6. The fact that these findings add to the complexity of the problem of determining the composition and the natural function of tannin extracts is emphasized.

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REFERENCES

Allan, J. & Robinson, R. (1924). *J. chem. Soc.* p. 2192.
Bate-Smith, E. C. & Westall, R. G. (1950). *Biochim. biophys. Acta*, **4**, 427.
Charlesworth, E. H. & Robinson, R. (1933). *J. chem. Soc.* p. 268.
Easterly, D. G., Hough, L. & Jones, J. K. N. (1951). *J. chem. Soc.* p. 3416.
Freudenberg, K. & Blummel, L. (1924). *Liebigs Ann.* **440**, 51.
Freudenberg, K. & Maitland, P. (1934). *Liebigs Ann.* **510**, 193.
Hough, L., Jones, J. K. N. & Wadman, W. H. (1949). *J. chem. Soc.* p. 2511.
Kafuku & Sebe (1932). *Bull. chem. Soc. Japan*, **7**, 114.
Kirby, K. S., Knowles, E. & White, T. (1951). *J. Soc. Leath. Tr. Chem.* **35**, 338.
Kirby, K. S., Knowles, E. & White, T. (1952). *J. Soc. Leath. Tr. Chem.* **36**, 45.
Kirby, K. S., Knowles, E. & White, T. (1953). *J. Soc. Leath. Tr. Chem.* **37**, 283.
Kirby, K. S. & White, T. (1954). *J. Soc. Leath. Tr. Chem.* **38**, 215.
Kuhn, R. & Low, I. (1944*a*). *Ber. dtsch. chem. Ges.* **77**, 202.
Kuhn, R. & Low, I. (1944*b*). *Ber. dtsch. chem. Ges.* **77**, 211.
Leopold, B. (1952). *Acta chem. scand.* **6**, 38.
Mayer, F. & Cook, A. H. (1943). *The Chemistry of Natural Coloring Matter.* New York: Reinhold.
Nierenstein, M. (1907). *Ber. dtsch. chem. Ges.* **40**, 4575.
Perkin, A. G. & Gunnell, O. (1896). *J. chem. Soc.* p. 1303.
Swain, T. (1953). *Biochem. J.* **52**, 200.
White, T. (1949). *J. Soc. Leath. Tr. Chem.* **33**, 39.
White, T., Kirby, K. S. & Knowles, E. (1952). *J. Soc. Leath. Tr. Chem.* **36**, 148.

A Hexose-1-Phosphatase in Silkworm Blood*

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A number of phosphatases have been found in insects; however, little is known concerning their substrate specificity and the part they play in intermediary metabolism. Drilhon (1943) showed that the gut of certain species of Coleoptera, Neuroptera, Orthoptera and Lepidoptera contains an alkaline phosphatase, while an acid phosphatase is present in the Malpighian tubules. The presence of an acid phosphatase has also been observed in the honey-bee, housefly, and cockroach (Rockstein & Levine, 1951). Day (1949), applying histological methods, studied the distribution of alkaline phosphatase in several insects. With β-glycerophosphate as substrate, activity was found in the alimentary tract, in muscle, and in storage, nervous, and reproductive tissue. The silk glands of the silkworm (*Bombyx mori* L.) contain a strong phosphomonoesterase (maximum activity, pH 4·2–5·0) and a feebler alkaline phosphatase (pH maximum, 7·5–8·5). An alkaline pyrophosphatase is also present (Denucé, 1952). A specific adenosinetriphosphatase (ATP-ase) is present in the mitochondria of the housefly (Sacktor, 1953). This enzyme is activated by magnesium and inhibited by azide but not fluoride. A fluoride-sensitive ATP-ase is present in the 'soluble' fraction of the housefly preparation.

The present paper is concerned with some of the properties of a specific hexose-1-phosphatase which has been found in silkworm blood.

* Contribution No. 190, Division of Forest Biology, Science Service, Department of Agriculture, Ottawa, Canada.

EXPERIMENTAL

Silkworm-blood preparation

Silkworm-blood preparations were obtained from pure-line strains of white, yellow, and zebra varieties of *B. mori* reared at this laboratory. Fifth-instar larvae within 2–3 days of pupation were used throughout the study. The larvae were bled into a test tube embedded in crushed ice. Blood collected in this manner can be stored for at least 3 months at −28° without loss of hexose-1-phosphatase activity. In the experiments to be described here 0·5ml. of a 1:10 (v/v) dilution of blood in water was used routinely. This represents, on the average, 250 μg. total N in non-dialysed or 75 μg. total N in dialysed blood.

Materials

All materials used were reagent grade. Glucose 1-phosphate, glucose 6-phosphate, fructose 6-phosphate, ribose 5-phosphate, hexose diphosphate and adenosine

triphosphate were obtained from Schwarz Inc., New York, U.S.A. A sample of galactose 1-phosphate was generously supplied by Dr D. J. Kushner. Sugar phosphates supplied as the Ba salt were converted into Na salts. Hexose diphosphate was purified by washing the Ba salt at pH 8·0 with water several times. *p*-Nitrophenyl phosphate was obtained from the Sigma Chemical Company, Saint Louis, Mo., U.S.A.

Incubations

Incubations were carried out in open test tubes (3·5 ml. capacity) in a water bath at 30°. The total volume incubated in each tube was 2·0 ml. 50 mM sodium acetate, pH 5·0, was present in all experiments except where the pH/activity relationship was studied. All substrates and test substances were brought to pH 5·0 by addition of dilute HCl or KOH before addition to the incubation tubes.

The amount of enzymic activity in a particular system was represented by the difference between the amounts of reducing sugar and/or inorganic phosphate in an incubated sample and its control. The control contained a heated enzyme preparation in place of the active enzyme and it was incubated and treated similarly to the active system. Using this technique it was found that glucose 1-phosphate was stable under the experimental conditions and that the concentration of inorganic phosphate in the non-dialysed enzyme preparation accounted for 0·3 mM/100 ml. whole blood, in agreement with the figures given by Bialaszewicz & Landau (1938).

After incubation the protein was precipitated by addition of 0·5 ml. 12% (w/v) trichloroacetic acid and mixing. Samples of the supernatant were taken for inorganic phosphate determination by the method of Fiske & Subbarow (1925) and reducing sugar by the LePage (1951) modification of the Folin & Malmros (1929) method. A Beckman DU spectrophotometer was used for all colorimetric readings. In some instances, e.g. in obtaining the kinetic data for fluoride inhibition, a 5 cm. light path was used instead of a 1 cm. path.

RESULTS

Correlation between release of inorganic phosphate and reducing sugar

Reducing sugar and inorganic phosphate are formed at equal rates when glucose 1-phosphate is incubated with the silkworm-blood preparation. This observation is illustrated by the results given in Table 1. Three experiments are reported. (i) The relationship between the time of incubation and the amounts of end products accumulating was determined. (ii) The concentration of the substrate, glucose 1-phosphate, was varied between 0·5 and 4 mM and the amounts of glucose and inorganic phosphate produced were determined. (iii) The relationship between the rate of reaction and the enzyme concentration was investigated. The results show that in each instance the ratio (inorganic phosphate) : (reducing sugar) is approximately unity indicating a parallel release of glucose and inorganic

Table 1. *Relationship between rates of reducing sugar and inorganic phosphate release*

Standard test system as in text with glucose 1-phosphate, 10 mM, in (i) and (iii). Incubated 20 min. at 30°. In (i) the time of incubation was varied as indicated, in (ii) glucose 1-phosphate concentration was varied, in (iii) the enzyme concentration was varied.

		Incubation time (min.)	Inorganic phosphate (μmoles)	Reducing sugar (μmoles)	Inorganic phosphate / Reducing sugar
(i)	Expt. 1	10	0·45	0·50	0·90
		20	1·07	1·08	0·99
		30	1·72	1·51	1·14
	Expt. 2	5	0·55	0·56	0·98
		10	0·76	0·78	0·97
		15	1·20	1·21	0·99
		Glucose 1-phosphate concn. (mM)			
(ii)		0·5	0·69	0·68	1·01
		0·75	0·82	0·83	0·99
		1	1·04	0·93	1·12
		1·5	1·23	1·19	1·03
		2	1·36	1·40	0·97
		4	1·96	1·90	1·03
		Silkworm blood, 1:10 dilution (ml.)			
(iii)		0·10	0·93	1·00	0·93
		0·20	1·88	1·72	1·09
		0·40	3·48	3·05	1·14
		0·80	5·38	5·30	1·02
		1·20	7·06	7·35	0·96

phosphate. The data suggest that the breakdown of glucose 1-phosphate can be represented as follows:

glucose 1-phosphate → glucose + inorganic phosphate (1)

The presence of glucose in incubated mixtures was demonstrated by paper chromatography.

Tubes containing glucose 1-phosphate enzyme and buffer were incubated with active or heated enzyme preparations. Samples (0·05 ml.) were applied to the origin of a sheet of Whatman no. 1 paper and developed overnight, either in *n*-propanol–ammonia–water (6:3:1, by vol.) or in *tert.*-butanol–acetic acid–water (60:25:15, by vol.). After drying, the papers were sprayed with aniline hydrogen phthalate reagent (Partridge, 1949) for reducing sugars. Glucose could be detected only when the complete system had been incubated. The reducing substance found during incubation moved to the same position as a test sample of glucose and, when mixtures of glucose and the complete incubation mixture were chromatographed, only one spot appeared.

The results given in Table 1 also illustrate that the amounts of the end products produced are proportional to the time of incubation and to the quantity of enzyme present, and provide further evidence that a single one-step reaction is involved.

Substrate specificity

The substrate specificity of the silkworm-blood enzyme towards a number of phosphate esters was investigated. Results given in Table 2 show that, of the naturally occurring phosphate esters tested, only glucose 1-phosphate and galactose 1-phosphate are hydrolysed to any considerable degree. The preparation also hydrolyses synthetic *p*-nitrophenyl phosphate, but at a rate slightly greater than that for glucose 1-phosphate.

Table 2. *Substrate specificity of silkworm-blood phosphatase*

Standard test system as in text with substrate as indicated. Incubated 15 min. at 30°. The control with each substrate contained the complete system with heated enzyme.

Test substance (10 mM)	Inorganic phosphate released by test substance expressed as % of inorganic phosphate from glucose 1-phosphate in same experiment
Glucose 1-phosphate	100·0
Galactose 1-phosphate	82·0
Glucose 6-phosphate	0·0
Fructose 6-phosphate	6·0
Hexose diphosphate	4·0
Ribose 5-phosphate	8·0
Na pyrophosphate	1·0
3-Phosphoglycerate	2·0
β-Glycerophosphate	0·3
Adenosine triphosphate (5 mM)	2·5
p-Nitrophenyl phosphate	135·0

When mixtures of glucose 1-phosphate with *p*-nitrophenyl phosphate or galactose 1-phosphate are incubated with the enzyme, the quantity of inorganic phosphate liberated is not the sum of the amounts produced when each substrate is incubated separately, as would be expected if two enzymes saturated with substrate were present (Table 3). Furthermore, the hydrolysis of both substances is inhibited by low concentrations of fluoride (Table 3). On the basis of these results it is concluded that glucose 1-phosphate, galactose 1-phosphate, and *p*-nitrophenyl phosphate are decomposed by a single enzyme.

Table 3. *Effect of fluoride on the breakdown of mixtures of glucose 1-phosphate (G 1-P) with p-nitrophenyl phosphate (p-NPP) and galactose 1-phosphate (Gal 1-P)*

Standard test system as in text with G1-P, Gal1-P, *p*-NPP, 10 mM; NaF, 0·2 mM. Incubated 20 min. at 30°.

Expt.	Additions to basic medium	Inorganic phosphate liberated (μmoles)	Inhibition by fluoride (%)
1	G1-P	1·52	—
	G1-P + NaF	0·87	43
	p-NPP	2·06	—
	p-NPP + NaF	1·46	29
	G1-P + *p*-NPP	2·21	—
	G1-P + *p*-NPP + NaF	1·60	28
2	G1-P	2·06	—
	G1-P + NaF	1·00	51
	Gal1-P	1·69	—
	Gal1-P + NaF	0·44	74
	G1-P + Gal1-P	2·16	—
	G1-P + Gal1-P + NaF	0·77	64

pH/*activity relationship*

The pH/activity diagram for the enzyme with glucose 1-phosphate as substrate is given in Fig. 1. Maximum activity is attained at pH 4·0–4·5 and thereafter it declines gradually as the pH approaches neutrality. The pH of silkworm blood falls in the range 6·2–6·6 (Epstein, 1930). It is evident, therefore, that the enzyme is active, though not at its maximum efficiency, at the physiological pH, and might be expected to be of importance in the metabolism of carbohydrates in the blood.

Substances affecting activity

A number of compounds were found to inhibit hexose-1-phosphatase activity. A list of some of the materials tested and their effects is given in Table 4. Fluoride is a strong inhibitor at low concentrations, producing approximately 50 % inhibition at 0·2 mM. Both arsenate and phosphate inhibit at 10 mM. It is interesting to note that while one of the reaction products, phosphate, inhibits strongly, the other

reaction product, glucose, has no effect on the enzyme activity. Other 1-substituted sugars, e.g. methyl α-glucoside, methyl α-mannoside, salicin, and αα-trehalose do not affect the rate of glucose 1-phosphate dephosphorylation. Citrate (10 mM) is a weak but consistent inhibitor. This latter result indicates that the new silkworm phosphatase is different in properties from mammalian prostate acid phosphatase which is stimulated by citrate (Anagnostopoulos, 1953).

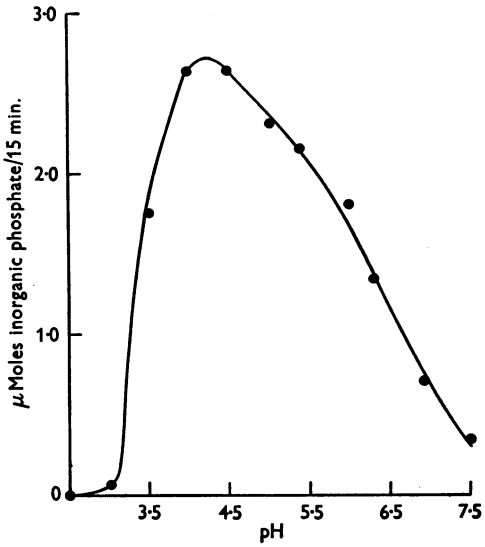

Fig. 1. Composite diagram of the pH/activity relationship of hexose-1-phosphatase. The buffers used were: glycine–HCl, 10 mM, pH 2·5–4·0; acetic acid–sodium acetate, 50 mM, pH 4·0–6·5; glucose 1-phosphate, 20 mM, pH 5·5–7·5. The complete system contained buffer at the stated concentration; glucose 1-phosphate, 10 mM except in the pH range 5·5–7·5 when it was also buffer. Other conditions standard. Incubated 15 min. at 30°.

It was important to compare the effects of fluoride, nitrite, and hydroxylamine on hexose-1-phosphatase as these three compounds are known to terminate the latency phenomenon in silkworm virus disease (Veneroso, 1934, Yamafuji & Cho, 1947). The possibility exists that these substances owe their action to an interference in an essential metabolic process connected with virus production. However, neither nitrite nor hydroxylamine alters hexose-1-phosphatase activity and it follows that other enzyme systems are affected by compounds terminating the latency.

High concentrations of potassium and sodium ions exert little effect on the phosphatase. At a concentration which uncouples oxidation and phosphorylation 2:4-dinitrophenol has no effect on enzyme activity.

Kinetics of fluoride inhibition

Results given in Fig. 2 illustrate the kinetics of the inhibition of hexose-1-phosphatase activity by two concentrations of fluoride when glucose 1-phosphate is substrate. The data are plotted according to the method of Lineweaver & Burke (1934). The form of the diagram indicates that fluoride is a competitive inhibitor of the enzyme. The K_m value in the experiment presented in Fig. 2 is 4 mM. The actual value varied from 3·3 to 5·5 mM

Table 4. *Effects of substances on hexose-1-phosphatase activity*

Standard test system as in text with glucose 1-phosphate, 10 mM. Incubated 20 min. at 30°.

Test substance	Concn. (mM)	Percentage inhibition by test substance of glucose 1-phosphate hydrolysis
Sodium fluoride	0·2	56·0*†
Sodium phosphate	10	54·0†
Sodium arsenate	10	80·0†
Glucose	10	7·0*
Methyl α-glucoside	10	0·0*†
Methyl α-mannoside	10	0·0*†
Salicin	10	0·0*†
αα-Trehalose	10	0·0*
Glucose 6-phosphate	20	0·0*
Sodium citrate	10	25·0*†
Potassium nitrite	25	3·0*
Hydroxylamine hydrochloride	10	6·0*
Potassium chloride	100	7·0*†
Sodium chloride	100	16·0*†
2:4-Dinitrophenol	0·5	0·0*†
Glycogen	2 mg./ml.	0·0*†
Potato starch	2 mg./ml.	0·0*†
Maltose	10 M	3·0*

The superscript symbols refer to the analytical method used to assay hexose-1-phosphatase. * Refers to inorganic phosphate release; † to reducing sugar release.

in a series of experiments. It is possible that the observed range is due to interfering substances present in the crude whole blood enzyme preparations.

Effects of magnesium and dialysis

Two 15 ml. lots of a 1:5 (v/v) dilution of silkworm blood in 50 mM sodium acetate, pH 5·0, were prepared. The first portion was stored overnight at 0° and the second was dialysed overnight at 0° against 5 l. of buffer. The hexose-1-phosphatase activities of the undialysed and the dialysed preparations were then compared under identical experimental conditions. The effect of magnesium on the two preparations was also tested. Results given in Table 5 show that magnesium sulphate (5 mM) has no effect on the enzyme activity of either preparation.

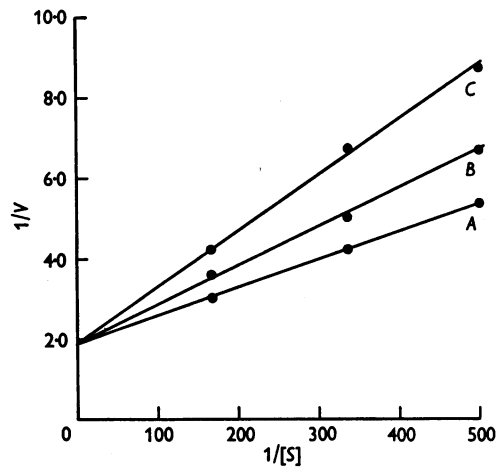

Fig. 2. Inhibition of hexose-1-phosphatase by sodium fluoride. Line *A* represents activity in absence of inhibitor. Line *B* with sodium fluoride, 0·125 mM; line *C* with sodium fluoride, 0·175 mM. Complete system as in text, glucose 1-phosphate as indicated. Incubated 10 min. at 30°. [*S*] refers to molar concentration of the substrate; *V* is the amount of reducing sugar liberated during incubation, expressed in optical density units.

Estimation of glucose 1-phosphate in mixtures with other phosphate esters

The feasibility of using the crude silkworm-blood enzyme for glucose 1-phosphate analysis was considered and some preliminary results are reported. Results given in Table 6 show the percentage recoveries obtained when mixtures of glucose 1-phosphate with glucose 6-phosphate and ATP were assayed using the enzyme. In these experiments the incubation time was increased to 60 min. but the final blood dilution was kept at 1:40. It will be seen that recoveries in the range 84–91 % can be obtained when 0·25, 0·5, or 1·0 μmole of glucose 1-phosphate is added to the silkworm preparation. When mixtures of glucose 1-phosphate with high ratios of glucose 6-phosphate or ATP were estimated using the enzyme the recovery range was 89–107 % (Table 6). The method can, therefore, be

Table 5. *Effects of dialysis and magnesium ions on hexose-1-phosphatase activity*

Standard test system as in text with glucose 1-phosphate, 10 mM. Incubated 20 min. at 30°.

	Inorganic phosphate due to enzyme activity (μmoles)	
$MgSO_4$	Non-dialysed	Dialysed
Nil	1·78	1·17
5 mM	1·79	1·02

Table 6. *Use of the silkworm-blood preparation for the estimation of glucose 1-phosphate in mixtures with ATP and glucose 6-phosphate (G 6-P)*

Standard test system as in text with additions as indicated. Incubated 60 min. at 30°. A control containing heated enzyme was run on each of the incubated tubes.

	Additions to basic medium (μmoles)		Inorganic phosphate due to enzyme action* (μmoles)	Percentage of added glucose 1-phosphate accounted for
Expt.	Glucose 1-phosphate	Admixture		
1	0·500	Nil	0·435	87
	1·000	Nil	0·840	84
	0·500	20 G6-P	0·536	107
	1·000	20 G6-P	0·895	89
2	0·250	Nil	0·211	84
	0·500	Nil	0·454	91
	0·250	5 ATP	0·237	95
	0·500	10 ATP	0·490	98

* The figures given in this column represent the amount of inorganic phosphate found after incubation, less that found in the heated enzyme control.

used to give a rough quantitative estimate of glucose 1-phosphate present in mixtures with other naturally occurring phosphates possessing several similar chemical properties.

DISCUSSION

Results given above indicate that silkworm blood contains an enzyme which splits glucose 1-phosphate according to Eqn. 1. The possibility may be considered that Eqn. 1 represents the sum of reactions (2) and (3):

glucose 1-phosphate →
polysaccharide + inorganic phosphate, (2)

polysaccharide → glucose. (3)

The reaction depicted in Eqn. 2 could be catalysed by a phosphorylase, and that in Eqn. 3 by an hydrolytic enzyme. However, it was found that neither glycogen (obtained from an invertebrate source), starch nor maltose is decomposed in preparations of silkworm blood in which there is rapid hydrolysis of glucose 1-phosphate. Also, glycogen, starch or maltose do not significantly alter the rate of splitting of glucose 1-phosphate as might be anticipated if they were intermediates (see Table 4). Moreover, if the hydrolysis of glucose 1-phosphate required two or more enzymes, different rates of accumulation of glucose and inorganic phosphate would be expected under certain experimental conditions. However, a strict 1:1 relationship between the amounts of glucose and inorganic phosphate liberated is found under all the experimental conditions examined, e.g. variation of enzyme and substrate concentration, time of incubation, pH variation and presence of inhibitors. For these reasons it is concluded that glucose 1-phosphate hydrolysis is catalysed by one enzyme only.

The phosphatase shows a high substrate specificity, since, of the naturally occurring phosphates tested as possible substrates, only glucose 1-phosphate and galactose 1-phosphate are hydrolysed. Some further work on the substrate specificity is necessary before the preparation can be used for analysis of complex mixtures of sugar phosphates.

The studies of Smolin (1952) have shown that glucose 1-phosphate and glucose 6-phosphate are present in considerable amounts in larval silkworm blood and, by analogy with other animal groups, it is not unlikely that they are involved in the intermediate carbohydrate metabolism.

Silkworm hexose-1-phosphatase is considered to take part in the enzymic breakdown of glycogen in larvae. In mammals, the conversion of glycogen into blood glucose by the liver is as follows (Duve, Berthet, Hers & Dupret, 1949; Swanson, 1950):

glycogen + inorganic phosphate —(phosphorylase)→ glucose 1-phosphate —(phosphoglucomutase)→ glucose 6-phosphate —(glucose-6-phosphatase)→ glucose + inorganic phosphate.

In silkworm the postulated pathway for the formation of blood glucose from glycogen is conversion of glycogen into glucose 1-phosphate as above, followed by hydrolysis according to Eqn. 1.

The essential difference from glycogen breakdown in mammalian liver is that a phosphoglucomutase is not involved and reaction is accomplished in two stages. All attempts to demonstrate the presence of phosphoglucomutase or phosphorylase in silkworm blood have given negative results. However, the presence of glycogen and fermentable sugar in blood was demonstrated by Kuwana (1937) and phosphorylase may be encountered in one of the other tissues.

SUMMARY

1. Some of the properties of an enzyme present in silkworm blood which hydrolyses glucose 1-phosphate are described.

2. Amongst a series of naturally occurring phosphate esters tested as substrates, only glucose 1-phosphate and galactose 1-phosphate are split. Synthetic *p*-nitrophenyl phosphate is hydrolysed by the same enzyme.

3. Enzyme activity is unaffected by addition of magnesium ions to a non-dialysed or dialysed preparation, but is inhibited by fluoride, phosphate and arsenate. Fluoride is a competitive inhibitor of the enzyme.

4. Optimum enzyme activity is at pH 4·0–4·5. Activity decreases sharply on the acid side, and more gradually on the alkaline side of the pH optimum.

5. The silkworm-blood preparation can be used to assay glucose 1-phosphate in mixtures with glucose 6-phosphate and adenosine triphosphate.

6. The possibility is considered that the physiological role of the enzyme in silkworm is analogous to that of glucose-6-phosphatase in mammals.

I should like to thank Dr G. H. Bergold for the interest he has shown in the present work.

REFERENCES

Anagnostopoulos, C. (1953). *Bull. Soc. Chim. biol., Paris*, **35**, 575.

Bialaszewicz, K. & Landau, C. (1938). *Acta Biol. exp., Varsovie*, **12**, 307.

Day, M. F. (1949). *Aust. J. sci. Res.* **2B**, 31.
Denucé, J. M. (1952). *Experientia*, **8**, 64.
Drilhon, A. (1943). *C.R. Soc. Biol., Paris*, **137**, 390.
Duve, C. de, Berthet, J., Hers, H. G. & Dupret, L. (1949). *1st Int. Congr. Biochem.* (Abstr. of Commun.) p. 403.
Epstein, W. (1930). *Russk. Zool. Zh.* **10**, 77.
Fiske, C. H. & Subbarow, Y. (1925). *J. biol. Chem.* **66**, 375.
Folin, O. & Malmros, H. (1929). *J. biol. Chem.* **83**, 115.
Kuwana, Z. (1937). *Japan J. Zool.* **7**, 273.
LePage, G. A. (1951). *Manometric Techniques and Tissue Metabolism*, p. 190. Minneapolis: Burgess Publishing Co.
Lineweaver, H. & Burke, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
Partridge, S. M. (1949). *Nature, Lond.*, **164**, 443.
Rockstein, M. & Levine, L. (1951). *Ann. ent. Soc. Amer.* **44**, 469.
Sacktor, B. (1953). *J. gen. Physiol.* **36**, 371.
Smolin, N. (1952). *Biochemistry, Leningr.*, **17**, 61.
Swanson, M. A. (1950). *J. biol. Chem.* **184**, 647.
Veneroso, A. (1934). *Boll. Staz. sper. Gelsic. bachi. Ascoli Piceno*, **13**, 1.
Yamafuji, K. & Cho, T. (1947). *Biochem. Z.* **318**, 95.

A Possible Structure for the Higher Oxidation State of Metmyoglobin

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In a previous paper (George & Irvine, 1954*a*) we concluded that there are two kinds of structure for the higher oxidation state of metmyoglobin (MetMb) which could explain the experimental observations. One is a radical structure formed by hydrogen atom removal from a methine carbon atom, a pyrrolic carbon atom or some other atom within the conjugated network of porphyrin ring and haemoprotein linkage; the other is a derivative of quadrivalent iron of the ferryl ion type, FeO^{2+}.

Further experiments have been undertaken to enable a choice to be made between these two structures, and the results of these are described and discussed below. The experiments fall into two sections. In the first, the hydrogen-ion changes accompanying the formation of the higher oxidation state in unbuffered solutions were measured, while in the second, the equilibrium constant for the reaction between MetMb and K_2IrCl_6 was determined under varying conditions of pH, temperature and ionic strength. The results of both classes of experiments are most simply interpreted on the basis of the ferryl ion structure.

A brief account of the pH measurements has already been published (George & Irvine, 1954*b*).

MATERIALS AND METHODS

Potassium chloroiridate. This was the same as that used previously (George & Irvine, 1954*a*). Spectrophotometric and potentiometric titrations with $K_4Fe(CN)_6$ showed the sample to be 99% pure. The titrations were carried out in acid solution using glass-distilled HCl, and the solutions were made up with water distilled from dil. $KMnO_4$. In this way reduction of the K_2IrCl_6 by trace reducing matter was prevented. In the experiments using unbuffered solutions, the K_2IrCl_6 was made up in CO_2-free water as described below, and used immediately to minimize any effect resulting from its hydrolysis. In the equilibrium experiments K_2IrCl_6 was made up in dilute glass-distilled HCl ($\sim 10^{-4}$ M) to prevent hydrolysis, although, as was shown previously (George & Irvine, 1954*a*) if hydrolysis occurs to any extent, its effect is almost negligible. The strength and volumes of the buffer solutions used were such that their pH values were not affected by the acid in the K_2IrCl_6 solution.

Buffer solutions. In the experiments with K_2IrCl_6, phosphate buffers ($NaOH + NaH_2PO_4$) were used, the ionic strengths being adjusted to the value required by the addition of AR NaCl. The pH of each solution was measured using a Cambridge pH meter calibrated with 0·05 M potassium hydrogen phthalate buffer.

Metmyoglobin. MetMb was prepared and standardized as in previous papers (George & Irvine, 1952, 1953*a*).

Hydrogen peroxide. This was kindly supplied by Laporte Chemicals Ltd., as 97% (w/w) H_2O_2, free from inhibitors. Stock solutions of approximately 0·1 N were prepared by dilution, standardized against $KMnO_4$, and then further diluted to the desired concentration.

Measurement of pH *in unbuffered solutions.* The measurements were made with a Cambridge pH-meter using a glass electrode and the usual calomel reference electrode. The instrument was calibrated with 0·05 M borax solution made up in CO_2-free-distilled water (pH = 9·25 at $t = 20°$). Solutions of NaOH, H_2O_2, and K_2IrCl_6 used in these experiments were also made up in CO_2 free water, and CO_2 was removed from MetMb solutions by evacuating for about 10 min. with a water pump.

In order to prevent errors during measurement due to absorption of CO_2 by the solutions, the following experimental procedure was adopted. The solution of MetMb was placed in a glass cell sealed with a Perspex cover into which were fitted the two electrodes. A steady flow of nitrogen was maintained over the surface of the liquid by means of a side arm in the cell. A Perspex screw with a small hole, fitted in the cell cover, provided an outlet for the nitrogen and, when unscrewed, enabled liquid to be pipetted into the cell.