarized recently by Keilin (1944) and by Keilin & Wang (1946), and a full account of its anatomy and bionomics, together with a complete bibliography, is given by Dinulescu (1932); only a very brief description need therefore be given here.

The free-living adult fly fixes its eggs during the summer months to the hairs of the horse, and in about 5–6 days the larvae are completely formed. As a result of the horse licking itself, hatching is induced, and those larvae which are taken into the buccal cavity survive. These first-stage larvae penetrate into the tongue and migrate towards the pharynx, where they moult and emerge to attach themselves for a short period to the pharyngeal mucosa. Subsequently, they pass down into the stomach and re-attach themselves to the mucosa of the cardiac region. The second moult now occurs, and third-stage larvae first appear about September and are fully grown by October or November. For the next 7–8 months, until the following May or June, the larvae take little if any nourishment, and their general metabolism is at its lowest (Dinulescu, 1932). This quiescent period, therefore, may be likened to diapause in certain free-living forms, and it might be expected that under these conditions the composition of the blood would be relatively constant. Eventually, the larvae leave the alimentary tract, and pupate some inches down in the soil. The adult fly emerges in about 20–30 days.

It is becoming increasingly clear that for the analysis of insect haemolymph to have any biological meaning, it is essential to know the age of the insect, its physiological state, and the environmental conditions under which it was kept prior to analysis. The age cannot be ascertained accurately for *Gastrophilus* on account of its parasitic habitat, but the fact that the larvae used for the present work were all in a state of diapause makes any variations in age in this particular case relatively less important. Certain effects on the blood composition of differences in environment are described below.

On account of the ease with which the larvae may be obtained, and the relatively large quantities of blood which they yield, 25–30 ml. of haemolymph may be collected from about 200 larvae in 1 hr., these insects form excellent material for the present type of study; but it needs to be emphasized that, from a comparative point of view, the extent to which the data obtained may be applied to other dipterous larvae is quite uncertain.

EXPERIMENTAL

Material. Larvae of *Gastrophilus* were obtained from the local knackery. As soon as possible after the killing of a horse the stomach was removed and opened up; if larvae in sufficient numbers were present, that portion of the stomach wall to which they were attached was cut out and placed in a large vacuum flask containing water at either 37–38° or iced water at 0°. Depending upon this difference in temperature, larvae will be subsequently referred to as either 'warm' or 'iced', respectively. About 30 min. after their removal from an infected host the larvae arrived at the laboratory, where they were rinsed with either warm or ice-cold water, and gently picked off the mucosa with forceps taking particular care not to crush the large tracheal cells at the posterior end of the body. Mucus and adhering particles of grain, chaff, etc., were removed by two or three further washings with water and the larvae, dried between large sheets of filter paper, were now ready to be bled, or could be kept for a few days in a large dish of acidified tap water.

The blood was obtained by making a small incision with scissors at the anterior end of the larva and immediately dropping them into a funnel covered with fine gauze through which the blood drained into a test tube, generally kept on ice. When it was necessary to collect haemolymph unexposed to air, the incision was made in larvae kept submerged beneath a 3 cm. deep layer of heavy paraffin oil in a narrow tube.

In general, the analyses were begun between 1 and 2 hr. after the larvae had been removed from their host, but, nevertheless, marked variations were found in the concentration of certain of the more labile blood constituents as between 'warm' and 'iced' larvae. This is probably due to differences in activity, since at 37° the larvae, when removed from the stomach, are very active (particularly so when no longer attached to the mucosa), at room temperature they show only slow undulating movements, while at 0° they are limp and entirely motionless, and generally survive for less than 24 hr.

Analytical methods. The techniques described in the literature for use with mammalian blood were applied directly to *Gastrophilus* haemolymph. Robertson & Webb (1939) have pointed out the dangers of applying to marine invertebrates analytical methods primarily evolved for mammalian tissues, and their criticisms are almost certainly also valid in the case of insects. In the present work no special study was made of the accuracy of the methods chosen, except in so far as in the majority of the determinations satisfactory recoveries of added known amounts of the substance to be determined were obtained in preliminary experiments.

Total blood nitrogen and non-protein nitrogen (N.P.N.) were estimated by micro-Kjeldahl before and after sodium tungstate deproteinization. In agreement with the observations of Heller (1932) and Leifert (1935) on lepidopteran blood, the N.P.N. values were some 5–10% higher when trichloroacetic acid (TCA) was used for deproteinization. The sodium tungstate filtrate was also employed for α-amino N determination in the Van Slyke apparatus, and for amide N determination, estimated as the extra NH_3 formed after hydrolysis with 10% (v/v) H_2SO_4. Na and K were determined according to King (1946), Ca by the method of Kramer & Tisdall (1921), and the solution remaining after twice repeated precipitation of Ca used for the analysis of Mg (Greenberg & Mackey, 1932; Greenberg, Anderson & Tufts, 1935). Cu and Zn were determined in both whole blood and the sodium tungstate filtrates according to Eden & Green (1940) and the mixed colour dithizone method of Sandell (1944), respectively. S was estimated in the deproteinized filtrate as described by Power & Wakefield (1938), Cl was determined according to Sendroy (1937) and P by the method of Allen (1940). NH_3 was estimated in the Parnas-Heller apparatus (Parnas & Heller, 1924), and similarly urea, as the extra NH_3 formed by the action of urease. For uric acid the method of Brown (1945) was employed, the combined fatty acids + sterols were determined by Bloor's dichromate oxidation technique as described by Boyd (1931), and bisulphite-binding substances by the Clift & Cook (1932) procedure. Analyses for lactic acid were carried out by the colorimetric method of Barker & Summerson (1941) where, contrary to the directions given by these authors but in agreement with Umbreit, Burris & Stauffer (1945), it was found essential to cool the samples in ice prior to addition of H_2SO_4. Succinic and citric acids were estimated according to Krebs (1937) and Krebs & Eggleston (1944) respectively. The total reducing value was measured by the Miller & Van Slyke (1936) procedure as modified by Bacon & Bell (1948), true glucose being determined with glucose oxidase (Keilin & Hartree, 1948), and fructose by the Seliwanoff colour (Roe, 1934). The method of Mann (1946) was employed for determinations of yeast-fermentable reducing values, and that of Genkin (1938) for blood glycogen.

RESULTS

General reactions

The blood of *Gastrophilus* larvae is a clear, yellowish, slightly viscous fluid, of which 0·1–0·15 ml. may be obtained from a single fully grown larva. On being allowed to stand, either at room temperature or at 0°, it soon becomes opalescent and gradually forms a heavy flocculent precipitate (cf. Keilin & Wang, 1946). This precipitate, insoluble in either water or acids, dissolves readily in dilute alkali to give a strongly positive biuret reaction. This change appears to be of the type described by Yeager & Knight (1933) as blood protein coagulation, and to be independent of any blood cells present. On further standing in the presence of air, the blood darkens from the exposed surface downwards due to the formation of melanin. This reaction can be prevented by either warming the blood to 60–65° (at a slightly higher temperature, 65–70° complete coagulation occurred) or by addition of cyanide, both of these treatments inhibiting the blood tyrosinase.

The haemolymph gave all the familiar colour tests for proteins, e.g. the biuret, xanthoproteic, Millon, Molisch, ninhydrin and Sakaguchi reactions. However, the absence of any substances containing sulphydryl groups such as reduced glutathione or cysteine, and of tryptophan, was demonstrated by consistently negative nitroprusside and bromine tests; the diazobenzenesulphonic acid reaction for histidine was also negative. Ammoniacal silver nitrate was not reduced, indicating absence of appreciable amounts of reducing compounds such as ascorbic acid or free *o*- or *p*-quinols, and no blue colour was obtained with the lanthanum nitrate test for acetate or propionate.

The blood of most insects contains suspended cells or haemocytes, which generally number some tens of thousands/μl. (Tauber & Yeager, 1935, 1936). In *Gastrophilus* blood obtained as described above, the average of 20 cell counts with the Thoma haemocytometer gave the extremely low figure of 242 cells/μl. For the purpose of the present analysis, therefore, the blood may be considered as a virtually cell-free fluid analogous, for purposes of comparison, to mammalian blood plasma.

The specific gravity of the blood measured with a micropyknometer had a value of 1·062, which is the highest yet recorded for insect haemolymph. The depression of the freezing point, Δ, measured cryoscopically, gave a mean value of $-0·872°$, which corresponds approximately to 0·25 M-sodium chloride. Samples of blood dried overnight at 110° contained on the average 15·7% of solid matter. For blood collected at 20° without exposure to air, the average pH measured with the glass electrode was 6·80, but the actual pH *in vivo* at 37° is somewhat lower (Levenbook, 1950).

Microspectroscopic examination of a layer of blood about 1 cm. or more thick showed very faint absorption bands near 580 and 545 mμ.. which correspond to the α and β bands for *Gastrophilus* haemoglobin described by Keilin & Wang (1946). Whether this haemoglobin is a normal blood constituent or was present as a tissue contaminant is difficult to determine, but in any case its concentration is so low, that it cannot have any respiratory function. This is confirmed by measurements of the oxygen content of the blood which, determined in the usual manner in the Van Slyke apparatus on samples unexposed to the air, gave a mean value of 0·15 vol. %. The addition of ferricyanide was without effect on the values so obtained, and only served to produce a semi-solid gummy mass in the extraction chamber. Further, equilibration of the blood samples with air prior to analysis only produced small increases in the oxygen content. It is of interest that the quantity of oxygen carried by the blood appears to be substantially less than would be present in an isotonic salt solution (Babers, 1938).

Chemical analysis

The results of the chemical analyses are summarized in Table 1; unless otherwise stated each value is the mean of at least three determinations carried out on 'warm' larvae.

Table 1. *The composition of* Gastrophilus *larva blood*

	mg./100 ml. blood
Total reducing value, expressed as glucose[a]	356
Reducing value after yeast fermentation[a]	95
Apparent fructose ('fructose')[a]*	280
True glucose ('warm' larvae)	64·5
True glucose ('iced' larvae)	10
Apparent glycogen ('glycogen')*	84·5
Total N	1850
Non-protein N	130·1
Total protein (protein N × 6·25)	10·75 g./100 ml.
Amino N in sodium tungstate filtrate	93·8
Amide N in sodium tungstate filtrate	11·5
NH_3 (resting value in 'warm' larvae)	4·5
NH_3 (resting value in 'iced' larvae)	1·83
Uric acid	2·2
Urea	20·4
Lipid (fatty acids + sterols)	137
Bisulphite-binding substances, as pyruvic acid	10
Lactic acid ('warm' larvae)	44·5
Lactic acid ('iced' larvae)	12·3
Succinic acid[b]	242
Citric acid	45
Na	402·5
K	45
Ca	11·4
Mg	38·8
Cl	52·5
HCO_3^- at pH 6·8[c]	88·5
Total P	109
Total acid-soluble P ('iced' larvae)	81·4
Inorganic P ('iced' larvae)	12·5
Total S (in TCA filtrate)	9·7
Inorganic S (in TCA filtrate)	6·8
Total Cu	0·55
Cu in sodium tungstate filtrate	0·22
Total Zn (two estimations)	0·88
Zn in sodium tungstate filtrate (one estimation)	0·57

[a] Levenbook (1947); [b] Levenbook & Wang (1948); [c] Levenbook (1950).

* For the true compounds in these cases correct values could not be determined owing to the presence of interfering substances which could not be differentiated by the methods employed. For further details see text.

Reducing value of the blood. In *Gastrophilus* blood the average total reducing value (t.r.v.) of 356 mg./100 ml., expressed as glucose, was lowered by 60–70 % after yeast fermentation, and accordingly, some 210–260 mg./100 ml. might be attributed to glucose. Since the time for complete yeast fermentation of the blood sugar was far longer than would have been required to ferment an equivalent amount of glucose, and since Mann (1946) had found the local brewer's yeast to ferment fructose much more slowly than glucose, the Seliwanoff (1887) colour test for ketoses was tried and a strongly positive reaction obtained.

The presence of fructose in *Gastrophilus* has already been reported briefly elsewhere (Levenbook, 1947), and fuller details are now presented below.

Following the procedure of Babers (1941), a pooled sample of 5 ml. of blood was used for the preparation of the phenylosazone; characteristic sheaves of yellowish glucosazone crystals were obtained which, following recrystallization from ethanol, had m.p. (uncorr.) 205°. For definite characterization, the 2:3–4:5-di-*iso*propylidene compound of fructose was prepared from the blood after the method of Bell (1947) and Bacon & Bell (1948).

Blood (60 ml.) was centrifuged to remove cellular and fatty materials, and deproteinized by the addition of 3 vol. acetone. The precipitate was washed with 75% (v/v) acetone, and the combined filtrates evaporated to dryness under reduced pressure at a temperature not exceeding 40° in the presence of about 1 g. of Hyflo-Supercel. The resulting hard, gummy precipitate was dissolved in 50 ml. warm water to give an opalescent, light-orange solution. This was treated with charcoal, filtered through a sintered glass filter, and the clear filtrate evaporated to dryness as above. The residue was kept in a high-vacuum desiccator over conc. H_2SO_4 for several hours, and the drying completed by refluxing with absolute ethanol-benzene (2:1, v/v) for 2 hr. and evaporating off the solvent. On testing the distillate for its Seliwanoff reaction, a strong red colour was obtained, the intensity of which accounted for about half the Seliwanoff positive material originally present in the blood. Since a similar distillate obtained by refluxing pure fructose with ethanol-benzene gave no trace of a red colour, it was concluded that some volatile substance giving a positive Seliwanoff reaction other than fructose had been extracted from the blood.

The dried powder remaining after the above treatment was shaken for 6 hr. with 300 ml. dry acetone containing 15 ml. of conc. H_2SO_4. To the dark brown solution so formed, anhydrous Na_2CO_3 was added with continuous mechanical stirring until the colour changed to pale yellow. The mixture was filtered, the precipitate washed with acetone, a few drops of 30% (w/v) KOH added to the combined filtrates, and these then evaporated under reduced pressure until some 10 ml. of a thick, oily brown liquid remained. To this syrup, which was only slightly miscible with water and gave a very intense Seliwanoff colour, 100 ml. of water were added and the mixture distilled under reduced pressure until about half the water had passed over. The aqueous layer was then decanted and the syrupy residue washed three times with small amounts of warm water. The combined aqueous portions were acidified, treated with charcoal and filtered; on neutralization to pH 9, a gelatinous precipitate gradually formed which was removed by centrifugation. The supernatant was extracted three times with equal volumes of $CHCl_3$, the extract dried with anhydrous Na_2SO_4, evaporated almost to dryness, and isomers of the desired diacetone derivative hydrolysed overnight by the addition of 5 ml. 0·1 N-HCl.

The acid solution was treated with charcoal, filtered and neutralized to pH 9, whereupon a gelatinous precipitate again formed. This was centrifuged off, the supernatant extracted with $CHCl_3$ as above and evaporated to dryness. A semi-crystalline residue was obtained which, following repeated recrystallization from ligroin (b.p. 40–60°), yielded 18 mg. of white crystals of m.p. (uncorr.) 94·5°, mixed melting point with an authentic sample of di-*iso*propylidene fructose prepared in the above manner from pure fructose, was 94·2°.

With the kind assistance of Dr S. M. Partridge, additional evidence for the presence of fructose in the blood was obtained from paper chromatography. Blood was deproteinized by ethanol-$CHCl_3$ treatment, the solvent evaporated off and the residue taken up in water. Salts were removed by passage through ion-exchange columns, and the chromatograms run in a variety of solvents as described by Partridge (1948). In all cases a strong spot was developed corresponding very closely to the position of fructose, generally together with a weaker one due to glucose. No other spots were obtained, indicating the absence of other reducing sugars or related substances.

It is clear from the above that fructose is undoubtedly present in *Gastrophilus* haemolymph, but the exact amount is uncertain, as will be apparent from the following data.

A very considerable difference exists in the glucose concentration in the blood depending upon whether 'warm' or 'iced' larvae are employed (Table 1). If blood was collected from 'iced' larvae, taking the greatest possible care to cause the minimum of tissue damage, and was deproteinized immediately, the true blood glucose was generally too low to be estimated with glucose oxidase. But for most of the present determinations 'warm' larvae were used, and in such cases a considerable amount of glucose, amounting to 64·5 mg./100 ml. (mean of nine estimations) was found in the blood. Similar considerations apply also to the blood 'glycogen', an alkali-stable material of uncertain structure which, on mild acid hydrolysis, had reducing properties and which was usually present in the blood of 'iced' larvae. The bulk, if not the whole of this substance, is not true glycogen, since it was scarcely soluble in hot water, did not give a typical brown colour with I_2 and did not yield glucose (as tested with glucose oxidase) after hydrolysis. It was present to the extent of 10–20 mg./100 ml. calculated as glucose. However, the value for 'glycogen' in the blood of 'warm' larvae as generally collected, was considerably higher, in the range of 40–127 mg./100 ml., and this included true glycogen as judged by the criteria enumerated above. The differences in the glucose content, therefore, are most probably due to the action of the powerful blood diastase (Kemnitz, 1916; Roy, 1937) acting upon glycogen either already present in the blood, or introduced artificially through tissue damage.

A non-fermentable fraction of the t.r.v. was found in all the samples of blood examined, the amount varying from 33 to 200 mg./100 ml. expressed as glucose. The sum of glucose + non-fermentable reducing substances from 'warm' larvae amounted to, on the average, 160 mg./100 ml., the difference between this figure and the average t.r.v. of 356 mg./100 ml. (i.e. 196 mg./100 ml.) presumably being accounted for by fructose. But the actual concentration of 'fructose' in the blood, as determined by the intensity of the resulting Seliwanoff colour (Roe, 1934), was 280 mg./100 ml., thus showing a discrepancy of 84 mg./100 ml. as compared with the calculated value.

Whilst any error in the determinations of glucose and non-yeast-fermentable reducing substances is probably small, the Roe method would appear to overestimate considerably the amount of true fructose present in *Gastrophilus* blood. Impossibly high values for true fructose (i.e. expressed as 'fructose'), almost equal to, or on occasion even exceeding the t.r.v., were not infrequently obtained (Table 2). It may be noted in this connexion that following complete yeast fermentation, a positive Seliwanoff reaction was never obtained, and that added fructose was satisfactorily recovered either by its Seliwanoff colour or by reducing value (Table 2); this incidentally confirms the observation of

Table 2. *Total reducing value (t.r.v.), yeast-fermentable reducing substances, 'fructose' and recovery of added fructose determinations on* Gastrophilus *blood*

Exp. no.		
1	T.r.v. of *Gastrophilus* blood	322 mg./100 ml.
	'Fructose' (Roe method)	325 mg./100 ml.
	Non-yeast-fermentable reducing value	92 mg./100 ml.
	Excess 'fructose', excluding any glucose undoubtedly present	95 mg./100 ml.
2	T.r.v. of *Gastrophilus* blood	302 mg./100 ml.
	'Fructose'	261 mg./100 ml.
	Above sample with 50 mg. fructose/100 ml. added:	
	T.r.v.	350 mg./100 ml.
	Fructose recovery measured by reducing value	96%
	Total fructose (i.e. 'fructose' + added fructose) estimated by Seliwanoff colour	308 mg./100 ml.
	Fructose recovery by above method	95%
3	T.r.v. of 2 ml. yeast-fermented blood	0·164 mg. as glucose
	'Fructose'	No reaction
	A similar 2 ml. fermented sample + 0·2 mg. fructose added:	
	T.r.v.	0·355 mg. as glucose
	Fructose recovery by reducing value	96%
	Fructose estimated by Seliwanoff colour	0·196 mg.
	Fructose recovery by Seliwanoff colour	98%

Bacon & Bell (1948) that fructose has the same reducing value as glucose when determined by the Miller & Van Slyke (1936) method.

The presence in the blood of some substance which interferes in the determination of fructose by the Roe method would also account for two other observations. Thus, following deproteinization with $Cd(OH)_2$, it was found necessary to wash the precipitate four to six times before the washing fluid no longer gave a positive Seliwanoff reaction; true fructose is quantitatively extracted in at the most two washings. Then, in the preparation of the diacetone fructose derivative described above, the final yield was only about one-tenth of that to be expected if the whole of the Seliwanoff colour in the blood were due to fructose.

The absence of phosphorylated fructose which, together with a variety of other keto compounds, might give positive Seliwanoff colours is shown in Table 3. Any phosphorylated fructose would be precipitated by the addition of excess barium acetate and 4 vol. ethanol to a neutralized (pH 8) TCA blood filtrate (Umbreit *et al.* 1945).

Table 3. *Absence of phosphorylated fructose in* Gastrophilus *blood*

	mg./100 ml. as glucose
T.r.v. of blood sample	359
'Fructose'	312
'Fructose' in supernatant after precipitation with barium acetate and ethanol	306
'Fructose' in precipitate from above	1·5

Free amino-acids in the blood

A high value for free amino-acids has invariably been found in insect haemolymph, and the nature of these amino-acids in *Gastrophilus* has been investigated by paper chromatography as follows.

Blood was diluted with an equal volume of water, and deproteinized with either sodium tungstate or by ethanol-$CHCl_3$ treatment. With either method the results were the same, but the latter produced sharper chromatograms, possibly owing to the absence of added salts. The extract was evaporated to dryness in a vacuum desiccator and the residue taken up in a minimum of water. The aqueous solution was then analysed by two-dimensional paper chromatography (Consden, Gordon & Martin, 1944), using *n*-butanol-acetic acid-water (Partridge, 1948), followed by phenol in an atmosphere of coal gas and NH_3 as solvents. Following the recommendation of Dent (1948), a portion of each sample was hydrolysed with approx. 6 N-HCl in a sealed tube for 2–3 hr. at 150°, and the hydrolysate evaporated to dryness and redissolved in a minimum of water. The untreated and hydrolysed samples were then run simultaneously on sheets of Whatman no. 1 filter paper, and the position of spots due to peptides or amides thereby definitely localized.

The data obtained from the chromatography of *Gastrophilus* haemolymph, together with that from other insects, is presented in Table 4. For *Gastrophilus* some thirteen free amino-acids and four polypeptides were found, and despite occasional minor variations, this picture was rather constant for the different batches of larvae examined.

Special interest attaches to two spots on the chromatograms, the intensity of which was far greater than any of the others. The first of these is due to glutamine, and its identity is proved by the following facts. (1) The most noticeable changes after acid hydrolysis were the disappearance of the strong glutamine spot and certain weaker (peptide) spots, and the appearance of a strong spot in the position of glutamic acid together with weaker spots corresponding to aspartic acid, histidine and threonine. Other pre-existing spots, noticeably lysine, were intensified after hydrolysis. It may therefore be concluded that conjugated amino-acids gave rise to free amino-acids on hydrolysis. (2) The glutamine spot disappeared and was replaced by an equally intense one due to glutamic acid after very mild treatment, i.e. heating at pH of approx. 6

Table 4. *Free amino-acids in insect haemolymph*

(The presence or absence of an amino-acid is denoted by + or − respectively. ? denotes that the result is only tentative.)

Amino-acid	*Gastrophilus intestinalis* Before hydrolysis	*Gastrophilus intestinalis* After hydrolysis	*Melolontha vulgaris*[a]	*Oryctes nasicornis*[a]	*Dytiscus marginalis*[b]	*Aeschna cynea* nymph[c]	*Calliphora erythrocephala* larva[d]	*Calliphora erythrocephala* pupa[e]*	*Phalera bucephala* pupa[e]*
Glycine	+	+	−?	−?	.	+	+	++	+
Alanine	+?	+?	+?	.	.	+	++	++	+
β-Alanine	.	.	.	.	.	.	.	+	.
Valine	+	+	+	+	.	+	+	+	+
Leucine and/or isoleucine	+	+	++	+	.	+	+	+	+
Serine	+	+	+?	+?	.	+?	+	+	+
Threonine	−	+	.	.	.	.	.	+	.
Proline	+	+	−?	−?	.	+	+	+	+
Hydroxyproline	−	−	++	++	.	.	.	−	−
Phenylalanine	+	+	.	.	−	.	+	.	.
Tyrosine	+	+	++	−	++	+	++	+	+
Tryptophan	−	−	+	+?	−	.	.	−	−
Lysine	+	+	+	+	.	+?	+	+	++
Arginine	+	+	+	+	−	+?	.	+	++
Histidine	−	+	+	+	+	.	+	−	−
Methionine	−	−	.	.	.	.	−	+	−
Cystine or cysteine	−	−	.	.	−	.	−	−	−
Aspartic acid	Trace	+	Trace	Trace	.	.	+	+	+
Glutamic acid		++						++†	+
Asparagine	−	−	+	−	.	.	.	.	.
Glutamine	++	−	++	++	.	+?‡	.	+	−
Taurine	+?	+?	.	.	.	.	.	+	−
Polypeptide	++	−	+	+	.	.	.	+	+
Hydroxyphenyl amino-acid	++	++	.	.	.	.	.	.	.

[a] Ussing (1946); [b] Florkin & Duchâteau (1942); [c] Raper & Shaw (1948); [d] Finlayson & Hamer (1949); [e] Agrell (1949).
* These data are for the whole pupa extract, but the amino-acids are probably fairly equally divided between the pupal fluids and tissues (cf. Heller, 1932).
† Probably formed from some labile precursor (Agrell, 1949). ‡ Personal communication.

for 2 hr. at 100°; this is typical for glutamine (Vickery, Pucher, Clark, Chibnall & Westall, 1935). (3) The enzymic method employed for its estimation (Krebs, 1948), which appears to be entirely specific for glutamine, was applied and the glutamine content of the blood of three species of insects, as found by this technique, is given in Table 5. By this

Table 5. *Concentration of glutamine in insect blood*

Species	No. of samples	Glutamine (mg./100 ml.)
Gastrophilus intestinalis, 3rd instar larva	4	98·6
Arctia caia (Lep.) larva, shortly before the pupal moult	2	302
Calliphora erythrocephala (Dip.), fully grown 3rd instar larva	2	89·5

method, very little or no free glutamic acid was found, thus confirming the evidence of the chromatograms. The glutamine content of 98·6 mg./100 ml. agrees reasonably well with the value calculated from the amide nitrogen figure, assuming the whole of this is due to glutamine; the approx. 11·5 mg./100 ml. amide nitrogen found in the blood would be formed after hydrolysis from some 119 mg./100 ml. of glutamine. It may be noted also that Ussing (1946) found 150 and 196 mg./100 ml. of glutamine in two species of beetles.

The second intense spot obtained from the *Gastrophilus* chromatograms is referred to in Table 4 as hydroxyphenyl amino-acid, and has not yet been identified; both on single- and two-dimensional chromatograms its R_F value is very close to that of alanine, which has prevented the certain identification of the latter. That the spot is not due to alanine has been demonstrated as follows. Bands, as opposed to spots of the amino-acid concentrate, were run on large sheets of filter paper in one direction using *n*-butanol-acetic acid-water. After 16–18 hr. the paper was dried, the band due to the unknown material (located on the paper by its fluorescence in ultraviolet light) cut out, and eluted off the paper with water. The solution so obtained was presumably pure, and was found to absorb strongly in the ultraviolet as measured in the Beckman spectrophotometer (Table 6), and to be rapidly oxidized by the tyrosinase present in *Gastrophilus*

blood. Alanine does not absorb in the ultraviolet, and is not attacked by tyrosinase.

Table 6. *Absorption maxima of the unidentified amino-acid from* Gastrophilus *blood*

pH	Absorption maxima (mμ.)
1	265
6	270
10	283

The absorption figures (Table 6) are similar to those of other hydroxyphenols, e.g. tyrosine (Holiday & Ogston, 1938), 3:4-dihydroxyphenylalanine (DOPA) (see Mason, 1948, for references), and since the R_F value in *n*-butanol-acetic acid-water was closer to that of DOPA than of tyrosine, it is tentatively inferred that two hydroxyl groups are involved. However, none of the colour tests employed for characterizing phenols, e.g. those with ferric chloride, Millon's reagent, Liebermann's potassium nitrite-sulphuric acid and Pauly's diazobenzenesulphonic acid were positive. In addition, no red colour was obtained following oxidation of blood filtrates with iodine, which thus excludes the presence of either DOPA or adrenaline (Evans & Raper, 1937; Schild, 1933), and the unknown substance differed from DL-2:5-dihydroxyphenylalanine (Neuberger, 1948) in its R_F values and the rate of oxidation by the blood tyrosinase. The nature of this compound is therefore uncertain.

The tyrosine content of the haemolymph was estimated by the tyrosine decarboxylase method of Gale (1945), which is considerably more specific than the usual colorimetric techniques. The mean of three series of estimations gave a tyrosine content of 25 mg./100 ml.

Phosphorus compounds

The true inorganic phosphorus level in *Gastrophilus* blood can only be obtained by using 'iced' larvae with due precautions to keep the TCA blood filtrate cold until neutralized. Inorganic phosphorus values from 'warm' larvae, or of blood that had been allowed to stand at room temperature for 15–20 min. before deproteinization, were up to four times as high as those for 'iced' larvae. This is probably due, at least in part, to the presence of a very active blood phosphatase which was demonstrated by the rapid liberation of inorganic phosphorus from added adenosinetriphosphoric acid (ATP), and whose action could largely be inhibited by fluoride. The greater part of the phosphorus esters representing the difference between the inorganic phosphorus and the total acid-soluble phosphorus (about 70 mg./100 ml.) was not hydrolysed after 3 hr. in N-hydrochloric acid at 100°. The amount of easily hydrolysable phosphorus esters is small, as shown in Fig. 1; the values would probably have been a little higher if 'iced' larvae had been employed. From three such hydrolysis curves the values calculated for the categories of hydrolysable phosphorus esters in the blood are shown in Table 7. Since the phosphorus compounds have not been investigated further, any more precise description of their nature is at present unwarranted.

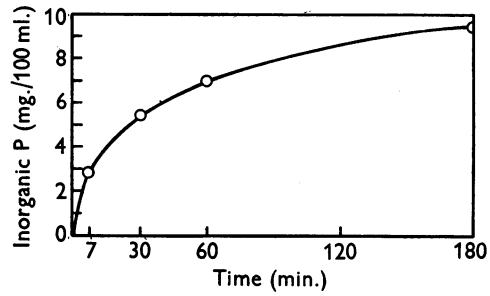

Fig. 1. Hydrolysis of P esters from *Gastrophilus* blood in N-HCl at 100°.

Table 7. *Hydrolysable phosphorus esters in* Gastrophilus *blood*

(P_0 = inorganic P, P_7, P_{30}, P_{60}, and P_{180} indicate the amount of inorganic P liberated by acid hydrolysis after 7, 30, 60 and 180 min.)

	(mg./100 ml.)
P_0	22·8
$P_7 - P_0$	2·4
$P_{30} - P_7$	1·7
$P_{60} - P_{30}$	1·5
$P_{180} - P_{60}$	1·1
$P_{\text{total acid-sol.}} - P_{180}$	49·9

Lactic acid

Lactic acid was another substance, the concentration of which changed with temperature. At either 0 or 37° the variation in lactate was high, 12·3 mg./100 ml. (S.D. ± 9·3 mg./100 ml.) at 0° and 44·5 mg./100 ml. (S.D. ± 15·9 mg./100 ml.) at 37°. Thus the values obtained from the active 'warm' insects were three to four times higher than those from immobile larvae. The concentration of other organic acids, e.g. succinic (Levenbook & Wang, 1948) and citric acids, was but slightly, if at all, affected by temperature.

Ammonia

In their investigations on the ammonia content of the blood in certain aquatic invertebrates, Florkin & Frappez (1940) found that the resting ammonia levels for the beetles *Dytiscus* and *Hydrophilus* were extremely low, but increased rapidly after shedding the blood, whereas in the crayfish the ammonia content of the blood as drawn was already relatively high, and there was no further increase on standing. A similar investigation on *Gastrophilus* haemolymph

was complicated by the fact that it was not possible to employ the Conway (1947) diffusion technique as used by the above authors; all the salts recommended by Conway caused coagulation of the haemolymph, and determinations were therefore made by distillation in the Parnas-Heller apparatus.

The resting ammonia values in the blood of either 'warm' or 'iced' larvae increased rapidly on being allowed to stand at room temperature, but the initial level was considerably lower in the latter. In either case the final value about 1 hr. after shedding was approximately the same although, as shown in Fig. 2, the rate of increase was different. There was

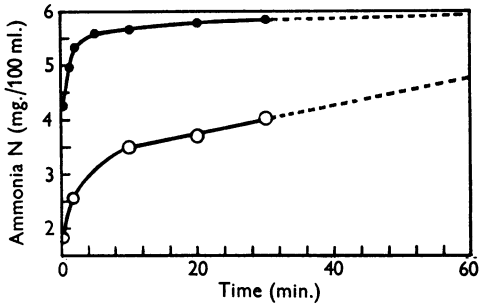

Fig. 2. Formation of NH_3 in *Gastrophilus* blood allowed to stand at room temperature. ●—●, 'warm' larvae; ○—○, 'iced' larvae.

no increase in the resting ammonia level if the blood was collected and allowed to stand for up to 15 min. under a layer of 10 cm. of heavy paraffin oil. The labile precursors responsible for the ammonia formation are at present unknown.

DISCUSSION

The interesting features in the composition of *Gastrophilus* haemolymph may be presented best by a comparison with the blood of other species, and with human plasma, on which a great deal of careful analytical work has been performed.

Osmotic pressure and mineral content of Gastrophilus *blood*

The mineral content of *Gastrophilus* blood has no outstanding features as compared with other species. Thus the value for sodium was high and that for potassium low, which agrees with the general finding of Boné (1944) that in terrestrial insects potassium exceeds sodium in the haemolymph of phytophagous species, while the converse is true for carnivorous or haematophagous forms. The sodium content in *Gastrophilus* is only slightly higher, and the potassium content about twice as high as in human plasma. But the calcium content, about the same as for plasma, is distinctly lower than in most other insects, whereas the magnesium, which is about the same as found in various Lepidoptera, is over twelve times that present in human plasma. Marine invertebrates excepted, a high magnesium content in insect haemolymph is characteristic of the class, but the state of this electrolyte, as indeed of all the others, is quite unknown, and an investigation would undoubtedly be difficult. Thus to consider only calcium and magnesium, both of these can form unionized combinations with proteins, amino-acids, carbonate, phosphate, succinate and citrate, all of which are present in *Gastrophilus* blood. Values for the dissociation constants of such complexes have been worked out for mammalian blood, but it is, nevertheless, still uncertain what proportion of these cations is in the free ionized form. Since the general constitution, and particularly the nature of the proteins (Florkin & Duchâteau, 1943) of insect blood, is different from that of the mammal, it would appear to be premature to apply to the former the equations expressing the ionic interrelationships existing in the latter (see, for example, Conway, 1945).

In mammalian plasma two-thirds of the total cations are maintained at electroneutrality by the predominant chloride anion. For insect haemolymph in general, and *Gastrophilus* in particular, the position is quite different. In the silkworm, Bialaszewicz & Landau (1938) found chloride to account for 12–18% of the total blood base, while Bishop, Briggs & Ronzoni (1925) postulated the presence of organic acids to make up for the deficit of anions which they found in bee larva blood. The picture for *Gastrophilus* is shown in Table 8, where the major ionic constituents have been recalculated from Table 1 on the basis of a water content of 85% in m-equiv./l. of water.

Table 8. *Anion-cation balance in* Gastrophilus *blood*

Cations	m-equiv./l. water
Na^+	206
K^+	13
Ca^{++}	7
Mg^{++}	38
Sum of cations	264
Anions	
Cl^-	17
HCO_3^- (pH 6·8)	17
SO_4^{--}	6
Phosphate (assuming each mol. of total P binds 1 equiv.)	40
Proteinate$^-$	17
Succinate^{--}	48
Citrate^{---}	8
Lactate$^-$	2
Sum of anions	155

The figures in Table 8 have been calculated to the nearest m-equiv. and, particularly in the case of proteinate and phosphate, are to be considered as a first approximation only. They do show, however,

that chloride amounts to less than 7 % of the total base, whereas organic acids represent about 22 %. It is clear that there is a large deficit of anions amounting to some 110 m-equiv.; this is probably to be explained by the considerable proportion of undissociated bivalent base, a small amount of base bound by amino-acids and other acidic substances still to be detected.

Reducing substances in Gastrophilus *blood*

The t.r.v. of mammalian plasma is predominantly due to glucose and its value is relatively constant. In insect blood the t.r.v. varies widely, not only from one species to another, but also in the same insect at different stages of its life history as shown by Levenbook (1947) for *Gastrophilus*, Demjanowski & Prokoffjewa (1935) and Florkin (1937) for the silkworm, and by other workers. Further, glucose is only of minor importance in insects, since the greater part, generally over 70 %, of the t.r.v. is not fermentable by yeast. However, incubation of *Gastrophilus* blood with yeast produces a very substantial decrease in the t.r.v., but the substance fermented is not glucose but fructose, together with one or more substances giving a positive Seliwanoff reaction. Fructose is absent from the blood of other insects (Levenbook, 1947) and this is another demonstration of the irregular distribution of this sugar in animal tissues. Fructose is, for example, found in various embryonic fluids and foetal blood (Needham, 1942; Bacon & Bell, 1948), in seminal plasma (Mann, 1946) and in the reproductive organs of the male locust (Humphrey & Robertson, 1949), but is not normally present to any extent in the blood of the adult mammal. The function of the fructose in *Gastrophilus* is still under investigation, but it is of interest that the sugar gradually disappears during pupation, and that the larval glycogen is composed of the usual glucose, and not fructose, units.

Amino-acids

The α-amino nitrogen content (93·8 mg./100 ml.) of *Gastrophilus* haemolymph is relatively low as compared with other insects, but it is still about twenty times greater than the concentration of free amino-acids in mammalian plasma. Florkin (1949) considers that 'the osmotic pressure of insect blood is to a significant extent largely maintained by the presence of a high concentration of free amino-acids' and suggests that this is a biochemical characteristic of insect haemolymph, and as such it should also apply to *Gastrophilus*. To what extent this is so may be seen from the following.

As shown in Table 4, the concentration of basic amino-acids arginine, lysine and histidine is low in this insect, and the isoelectric point of the remainder is not far removed from the pH of the blood. Hence the bulk of these acids is in the form of free 'zwitterions', $^{+}NH_3.R.COO^{-}$, the osmotic effect of which is comparable to that of a non-electrolyte. It may be assumed that each atom of amino nitrogen is equivalent to a molecule of amino-acid, corresponding to a molar Δ value of $-1{\cdot}86°$. The presence of considerable amounts of free glutamine necessitates a small correction. Hamilton (1945) showed that some 80 % of the amide nitrogen of glutamine reacts as α-amino nitrogen in the Van Slyke manometric method. Since the glutamine content has been determined in the present case, this effect may be allowed for by subtracting approx. 7 mg./100 ml. from the 93·8 mg./100 ml. of total amino nitrogen. The Δ value due to amino-acids is then calculated as $-0{\cdot}87$ (g. amino nitrogen/l.) $\times 1{\cdot}86/14 = -0{\cdot}115°$, or only some 13 % of the total osmotic pressure. But it is relevant in this connexion that Bishop *et al.* (1925) found a known mixture of amino-acids to give a Δ value over twice that computed on the basis of the above assumptions, so that in most insects with their considerably higher free amino-acid content, these acids may well play a major part in maintaining the osmotic pressure.

In comparing the nature and distribution of the amino-acids in insects, it would appear from Table 4 that glycine, alanine (probably), valine, leucine or isoleucine, serine, proline, tyrosine, arginine, lysine, glutamine and polypeptides occur in the blood of most, if not all, insects; the dicarboxylic acids, threonine, phenylalanine, histidine, tryptophan and asparagine are either less widely distributed or else are present in traces, whereas methionine has only been found occasionally in one species and cyst(e)ine is apparently absent. In view of the high concentrations of glutamine found in the blood of different species as reported in the present work, it is of interest that this substance accounts for 18–25 % of the total α-amino nitrogen in mammalian plasma (Hamilton, 1945).

The nature of the unknown phenolic amino-acid

It has been shown above that an intense, unidentified chromatogram spot assumed to be a phenolic amino-acid is a major constituent in *Gastrophilus* blood, and it is considered to be of special significance for the following reason. Pryor (1940) and Pryor, Russell & Todd (1947) have shown that the darkening and hardening (tanning) of insect cuticle is due to the action of a phenolase on an *o*-dihydroxyphenol, and it was suggested that the phenol was produced by the deamination of blood DOPA, itself formed enzymically from tyrosine. However, Lafon (1943) concluded that tyrosine was not the phenolic precursor, whereas Fraenkel & Rudall (1947) presented evidence that it was. Now it has been demonstrated that DOPA is absent from *Gastrophilus* blood, and the tyrosine

content is far less than was found by Fraenkel & Rudall (1947) and by Finlayson & Hamer (1949) in the blood of two other dipterous larvae. The former authors gave values of over 0·5 %, and should these be confirmed by a different method, the question would arise as to how such a high concentration could be obtained since the solubility of tyrosine in water at 25° and physiological pH is less than 0·05 %. Their results could be explained, however, if some other more soluble phenolic amino-acid, also present in the haemolymph and giving a similar colour reaction, had been estimated together with the tyrosine. Despite the finding that the phenolic amino-acid from *Gastrophilus* blood did not give a positive Millon reaction after chromatographic isolation (for which a variety of explanations might be offered), the fact that only phenolic substances have been found to be rapidly oxidized by insect tyrosinase, the close similarity between the absorption curve of this material and other phenols and the strong ninhydrin reaction, all point to the possibility of this substance being the phenolic precursor in *Gastrophilus*. In addition, the chromatogram spot, always strong, becomes intensified shortly before pupation, which agrees with this view. As the R_F values of this amino-acid and of alanine are very close, the high concentration of the latter in the blood of *Calliphora* as reported by Finlayson & Hamer (1949) might well repay further study.

Urea and uric acid

In *Gastrophilus* blood the concentration of urea is the highest, and that of uric acid the lowest yet recorded for insect haemolymph; the values obtained are similar to those for mammalian plasma. These are probably environmental adaptations, especially as there is no necessity for the conservation of water. In considering the view that 'the very high uric acid content of the blood is a characteristic of insects which distinguishes them from all other invertebrates' (Florkin, 1949), it may be pointed out that Drilhon & Florence (1946) have presented spectrophotometric evidence that in the blood of Lepidoptera the so-called uric acid is not in fact this trihydroxypurine, but some as yet undetermined substance and the presence of true uric acid in insects would appear to require further investigation.

Physiological salt solution for insect tissues

Krebs & Henseleit (1932), in designing their widely used mammalian physiological salt solution, based its ionic composition on the analytical data for mammalian plasma. Although a number of salt solutions for use with insect tissues have been suggested, only that of Bishop *et al.* (1925) has borne any relation to the composition of the haemolymph of the insect for which it was devised.

From the data of Table 1 this has now been attempted for *Gastrophilus*, and a solution having a concentration of Na, K, Mg and Cl similar to that in the blood has been prepared. Phosphate was employed as the principal anion, and Ca was omitted, as it was found to have a deleterious effect on the insect tissues *in vitro*. This solution had the following composition: NaCl, 0·1 g.; Na_2HPO_4, 1·1 g.; $NaH_2PO_4.2H_2O$, 0·53 g.; KH_2PO_4, 0·15 g.; $MgSO_4.7H_2O$, 0·4 g.; distilled water to make up 100 ml. In certain cases 0·048 g. of anhydrous $NaHCO_3$ was added and CO_2 from the cylinder bubbled through the solution for 30 min. prior to use. The pH was 6·70 in the first case, and 6·65 in the second. The osmotic pressure could not be measured cryoscopically on account of the precipitation which occurred when the solution was supercooled, but Δ was calculated to be about 0·65° which makes this medium rather hypotonic compared with haemolymph. However, increasing the salt concentration had little, if any, effect on the *in vitro* endogenous O_2 uptake of the insect tissues when suspended in these solutions. On allowing the solution to stand at room temperature for 24–36 hr., insoluble magnesium polyphosphate precipitated out. The medium was therefore made up in bulk without either the $MgSO_4$ or the bicarbonate, and these salts added in the required proportion to a sample on the day of use. Experiments involving the use of this solution will be presented in a future publication.

SUMMARY

1. The general properties, certain physical characteristics and a quantitative investigation of the inorganic and organic constituents of *Gastrophilus intestinalis* haemolymph are presented.

2. The presence of fructose in the blood has been demonstrated, together with one or more unidentified reducing substances which give a positive Seliwanoff reaction and are destroyed by yeast fermentation.

3. The nature of the free amino-acids in the blood has been investigated by chromatography, and the results compared with those from other insects. Glutamine has been estimated quantitatively in the blood of a number of species, and this amide, together with an unidentified phenolic amino-acid which is probably of importance in the darkening and hardening of the cuticle, are the major amino-acid constituents in *Gastrophilus* haemolymph.

4. It is shown that in the maintenance of electroneutrality, chloride accounts for 7 % and the organic acids so far determined for 22 % of the total base. A considerable deficit in anions was found.

5. On the basis of the present analyses, a physiological *Gastrophilus* saline solution has been devised for metabolic experiments on tissues from this insect *in vitro*.

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