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Glutathione Metabolism

1. THE GLYOXALASE ACTIVITY OF MATURE MAMMALIAN ERYTHROCYTES

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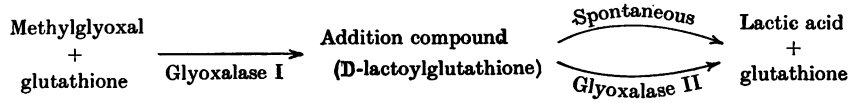
The glyoxalase system is frequently used in the assay of reduced glutathione and is considered by some (Bhattacharya, Robson & Stewart, 1955) to be one of the most reliable of the available methods. It has been noted on several occasions (Lohmann, 1932; Platt & Schroeder, 1934; Woodward, 1935) that the method is applicable only in the presence of relatively low concentrations of glutathione. At high glutathione levels, the glyoxalase activity is no longer proportional to the glutathione concentration. Thus with acetone-dried yeast, Platt & Schroeder (1934) observed that a steady increase in the glutathione concentration above a critical level resulted in a progressively smaller increment in glyoxalase activity until a maximum had been reached. Indeed, a further increase in glutathione concentration beyond the level required to produce a maximum glyoxalase activity resulted in a slight decrease in activity. These investigators noted further that the maximum glyoxalase activity produced with optimum glutathione levels was not constant for a particular enzyme concentration, but was raised still further by an increase in the methylglyoxal concentration. This latter observation suggests that factors which are non-enzymic in nature may influence the glyoxalase activity at high glutathione concentrations.

In the course of an investigation carried out in this laboratory on factors affecting the oxidation and reduction of glutathione in the mammalian erythrocyte, it became evident that in this tissue, also, the behaviour of the glyoxalase system in the presence of relatively large concentrations of glutathione was not easily understandable in terms of ordinary enzyme kinetics. Jowett & Quastel (1933) were led earlier to a similar conclusion. Recently, the glyoxalase system has been shown to consist of two distinct enzymes, glyoxalase I, which catalyses the formation of an addition compound between methylglyoxal (and other glyoxals) and glutathione, and glyoxalase II, which catalyses the breakdown of the addition compound to form lactic acid and glutathione. The addition compound also breaks down spontaneously under the influence of a number of factors (Racker, 1951; Crook & Law, 1952). In view of the recent findings, a reinvestigation of the conversion of methylglyoxal into lactic acid in intact and lysed erythrocytes was undertaken.



EXPERIMENTAL

Material. Venous blood was freshly drawn from normal human subjects, heparinized [1 drop of 1:1000 solution of heparin (Connaught Laboratories)/10 ml. blood], the plasma and buffy coat were removed by centrifuging, and the erythrocytes washed three times with 0·16 M-NaCl.

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After the last washing, saline was added to double the packed-cell volume.

Methylglyoxal was obtained from Bios Laboratories Inc., and reduced glutathione from Fisher Scientific Co.

Determination of glyoxalase activity in intact erythrocytes

The glyoxalase activity of the washed erythrocytes was estimated manometrically in Warburg respirometers with single side-arm flasks. The principle, as utilized by Lohmann (1932), Jowett & Quastel (1933), Platt & Schroeder (1934) and many others, depends on the fact that in the presence of glyoxalase and reduced glutathione, methylglyoxal is quantitatively converted into lactic acid, the formation of lactic acid being associated with the release of CO_2, which is measured manometrically. Unless otherwise stated, the glyoxalase activity of intact erythrocytes was estimated in the following manner. Into the main compartments of duplicate Warburg flasks were placed 0·4 ml. of 0·2 M-$NaHCO_3$, 0·05 ml. of the erythrocyte preparation and 0·16 M-NaCl to a final volume of 2·0 ml. Into the side arm was placed 0·2 ml. of 1 % methylglyoxal (final concentration $13·9 \times 10^{-3}$ M). The flasks were gassed at 30° with $N_2 + CO_2$ (95:5, v/v) for 10 min., tipped, and the readings taken after an open period of 2 min. The temperature of the bath was maintained at 30°, and the rate of shaking at 120/min.

The activity of the enzyme was found to be unaffected by variations in sodium bicarbonate concentration from 20 to 60×10^{-3} M, despite a variation in initial pH from 7·4 to 7·9. Similarly, variation in the methylglyoxal concentration from 6·7 to $20·1 \times 10^{-3}$ M had no significant effect on the glyoxalase activity under the conditions employed. The reaction continued at a steady rate until approximately 80–85 % of the methylglyoxal was destroyed. The further addition of glutathione, at concentrations varying from $0·95$ to $7·6 \times 10^{-3}$ M, did not alter the glyoxalase activity of the intact cells. The effect of varying the erythrocyte concentration on the evolution of CO_2 is shown in Fig. 1. Increasing the volume of the erythrocyte preparation from

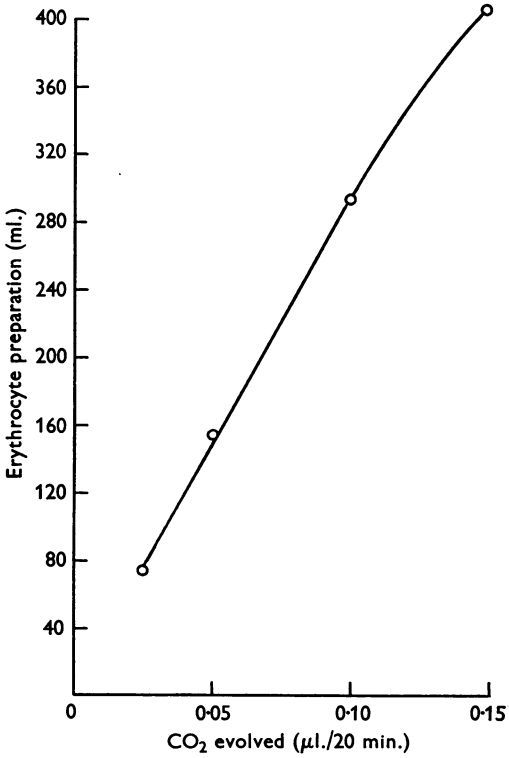

Fig. 1. Glyoxalase activity of intact erythrocytes; the main vessel contained 0·4 ml. of 0·2 M-$NaHCO_3$, 0·025–0·15 ml. of the erythrocyte preparation as indicated, and 0·16 M-NaCl in a final volume of 2·0 ml. The side arm contained 0·2 ml. of 1 % methylglyoxal.

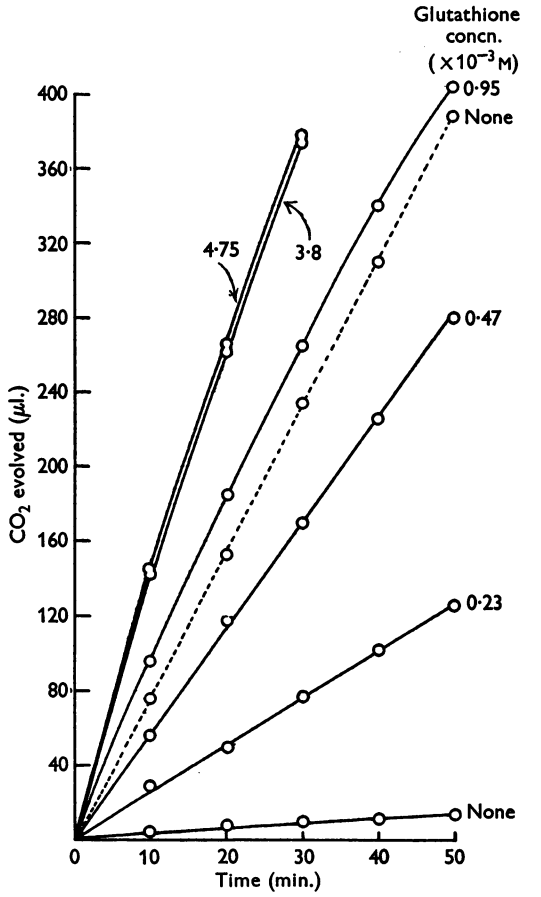

Fig. 2. Effect of glutathione concentration on the glyoxalase activity of lysed erythrocytes; the main vessel contained 0·4 ml. of 0·2 M-$NaHCO_3$, glutathione in concentrations ranging from 0·0 to $4·75 \times 10^{-3}$ M, and 0·05 ml. of the erythrocyte preparation. The side arm contained 0·2 ml. of 1 % methylglyoxal. In one flask (O- - - O), 0·16 M-NaCl was added to a final volume of 2·00 ml., thus preventing haemolysis, and in the remainder (O—O) the volume was made up with water.

Table 1. *Maximum CO_2 output by lysed erythrocytes and the required glutathione concentration at various methylglyoxal levels*

The main vessel contained 0·4 ml. of 0·2 M-$NaHCO_3$, 0·05 ml. of the erythrocyte preparation and water to a final volume of 2·0 ml. Methylglyoxal was added to the side arm. The glutathione concentration in the main vessel was increased until a maximum CO_2 output was obtained.

Methylglyoxal (mM)	Maximum CO_2 output (μl./20 min.)	Glutathione concn. at maximum CO_2 output (mM)
6·7	231	1·2
13·4	270	1·9
26·8	315	2·85
33·5	340	4·75

0·025 to 0·10 ml. resulted in a linear increase in the glyoxalase activity. Increasing the enzyme concentration beyond this point, however, did not increase the activity to the extent expected if a linear relationship were to hold. Thus the assay of the glyoxalase activity of intact erythrocytes as described would appear to be accurate for enzyme–coenzyme concentrations supplied by 0·025–0·10 ml. of the erythrocyte preparation.

Determination of glyoxalase activity in lysed erythrocytes

Jowett & Quastel (1933) noted that lysis of the erythrocytes results in an almost complete loss of glyoxalase activity and that the addition of glutathione to the lysed preparation leads to a return of activity. In the present study, lysis of the erythrocytes was produced by substituting water for saline in the manometer flask.

Effect of glutathione concentration. The effect of the addition of glutathione on the evolution of CO_2 by lysed erythrocytes is shown in Fig. 2. As the glutathione concentration is increased to high values, the rate of CO_2 output increases to a level considerably greater than that of the intact erythrocytes. However, the level of the maximum CO_2 output and the concentration of glutathione required to produce this maximum are not constant for a particular enzyme concentration but increase as the methylglyoxal concentration is increased (Table 1).

Effect of methylglyoxal concentration. The effect of increasing methylglyoxal concentrations on the glyoxalase activity in lysed erythrocytes is dependent on the glutathione level (Fig. 3). At glutathione levels greater than $2·85 \times 10^{-3}$ M an increase in the methylglyoxal concentration from 6·7 to $33·5 \times 10^{-3}$ M resulted in an increase in the evolution of CO_2. At glutathione levels below $0·47 \times 10^{-3}$ M, however, a similar increase in the methylglyoxal concentration resulted in a decrease in the CO_2 output. At intermediate glutathione concentrations ($0·47–2·8 \times 10^{-3}$ M), the evolution of CO_2 was either unaffected or slightly stimulated by an increase in the methylglyoxal concentration at relatively low levels, and an inhibition of activity occurred as the methylglyoxal concentration was increased further.

Effect of sodium bicarbonate concentration. The effect of the sodium bicarbonate concentration on the evolution of CO_2 by lysed erythrocytes is shown in Table 2. At a low glutathione concentration ($0·47 \times 10^{-3}$ M), the evolution of CO_2 was independent of the sodium bicarbonate concentration in the range $20–60 \times 10^{-3}$ M-$NaHCO_3$. However, at a relatively high glutathione concentration ($5·7 \times 10^{-3}$ M) a similar increase in the sodium bicarbonate concentration resulted in a distinct increase in the CO_2 output. The initial pH of the reaction mixtures as determined according to Umbreit (1947) is indicated.

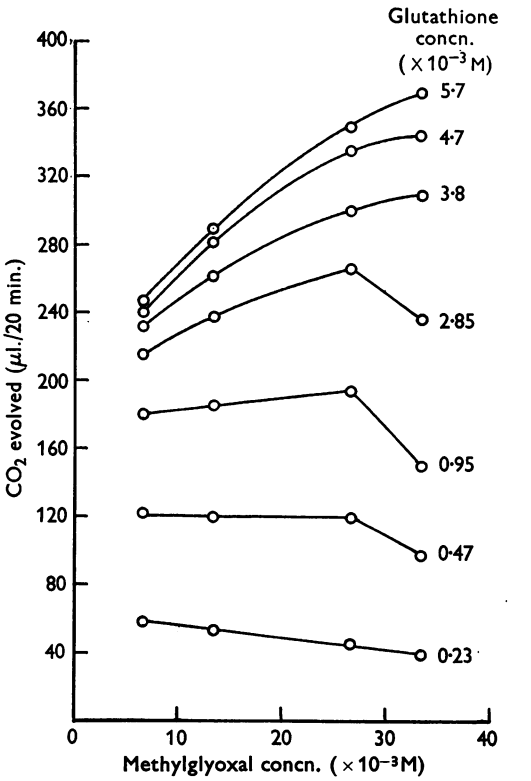

Fig. 3. Effect of methylglyoxal concentration on the glyoxalase activity of lysed erythrocytes; the main vessel contained 0·4 ml. of 0·2 M-$NaHCO_3$, glutathione in concentrations ranging from 0·23 to $5·7 \times 10^{-3}$ M, 0·05 ml. of the erythrocyte preparation, and water to a final volume of 2·0 ml.

Table 2. *Effect of sodium bicarbonate concentration on the glyoxalase activity in lysed erythrocytes*

The main vessel contained 0·05 ml. of the erythrocyte preparation and water to a final volume of 2·0 ml. The side arm contained 0·2 ml. of 1% methylglyoxal.

Sodium bicarbonate (mM)	Glutathione (mM)	Initial pH	Evolution of CO_2 (μl./20 min.)
20	0·47	7·4	120
40	0·47	7·7	120
60	0·47	7·9	120
20	5·7	7·4	234
40	5·7	7·7	279
60	5·7	7·9	311

Effect of enzyme concentration. The effect of varying the enzyme concentration on the evolution of CO_2 is shown in Fig. 4. At the low glutathione concentration of $0{\cdot}47 \times 10^{-3}$ M, an increase in the erythrocyte preparation from 0·0125 to 0·15 ml. resulted in a linear increase of the CO_2 output. However, at the high glutathione concentration of $5{\cdot}7 \times 10^{-3}$ M, a straight-line relationship between enzyme concentration and the CO_2 output was not observed. The glyoxalase system is less active at the higher concentrations than expected on the basis of a linear relationship.

Inhibition of the glyoxalase activity of lysed erythrocytes by methylglyoxal

The observation that, at low glutathione levels, an increase in the methylglyoxal concentration leads to a decrease in CO_2 output (Fig. 3) suggests that under certain conditions methylglyoxal may exert an inhibitory effect on the glyoxalase system. This is supported by the following experiment. Methylglyoxal was pre-incubated in the absence of glutathione with lysed erythrocytes in the main compartment of duplicate Warburg flasks for periods varying from 30 to 120 min. Under these conditions there is a negligible conversion of methylglyoxal into lactic acid (see Fig. 2) and we may, therefore, assume that the concentration of methylglyoxal remained largely unchanged. The subsequent addition of glutathione from the side arm reconstitutes the glyoxalase system. The resultant evolution of CO_2 was compared with that in the control flasks which contained both methylglyoxal and glutathione together (in the side arm) during the pre-incubation period. As can be seen from Fig. 5, the glyoxalase activity of the lysed erythrocytes in the control flasks was not significantly affected by pre-incubation for periods varying from 30 to 120 min. However, a considerable decrease in glyoxalase activity resulted from the pre-incubation of the lysed erythrocytes with methylglyoxal for a 30 min. period in the absence of added glutathione, with a further decrease in activity resulting from a prolongation of the pre-incubation period to 120 min. The addition of methylglyoxal or sodium bicarbonate from a second side arm did not increase the glyoxalase activity of the inhibited preparation, indicating

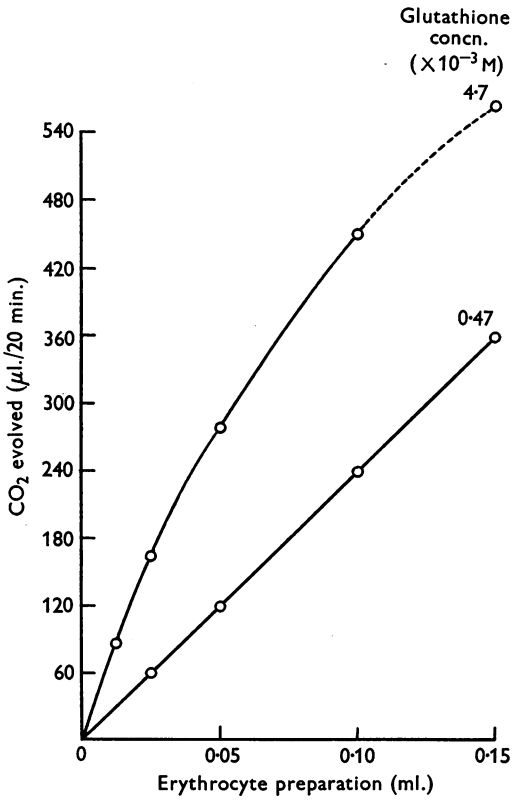

Fig. 4. Effect of erythrocyte concentration on the glyoxalase activity of lysed cells; the main vessel contained 0·4 ml. of 0·2 M-$NaHCO_3$, glutathione at two concentrations, $0{\cdot}47 \times 10^{-3}$ M and $4{\cdot}7 \times 10^{-3}$ M, and water to a final volume of 2·0 ml. The volume of the erythrocyte preparation varied from 0·0125 to 0·15 ml.; the side arm contained 0·2 ml. of 1% methylglyoxal.

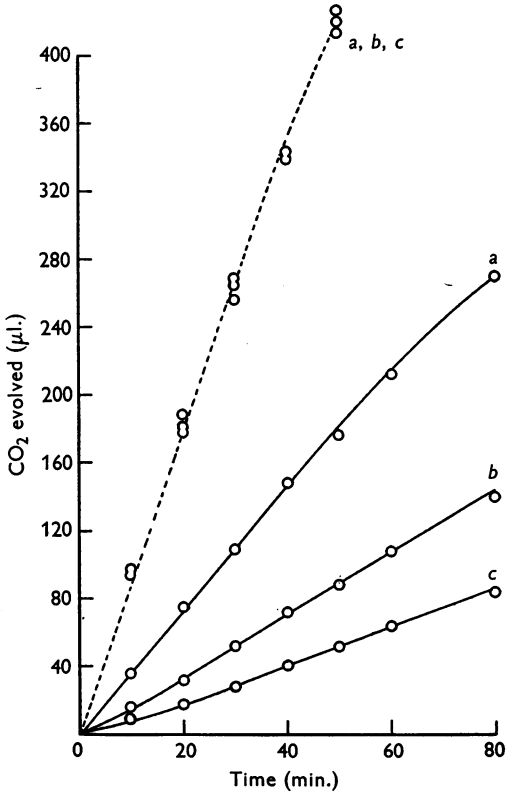

Fig. 5. Inhibition of glyoxalase activity by methylglyoxal; the main vessel contained 0·4 ml. of 0·2 M-$NaHCO_3$, 0·05 ml. of the erythrocyte preparation and water to a final volume of 2·0 ml. The side arm contained 0·2 ml. of 0·3% glutathione. Methylglyoxal (0·2 ml. of 1% solution) was added either to the main vessel (O—O) or to the side arm (O- - - -O). Flasks were pre-incubated for *a*, 30 min.; *b*, 60 min.; *c*, 120 min.

that the decline in activity is not the result of the destruction of either methylglyoxal or sodium bicarbonate during the pre-incubation period.

The effect of methylglyoxal on the glyoxalase activity is also shown in Table 3. Three experiments are presented with three different concentrations of glutathione added. In all instances, the evolution of CO_2 can be seen to be smaller in the experimental flask (methylglyoxal in the main vessel but glutathione in the side arm) than in the control flask (methylglyoxal and glutathione together in the side arm). At the low glutathione concentration of $0{\cdot}47 \times 10^{-3}$ M an increase in the methylglyoxal concentration resulted in a further decrease in the evolution of CO_2. However, as the glutathione level was increased to $0{\cdot}95 \times 10^{-3}$ M, the evolution of CO_2 became largely independent of the methylglyoxal concentration, and at a glutathione concentration of $4{\cdot}7 \times 10^{-3}$ M an increase in the concentration of methylglyoxal brought about an increase in the CO_2 output.

DISCUSSION

At low glutathione concentrations, the glyoxalase activity of lysed erythrocytes proceeds at a constant rate in direct proportion to the glutathione concentration. Variations in sodium bicarbonate and methylglyoxal concentration within the limits indicated (Table 2, Fig. 3) have no effect on the evolution of carbon dioxide, and a straight-line relationship exists between enzyme concentration and carbon dioxide output (Fig. 4). Intact erythrocytes exhibit similar properties. Under these conditions the conversion of methylglyoxal into lactic acid is entirely enzymic in nature and may be utilized for the assay of glutathione. However, it should be emphasized that the glyoxalase activity of lysed erythrocytes is independent of methylglyoxal concentration only within fairly narrow limits of glutathione and methylglyoxal concentration (Fig. 3).

As the glutathione concentration is increased, the glyoxalase activity of lysed erythrocytes assumes different characteristics from those observed at low glutathione concentrations. The evolution of carbon dioxide is initially greater than that observed with intact cells and decreases with time (Fig. 2). An increase in the methylglyoxal concentration results in an increase in the evolution of carbon dioxide even at optimum glutathione concentrations (Table 1). Further, an increase in the carbon dioxide output occurs on increasing the sodium bicarbonate concentration (Table 2), and the enzyme concentration is no longer directly proportional to glyoxalase activity (Fig. 4). In heart muscle as well as 'in most of the other tissues studied' (Racker, 1951) the conversion of methylglyoxal into lactic acid is limited by glyoxalase II activity (Racker, 1951; Crook & Law, 1952). If we assume a similar relationship in the erythrocyte, an increase in glutathione concentration will result in an increase in the addition compound to a level sufficient to saturate glyoxalase II. A further increase in glutathione concentration will lead to an accumulation of the addition compound and to its spontaneous breakdown. The observed evolution of carbon dioxide would then be a combination of that produced enzymically and that released spontaneously. The rate of spontaneous breakdown of the addition compound is increased by an excess of methylglyoxal (Crook & Law, 1952) and by an increase in pH (Yamazoye, 1936; Racker, 1951). This may account for the observed effect of methylglyoxal (Fig. 3, Table 1) and sodium bicarbonate (Table 2) concentration on the glyoxalase activity at high glutathione concentrations.

Evidence has been presented (Fig. 5, Table 3) which suggests that methylglyoxal exerts an inhibitory effect on the glyoxalase system in the presence of very low concentrations of glutathione. Phenylglyoxal also inhibits the conversion of methylglyoxal into lactic acid (Hopkins & Morgan, 1948; Crook & Law, 1952). However, in this instance, a preferential affinity for glutathione is believed to be involved (Crook & Law, 1952). The inhibition of a number of sulphydryl enzymes by methylglyoxal has been reported (Kun, 1950).

Table 3. *Effect of methylglyoxal on the glyoxalase activity at various concentrations of glutathione*

The main vessel contained 0·05 ml. of the erythrocyte preparation and water to a final volume of 2·0 ml. In the experimental flasks, methylglyoxal, in the concentrations indicated, was added to the main vessel and glutathione was placed in the side arm. In the control flasks, both methylglyoxal and glutathione were placed together in the side arm. The flasks were pre-incubated for 30 min. at 30°, the contents of the side arm tipped into the main compartment and the evolution of CO_2 was measured.

Glutathione (mM)	Methylglyoxal (mM)	Glyoxalase activity (μl. of CO_2 evolved/30 min.) Experimental flask	Control flask
0·47	6·7	130	186
0·47	13·4	125	172
0·47	26·8	116	168
0·95	6·7	142	258
0·95	13·4	140	265
0·95	26·8	138	273
4·7	6·7	164	300
4·7	13·4	184	370
4·7	26·8	196	400

SUMMARY

1. The glyoxalase activity of intact and lysed human erythrocytes has been studied.

2. Conditions are described under which the glyoxalase activity of intact erythrocytes proceeds at a constant rate, independent of the methylglyoxal and sodium bicarbonate concentrations and

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.

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The 'Malic' Enzyme in Insect Blood

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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