directly proportional to the erythrocyte concentration. The system may be used as a sensitive assay of the enzyme–coenzyme activity of the cell.

3. Lysed erythrocytes react similarly only in the presence of low concentrations of added glutathione. The rate of glyoxalase activity in the presence of relatively high concentrations of glutathione increases with the methylglyoxal and sodium bicarbonate concentrations and is no longer proportional to the enzyme concentrations. Further, the reaction rate decreases with time.

4. An inhibitory effect of methylglyoxal on the glyoxalase system is described.

I should like to acknowledge with gratitude the help and encouragement of Professor J. A. Dauphinee, and to thank the Banting Research Foundation for a grant-in-aid toward the expense of this investigation.

REFERENCES

Bhattacharya, S. K., Robson, J. S. & Stewart, C. P. (1955). *Biochem. J.* **60**, 696.
Crook, E. M. & Law, K. (1952). *Biochem. J.* **52**, 492.
Hopkins, F. G. & Morgan, E. J. (1948). *Biochem. J.* **42**, 23.
Jowett, M. & Quastel, J. H. (1933). *Biochem. J.* **27**, 486.
Kun, E. (1950). *J. biol. Chem.* **187**, 289.
Lohmann, K. (1932). *Biochem. Z.* **254**, 332.
Platt, M. E. & Schroeder, E. F. (1934). *J. biol. Chem.* **104**, 281.
Racker, E. (1951). *J. biol. Chem.* **190**, 685.
Umbreit, W. W. (1947). In *Manometric Techniques and Tissue Metabolism*, p. 25. Ed. by Umbreit, W. W., Burris, R. H. & Stauffer, J. F. Minneapolis: Burgess Publ. Co.
Woodward, G. E. (1935). *J. biol. Chem.* **109**, 1.
Yamazoye, S. (1936). *J. Biochem., Tokyo*, **23**, 319.

The 'Malic' Enzyme in Insect Blood

By P. FAULKNER
Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada

(*Received* 6 *March* 1956)

Insect blood appears to have two main metabolic functions: first, that of storage; and secondly, that of transporting nutritional metabolites between the specialized tissues. An important difference between insect and mammalian blood lies in the chemical composition of the plasma; this is extremely variable throughout the life of the insect and appears to depend on many factors, including the general nutritional state (Ludwig & Wugmeister, 1953), the stage of development (Wyatt, Loughheed & Wyatt, 1956) and the health of the insect (Drilhon & Vago, 1951, 1953; Ishimori & Muto, 1951).

The chemical composition of insects' blood has been reviewed by Yamafuji (1937), Buck (1953) and Hinton (1954). It has been found that many substances serving as intermediates in mammalian metabolism are present in substantial quantities in the blood of the silkworm (*Bombyx mori* L.), e.g. di- and tri-carboxylic acids (Tsuji, 1909), amino acids (see review by Hinton, 1954), keto acids (Venkatachala Murthy & Sreenivasaya, 1953) and sugar phosphates (Smolin, 1952; Wyatt *et al.* 1956). Some of these substances undergo biochemical changes in the blood, and in this paper some account of the metabolism of organic acids in the blood of the silkworm and of certain forest insects is given.

A preliminary account of this work has appeared (Faulkner, 1955*a*).

EXPERIMENTAL

Preparation of silkworm blood. Silkworm blood was collected as described previously (Faulkner, 1955*b*) and stored without dilution at −28°. Dialysed silkworm blood was obtained as follows. Whole blood was diluted with an equal vol. of water, transferred to a cellophan bag, and dialysed against 5 l. of water with constant stirring for 20 hr. at 1–3°. During dialysis, the blood, which was originally light yellow in colour, darkened and deposited a black material. After dialysis, the solution was filtered through Whatman no. 1 paper, and the filtrate, designated as 'dialysed blood', was stored at −28°.

Materials. Diphosphopyridine nucleotide (DPN) and triphosphopyridine nucleotide (TPN) (Nutritional Biochemicals Corp., Cleveland, Ohio), were 95% pure. Sugar phosphates (Schwarz Inc., New York) were obtained as barium salts and were converted into sodium salts.

Methods. Protein was determined according to the method of Lowry, Rosebrough, Farr & Randall (1951) and keto acid by the method of Friedemann & Haugen (1943). Malic-dehydrogenase activity was assayed by measuring the rate of reduction of TPN at 340 mμ. with silica cells (1 cm. light path) in a Beckman DU spectrophotometer. The standard test system contained the following in a total vol. of 2·2 ml.: aminotrishydroxymethylmethane (tris) buffer, pH 8·5, 18 mM; $MgSO_4$, 2·3 mM; L-malate, 9 mM; TPN, 0·23 mM; enzyme as indicated. All incubations were carried out at room temperature (20°). In the larger-scale experiments the cells were removed from the light-proof compartment between readings to prevent heating of incubation mixtures. One unit of enzyme is defined as the

amount that causes an increase of 0·01 optical density unit/min. in the test system (Ochoa, 1952). The rate was calculated during the third minute of incubation.

Incubations in which keto acid formation was measured were carried out in open test tubes (3·5 ml. capacity) in a water bath at 30°. After incubation, protein was precipitated by addition of trichloroacetic acid (final concn. 2·5%, w/v), and keto acid analyses were performed on the supernatant.

RESULTS

Dehydrogenase activity of silkworm blood. TPN is rapidly reduced when incubated with diluted silkworm blood and cannot be replaced by either DPN or a mixture of DPN and ATP. The results given in Fig. 1 show that TPN is not reduced when added to dialysed blood. However, if a heated extract of whole blood is added to an incubation mixture containing TPN and dialysed blood, reduced TPN is produced. The data given in Fig. 1 also indicate that reduction of TPN by silkworm blood is enzymic, since no reaction occurs when TPN is incubated with heated whole blood. These observations demonstrate that a TPN-linked dehydrogenase and its substrate are present in silkworm blood.

Substrate specificity. The substrate specificity of the blood dehydrogenase was investigated in a test system containing the following components: tris buffer (pH 8·0), Mg^{2+} ions, TPN, dialysed silkworm blood and the test substrate. Of the compounds tested, only L-malate and fumarate were sufficiently active as substrates to account for the reduction rates obtained in the non-dialysed blood. The results shown in Fig. 2 illustrate that the L-malic dehydrogenase of silkworm blood is specific for TPN.

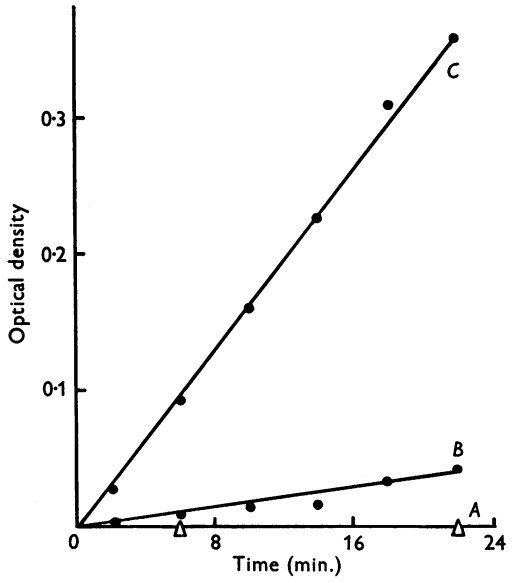

Fig. 1. Effect of dialysis on the activity of the silkworm-blood dehydrogenase. Tris, pH 7·5, 17 mM, was present in all incubations. The following additions were made as indicated: TPN, 0·38 mM; dialysed blood, 0·5 ml.; heated extract, obtained by heating diluted silkworm blood (1:4, v/v) for 5 min. at 100° and centrifuging, 0·5 ml. Total vol., 2·5 ml. Blank cell contained tris and dialysed blood. *A* contained TPN + heated extract; *B* contained TPN + dialysed blood; *C* contained TPN + dialysed blood + heated extract.

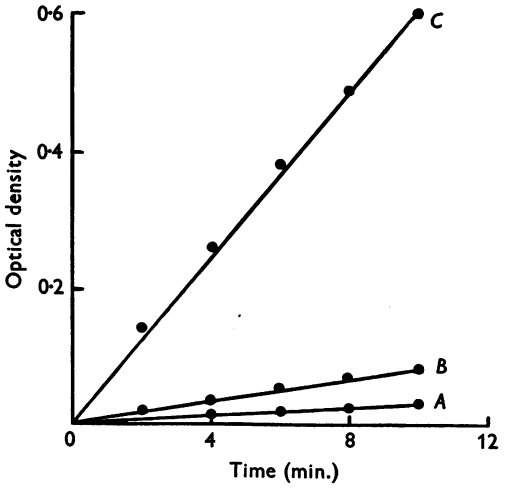

Fig. 2. Pyridine nucleotide specificity of the malic dehydrogenase. Basic medium, present in all cells, contained tris, pH 7·5, 34 mM; $MgSO_4$, 4·2 mM; dialysed blood, 0·5 ml. The following additions were made as indicated: L-malate, 4·2 mM; DPN, TPN, 0·25 mM. Total vol., 2·5 ml. Blank cell contained basic medium only. *A* contained L-malate + DPN; *B* contained TPN; *C* contained L-malate + TPN.

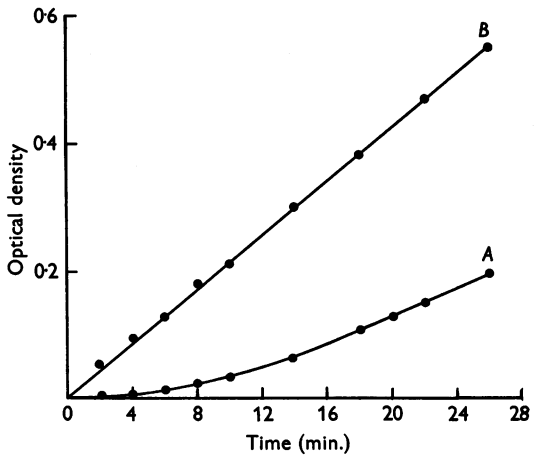

Fig. 3. Oxidation rates of L-malate and fumarate. Standard test conditions with L-malate and fumarate (9 mM) added as indicated; dialysed blood, 0·3 ml. In *A*, fumarate, and in *B*, malate, was added at zero time.

In the experiment shown in Fig. 3 the oxidation rates of L-malate and fumarate added at zero time to separate cells are compared. The oxidation of L-malate was immediate and its rate constant. However, a lag period of approximately 4 min. preceded oxidation in the cell containing fumarate. This suggests that fumarase is present in silkworm blood, and that fumarate added to the incubation mixture is converted into malate, which is subsequently oxidized by malic dehydrogenase.

Substances not oxidized under the test conditions include the following: D-malate, L-aspartate, L-glutamate, sorbitol, glucose, glucose 6-phosphate, ribose 5-phosphate, α- and β-glycerophosphate, D- and *meso*-tartrate, succinate and lactate.

Localization of the dehydrogenase in insect blood. Silkworm blood, in common with that of many other insects, contains few cells. Ikeda (1904) reports 6000 cells/ml. in fifth-instar silkworm larvae. The following experiment was carried out to determine whether the dehydrogenase found in whole blood was located in the plasma or in the cells.

Blood from forty larvae was divided into two parts. The first part was centrifuged at 20000 *g* at 1° for 30 min., and the supernatant diluted with cold water (1:4, v/v). The second, uncentrifuged, portion of blood was also diluted in the same manner, and the two samples were assayed for dehydrogenase activity in the standard test system. Enzyme activity in uncentrifuged blood was 25·5 units/ml., compared with 23·0 units/ml. in the supernatant of centrifuged blood. This result clearly indicates that most of the enzyme is present in the plasma, and that little of the total activity is associated with the blood cells.

pH–*Activity relationship.* Results given in Fig. 4 show that the pH optimum of the malic dehydrogenase of silkworm blood is 8·4. The average pH of fifth-instar silkworm blood is 6·77 (Heimpel, 1955).

End-product of malate oxidation. Two types of malic dehydrogenase requiring pyridine nucleotides have been described. The first is the 'malic' enzyme, which oxidatively decarboxylates L-malate to yield pyruvate (see Ochoa, 1952, for properties and distribution). The second enzyme, which was purified by Straub (1942), is malic dehydrogenase; it oxidizes malate to oxaloacetate. Sacktor (1953) studied a DPN-linked malic dehydrogenase in house-fly mitochondria, but the product of malate oxidation by this insect enzyme was not identified.

Experiments were performed to determine the identity of the keto acid end-product of malate oxidation in silkworm blood. Preliminary tests confirmed that pyruvate and oxaloacetate can be distinguished by the analytical procedure of Friedemann & Haugen (1943) for α-keto acids. In this method the 2:4-dinitrophenylhydrazones of the keto acids are extracted in different solvents. Ethyl acetate readily extracts the 2:4-dinitrophenylhydrazones of both pyruvate and oxaloacetate, but benzene selectively extracts the pyruvate derivative; under our conditions only 20% of the oxaloacetate derivative was extracted by benzene from an equimolar mixture with pyruvate. Thus by comparing the amounts of phenylhydrazone extracted by ethyl acetate and by benzene, it is possible to determine whether pyruvate or oxaloacetate is the predominating end-product.

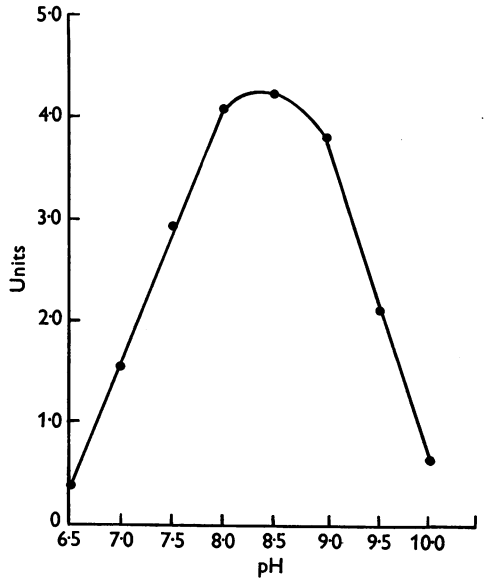

Fig. 4. pH–Activity relationship for L-malic dehydrogenase. Standard test system with 0·3 ml. of dialysed blood and the following buffers at 66 mM: sodium phosphate, pH 6·5–7·5; tris, pH 7·5–9·0; glycine–HCl, pH 9–10. At pH 7·5 and 9·0, where the buffers overlapped, the mean enzyme activity has been plotted. The difference of activity in the buffers at pH 7·5 and 9·0 did not vary more than 5% from the mean.

Table 1. *α-Keto acid production during malate oxidation*

Basic medium: tris buffer, pH 8·5, 20 mM; $MgSO_4$, 5 mM; dialysed blood, 0·5 ml. These additions were made as indicated: L-malate, 10 mM; TPN, 0·08 mM; methylene blue (MeB), 0·8 mM. Total vol., 2·0 ml. The mixtures were incubated for 60 min. at 30°.

Additions to basic medium			Pyruvate formed (μmoles)	
L-Malate	TPN	MeB	Ethyl acetate as extractant	Benzene as extractant
+	+	+	0·765	0·748
+	+	−	0·192	0·178
+	−	+	0·055	0·046
+	−	−	0·039	0·032
−	+	−	0·072	0·063
−	−	+	0·049	0·046

Table 2. *Purification of 'malic' enzyme in silkworm blood*

Fraction	Treatment	Total units	Percentage of DB-15	Specific activity (units/mg. of protein)
DB-15	Dialysis of whole blood	92	100	8·1
R_1	0–30% ammonium sulphate saturation	11	12	13·5
R_2	30–40% ammonium sulphate saturation	14	15	14·5
R_3	40–50% ammonium sulphate saturation	44	48	79·6
R_4	50–60% ammonium sulphate saturation	19	20	0·4
E_1	Adsorption of R_3 on $Ca_3(PO_4)_2$ gel at pH 5·0 in acetate buffer (2·5 mM). Elution with phosphate buffer, pH 7·3 (50 mM)	11	12	221·0

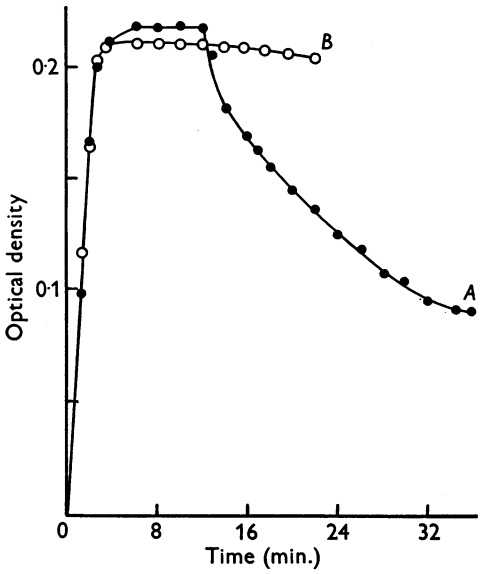

Fig. 5. Reversibility of the 'malic' reaction. Initial incubation conditions for both experiments: tris, pH 8·5, 10 mM; $MgSO_4$, 2·5 mM; L-malate, 2·5 mM; TPN, 0·09 mM; dialysed blood, 0·3 ml. In *A*, 25 μmoles of sodium pyruvate and 56 μmoles of $NaHCO_3$ were added at 12 min.; in *B*, 20 μmoles of oxaloacetate were added at 12 min. Final vol., 2·5 ml. Optical density readings have been corrected for dilution.

The results of a typical experiment with the silkworm-blood enzyme are given in Table 1. In this experiment the concentration of added TPN was kept low and methylene blue was added to reoxidize reduced TPN as it was formed in order to obtain maximum production of the keto acid end-product. The results show that roughly equivalent quantities of 2:4-dinitrophenylhydrazones were extracted with either benzene or ethyl acetate, indicating that pyruvate is the main end-product. These results also demonstrate that TPN, malate and an oxidizing agent such as methylene blue are necessary for maximum pyruvate production.

Reversibility of the enzyme reaction. The preceding experiments suggested that silkworm blood contains a 'malic' enzyme; however, the possibility had to be considered that oxaloacetate was the primary product of malate oxidation and that it subsequently yielded pyruvate by either enzymic or spontaneous decarboxylation. The strongest evidence for the non-participation of oxaloacetate in the reaction is based on the requirements for its reversibility.

TPN was reduced enzymically by a small quantity of L-malate, and when reduction of the TPN was complete pyruvate and bicarbonate were added (Fig. 5, curve *A*). The initial rate of reoxidation of reduced TPNH was rapid, showing that the reaction was readily reversed. In a second experiment (Fig. 5, curve *B*) oxaloacetate was added after the complete reduction of TPN, but in this case there was no rapid reoxidation as would be expected if oxaloacetate were an intermediate in L-malate oxidation.

Purification of the enzyme. Purification of the dialysed-blood preparation as a preliminary to a study of the effect of cations on the 'malic' enzyme was carried out by a combination of ammonium sulphate precipitation and fractional elution from calcium phosphate gel (Table 2). It will be seen that fraction R_3, precipitating between 40 and 50% of ammonium sulphate saturation, contained most of the 'malic' enzyme activity. Fraction R_3 was purified further by adsorption on calcium phosphate gel at pH 5·0 and subsequent elution at pH 7·3, yielding a solution whose specific activity was 221 units/mg. of protein. Veiga Salles & Harary (quoted by Ochoa, 1952) purified the 'malic' enzyme of pigeon liver by ethanol fractionation and fractional adsorption on calcium phosphate gel, and obtained a preparation more than three times as active as that obtained from insect blood.

Effect of cations and of ethylenediaminetetraacetate (EDTA). The 'malic' enzyme of pigeon liver has an absolute requirement for Mn^{2+} or Mg^{2+} ions (Veiga Salles & Ochoa, 1950). Silkworm-blood preparations that had been dialysed overnight against water usually did not require addition of Mg^{2+} or Mn^{2+} ions for maximum activity; however, Mg^{2+} ions were always added to the test system to offset any possible deficiency. The chelating agent EDTA

Table 3. *Effects of* Mn^{2+}, Mg^{2+} *and ethylenediaminetetraacetate (EDTA)*

Standard test system with Mn^{2+}, Mg^{2+} and EDTA as indicated.

Expt.	Enzyme preparation	EDTA (mM)	Cation tested	Activity of 'malic' enzyme (units)
1	Dialysed blood, 0·3 ml.	None	None	3·80
		2·0	None	0·20
		2·0	Mg^{2+} ($MgSO_4$, 2·5 mM)	3·75
2	Fraction DB-15-R_3, 0·1 ml.	None	None	0·25
		None	Mg^{2+} ($MgSO_4$, 0·25 mM)	0·60
		None	Mn^{2+} ($MnCl_2$, 0·25 mM)	1·4

Table 4. *Effect of* p-*chloromercuribenzoate (PCMB), oxaloacetate (OAA) and sugar phosphates on* L-*malate oxidation*

Basic medium: tris, pH 8·5, 20 mM; $MgSO_4$, 2·5 mM; TPN, 0·08 mM; dialysed blood, 0·3 ml. Other additions were made as indicated. Total vol., 2·2 ml.

Expt.	L-Malate (mM)	Inhibitor	Activity of 'malic' enzyme (units)	Inhibition (%)
1	10·0	None	4·90	—
	10·0	PCMB (2·0 mM)	2·55	48
	10·0	PCMB (4·0 mM)	1·70	65
2	2·5	None	4·65	—
	2·5	OAA (1·4 mM)	2·20	53
	10·0	OAA (1·4 mM)	3·25	30
3	10·0	None	4·90	—
	10·0	Glucose 6-P (10 mM)	3·45	30
	10·0	Ribose 5-P (10 mM)	1·20	76

strongly inhibits malate oxidation by preparations of dialysed blood, but if both Mg^{2+} ions and EDTA are present in the incubation mixture full activity is retained (Table 3, Expt. 1).

If a preparation of fractionated blood is used as the enzyme source, Mn^{2+} and Mg^{2+} ions have marked stimulatory effects on the rate of malate oxidation, Mn^{2+} ions being more effective (Table 3, Expt. 2).

Inhibitors of malate oxidation. A number of compounds were found to inhibit the 'malic' enzyme of the blood. These include *p*-chloromercuribenzoate, oxaloacetate and certain sugar phosphates. Examples of the experimental conditions under which these substances were effective are given in Table 4. The inhibition by *p*-chloromercuribenzoate (Table 4, Expt. 1) suggests that the 'malic' enzyme, in common with several other dehydrogenases, contains sulphydryl groups that are essential for its function. It is of interest to note that iodoacetate had no effect on the rate of TPN reduction when present at equimolar concentrations with L-malate.

Oxaloacetate is an inhibitor of the 'malic' enzyme of the silkworm; roughly 50 % inhibition is observed when oxaloacetate is present at half the substrate concentration (Table 4, Expt. 2). The inhibition is decreased when the L-malate concentration is raised, indicating that it may be competitive. Straub (1942) found that the DPN-linked malic dehydrogenase of pig-heart muscle is also inhibited by oxaloacetate. The inhibition of the rate of TPN reduction by oxaloacetate is not due to the reoxidation of TPNH by oxalacetate, as no reaction is observed when TPNH and oxaloacetate are incubated in the presence of the enzyme.

The data in Table 4 also show that glucose 6-phosphate and ribose 5-phosphate diminish the rate of reduction of TPN by malate. The apparent inhibition by sugar phosphates is due to the activity of a polyhydric alcohol phosphate dehydrogenase in silkworm blood (Faulkner, 1956) that reduces certain sugar phosphates in the presence of reduced TPN.

Occurrence of the 'malic' enzyme in blood of other insects. The 'malic' enzyme has been detected, by the standard test system, in the blood of the following forest insects: *Neodiprion banksianae*, the jack-pine sawfly; *Nymphalis antiopa*, the spiny-elm caterpillar; *Malacosoma disstria*, the forest-tent caterpillar, and *Choristoneura fumiferana*, the spruce budworm.

DISCUSSION

The malic dehydrogenase of insect blood possesses several properties in common with the 'malic' enzyme of pigeon liver, e.g. its nucleotide specificity, its activation by Mg^{2+} and Mn^{2+} ions, and the incubation conditions necessary for the reduction of pyruvate. The activities of the 'malic' enzyme and

oxaloacetic decarboxylase are associated with the same protein fraction in the pigeon-liver enzyme (Ochoa, 1952). Preliminary experiments with the 'malic' enzyme of silkworm blood suggest that no oxaloacetic decarboxylase is present.

The physiological significance of the 'malic' enzyme in insect blood is at present a matter for speculation. Previous studies have demonstrated that both malate (Tsuji, 1909) and pyruvate (Venkatachala Murthy & Sreenivasaya, 1953) are to be found in silkworm blood and it is likely that equilibrium between these two substances is maintained by the 'malic' enzyme. The enzyme is probably concerned with the further metabolism of malic acid, which is contained in the silkworm's food. Mulberry leaves, which are the exclusive food source of the silkworm, have been reported to contain 0·13 g. of malic acid/100 g. of fresh leaves (Suzuki, 1920). It is conceivable that the malic acid passes through the walls of the alimentary canal without chemical change and is metabolized in the blood.

A further function of the 'malic' enzyme may be its contribution to the maintenance of the anion–cation balance in the blood. In some insects it has been demonstrated that dicarboxylic acids make an important contribution to the total anionic strength. For example, the total cationic strength in *Gastrophilus* blood was found by Levenbook (1950) to be 264 m-equiv./l.; malate concentration is 42–64 m-equiv./l. (Nossal, 1952), accounting for over 20 % of the anionic strength. The malic acid concentration in silkworm blood was given by Tsuji (1909) as 0·5 %, i.e. 75 m-equiv./l. On the basis of Bialaszewicz & Landau's (1939) studies of the total anionic strength of silkworm blood, malic acid accounts for 43 % of the total number of anions. Should the number of anions in the blood decrease, or the cationic concentration increase, then by functioning in reverse, i.e. by malate synthesis from carbon dioxide and pyruvate, the 'malic' enzyme would be accomplishing the synthesis of further anionic carboxylic groups to offset the deficiency.

The 'malic' enzyme may also take part in the control of the redox potential of the blood, and may indirectly affect the extent of pigmentation and hardening of the insect's cuticle. Dennell (1947, 1949), working with *Sarcophaga falculata* and *Calliphora erythrocephala*, has shown that darkening of a new cuticle after moulting is due to melanin formation catalysed by the tyrosinase present in the blood and cuticle. He suggested that a dehydrogenase system present in insect blood determines the magnitude of the redox potential and hence the degree of tyrosinase activity and the rate of melanin formation. 'The liberation of the "pupation hormone" coincides with the complete and abrupt termination of dehydrogenase activity which leads to tyrosinase activity and hardening of the larval cuticle to form the puparium' (Dennell, 1949). Silkworm blood darkens rapidly upon exposure to air at room temperature owing to its phenoloxidase activity, and melanin particles are deposited. Under conditions *in vivo*, however, melanin formation is suppressed, although the blood is oxygenated. It is possible that the absence *in vivo* of tyrosinase activity is connected with the low redox potential of the blood, which may be maintained in part by the 'malic' enzyme system.

SUMMARY

1. Silkworm-blood plasma and the blood of several forest insects contain a triphosphopyridine nucleotide (TPN)-linked L-malic dehydrogenase. The enzyme has a pH optimum of 8·5–9·0 and requires Mn^{2+} or Mg^{2+} ions for full activity.

2. Pyruvate is the end-product of the L-malate oxidation. Reversibility of the enzyme has been demonstrated by addition of pyruvate and bicarbonate to a system containing reduced TPN.

3. The enzyme is inhibited by *p*-chloromercuribenzoate, oxaloacetate and some sugar phosphates.

4. The results suggest that fumarase is present in silkworm blood.

5. The possibility is considered that the 'malic' enzyme in insect blood helps to maintain the ionic balance, and may take some part in the control of the redox potential of the blood.

The technical assistance of Miss Lorna Callahan is gratefully acknowledged. This paper is Contribution no. 288, Forest Biology Division, Science Service, Department of Agriculture, Ottawa, Canada.

REFERENCES

Bialaszewicz, K. & Landau, C. (1939). *Acta Biol. exp., Varsovie*, **12**, 307.
Buck, J. B. (1953). In *Insect Physiology*. Ed. by Roeder, K. D. New York: John Wiley and Sons, Inc.
Dennell, R. (1947). *Proc. Roy. Soc.* B, **134**, 79.
Dennell, R. (1949). *Proc. Roy. Soc.* B, **136**, 94.
Drilhon, A. & Vago, C. (1951). *C.R. Acad. Sci., Paris*, **232**, 360.
Drilhon, A. & Vago, C. (1953). *Experientia*, **9**, 143.
Faulkner, P. (1955*a*). *Proc. 3rd Int. Congr. Biochem., Brussels*, p. 107.
Faulkner, P. (1955*b*). *Biochem. J.* **60**, 590.
Faulkner, P. (1956). *Biochem. J.* **64**, 436.
Friedemann, T. E. & Haugen, G. E. (1943). *J. biol. Chem.* **147**, 415.
Heimpel, A. M. (1955). *Canad. J. Zool.* **33**, 99.
Hinton, H. E. (1954). *Sci. Progr. Twent. Cent.* **42**, 684.
Ikeda, E. (1904). *Dainippon Sanshi Kaiho*, **151**, 11. [Quoted by Yamafuji (1937).]
Ishimori, N. & Muto, T. (1951). *J. seric. Sci., Tokyo*, **20**, 128.
Levenbook, L. (1950). *Biochem. J.* **47**, 336.
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). *J. biol. Chem.* **193**, 265.
Ludwig, D. & Wugmeister, M. (1953). *Physiol. Zool.* **26**, 254.

Nossal, P. M. (1952). *Biochem. J.* **50**, 349.
Ochoa, S. (1952). *The Enzymes*, vol. 2, p. 929. Ed. by Sumner, J. B. & Myrbäck, K. New York: Academic Press.
Sacktor, B. (1953). *Arch. Biochem. Biophys.* **45**, 349.
Smolin, A. N. (1952). *Biochemistry, Leningr.*, **17**, 61.
Straub, F. B. (1942). *Hoppe-Seyl. Z.* **275**, 63.
Suzuki, H. (1920). *Sakura Kaishi*, **8**. [Quoted by Yamafuji (1937).]
Tsuji, Y. (1909). *Sanji Hokoku*, **35**, 1. [Quoted by Yamafuji (1937).]
Veiga Salles, J. B. & Ochoa, S. (1950). *J. biol. Chem.* **187**, 849.
Venkatachala Murthy, M. R. & Sreenivasaya, M. (1953). *J. sci. industr. Res.* B, **12**, 314.
Wyatt, G. R., Loughheed, T. C. & Wyatt, S. S. (1956). *J. gen. Physiol.* **39**, 853.
Yamafuji, K. (1937). *Tabul. biol., Hague*, **14**, part 1.

Enzymic Reduction of Sugar Phosphates in Insect Blood

By P. FAULKNER
Laboratory of Insect Pathology, Sault Ste. Marie, Ontario, Canada

(*Received* 6 *March* 1956)

The presence of carbohydrate and phosphorus compounds in insect blood is now well established and data on their distribution have been summarized by Buck (1953). Smolin (1952) obtained evidence for the presence of glucose 6-phosphate and smaller quantities of glucose 1-phosphate in acid extracts of silkworm (*Bombyx mori* L.) blood. Wyatt, Loughheed & Wyatt (1956), using more refined techniques, have confirmed the presence of glucose 6-phosphate in acid extracts of freeze-dried silkworm blood but were unable to find glucose 1-phosphate. Of the seven phosphorus-containing materials separated by Wyatt *et al.* (1956) from silkworm blood, only glucose 6-phosphate and inorganic phosphate have been identified.

The present report is concerned with the finding that certain sugar phosphates are reduced to polyhydric alcohol phosphates by an enzyme in silkworm blood.

EXPERIMENTAL

Preparation of silkworm blood. The dialysed silkworm blood used in this investigation was obtained as described previously (Faulkner, 1956).

Materials. Glucose, glucose 6-, fructose 6- and ribose 5-phosphates were purchased from Schwarz Inc., New York; galactose 6-phosphate and triphosphopyridine nucleotide (TPN) from Nutritional Biochemical Co., Cleveland, Ohio, and reduced TPN from the Sigma Chemical Co., St Louis, Missouri. The galactose 6-phosphate was found to be contaminated with material reacting as a ketose, and was purified by several reprecipitations of the barium salt from ethanol–water (5:1, v/v).

Sorbitol 6-phosphate was prepared by reduction of the sodium salt of glucose 6-phosphate with a sodium borohydride solution by the procedure of Abdel-Akher, Hamilton & Smith (1951). The anthrone reagent, which does not give a colour with sorbitol, was used to determine when the sugar phosphate was completely reduced. The product was converted into the barium salt and recrystallized three times from ethanol–water (3:1, v/v) at pH 8·5. Periodate oxidation of the product gave a formaldehyde: phosphorus ratio of 1·16:1. The theoretical value for sorbitol 6-phosphate is 1:1.

The concentration of sugar phosphate solutions was calculated from the organic phosphorus content by the method of Fiske & Subbarow (1925).

Methods. Sugar phosphates were determined colorimetrically by means of the reaction with anthrone, as follows. To the sample, made up to 0·5 ml., 2 ml. of the anthrone reagent (Fairbairn, 1953) was added. After thorough mixing the solution was heated for 12 min. in a boiling-water bath and cooled immediately, and the colour read at 625 mμ.

Pentose was determined by the method of Meijbaum (1939), with 0·033% in place of 0·1% $FeCl_3$.

Polyhydric alcohols were determined by the periodate-oxidation method of West & Rapoport (1949) with the following modifications. Half volumes of samples and reagents were used throughout, and the solutions were treated with periodate for 4 min. only. The samples were heated in a boiling-water bath for 5 min. after addition of the chromotropic acid reagent. Sorbitol or ribitol (adonitol) was used as a standard.

Pyruvate was determined by the method of Friedemann & Haugen (1943), ethyl acetate being used to extract the 2:4-dinitrophenylhydrazone.

Experiments in which a change of reduced TPN concentration was measured were carried out at room temperature in silica cells (1·0 cm. light path) in a Beckman DU spectrophotometer at 340 mμ.

RESULTS

Reduction of sugar phosphates in dialysed silkworm blood

Dialysed silkworm blood contains a TPN-linked dehydrogenase capable of reducing a number of sugar phosphates. The reaction, whose equilibrium favours the formation of reduced-sugar phosphate, may be followed spectrophotometrically by observing the decrease in optical density at 340 mμ. of