over H_2SO_4 for 3 days. (Found: C, 33.3; H, 5.5; N, 11.7. $\text{C}_{10}\text{H}_{17}\text{O}_8\text{N}_3\text{S}$ requires C, 33.8; H, 4.8; N, 11.8%.) This was not a completely satisfactory analysis, but the identity of the compound was confirmed chromatographically. When run on paper with phenol-water, the compound gave a single spot, R_F 0.13, which reacted with ninhydrin but not with nitroprusside and cyanide nor with iodoplatinic acid. After being hydrolysed in 6N-HCl, the compound run in phenol-water in presence of NH_3 gave three ninhydrin-reacting spots corresponding to glutamic acid, cysteic acid and glycine.

L-Cysteinylglycine. This compound was prepared by partial hydrolysis of glutathione with N-HCl (Olson & Binkley, 1950).

SUMMARY

1. The three compounds γ -DL-glutamylglycylglycine, γ -DL-glutamyl-DL-alanylglycine and *S*(N-ethylsuccinimido)-L-cysteine have been synthesized.

2. From glutathione the following compounds have been prepared; *S*-methylglutathione, *S*(N-ethylsuccinimido)glutathione, γ -L-glutamyl-L- β -sulphoalanylglycine, L-cysteinylglycine and γ -L-glutamyl-L-alanylglycine, the last being obtained only in an impure state and in low yield.

3. On a paper chromatogram, phthalimido derivatives are detected by spraying with a reagent containing hydrazine hydrate. When the paper is dried, the phthalylhydrazide formed fluoresces strongly in ultraviolet light.

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The Effects of some Analogues of Glutathione on the Glyoxalase System

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It is now established that the enzymic conversion of methylglyoxal into lactic acid takes place in two stages. First, methylglyoxal reacts with glutathione (GSH) to form *S*-lactylglutathione, the reaction being catalysed by an enzyme which Racker (1951) has called glyoxalase I. Secondly, this thioester is hydrolysed by another enzyme, glyoxalase II, giving lactic acid and regenerating GSH. The GSH therefore acts as a coenzyme for the overall reaction whereby methylglyoxal is transformed into lactic acid. Glyoxalase I and II correspond to the 'enzyme' and the 'factor' respectively of Hopkins & Morgan (1948).

Behrens (1941) has shown that, in the glyoxalase reaction, glutathione may be replaced as a coenzyme

by either α -L-glutamyl-L-cysteinylglycine or β -L-aspartyl-L-cysteinylglycine, though both are less effective than GSH (γ -L-glutamyl-L-cysteinylglycine). Kögl & Akkerman (1946) found that γ -D-glutamyl-L-cysteinylglycine can also serve as a coenzyme for the glyoxalase system, but again it is less active than glutathione. Other substances tested as coenzymes but found ineffective include oxidized glutathione, cysteine, thioglycollic acid (Lohmann, 1932), γ -glutamylcysteine and cysteinylglycine (Woodward & Reinhart, 1942).

In the present work various analogues of GSH were tested as possible inhibitors of the glyoxalase reaction. Experiments with the complete glyoxalase system showed that certain of these analogues were indeed inhibitory but the simultaneous action of both glyoxalase I and II made quantitative interpretation of the results difficult. For this reason,

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most of the inhibition studies were carried out on glyoxalase I alone. GSH is a substrate for this enzyme, the other being methylglyoxal (or some other glyoxal derivative). The effect of any inhibitor which acts as an analogue of glutathione should therefore be primarily on glyoxalase I.

THEORETICAL

During the study of the effect of GSH analogues on glyoxalase I, it was found that certain of these compounds produced inhibition which was neither purely competitive nor purely non-competitive. A general equation of inhibition was therefore derived to cover competitive, non-competitive and intermediate types of inhibition (Matheson, 1954). Somewhat similar treatments have since come to our notice (Segal, Kachmar & Boyer, 1952; Botts & Morales, 1953) so that only the final equations will be given here.

In inhibition where the enzyme-substrate-inhibitor complex cannot break down to give the products of the reaction and the enzyme-substrate complex breaks down to yield the products of the reaction at a rate which is slow relative to the rate of formation of the complex, the initial velocity V is given by the general equation

$$\frac{V_{\max.}}{V} = 1 + \frac{I}{K_i} + \frac{K_m}{S} \left(1 + \frac{I}{K_i} \right), \quad (1)$$

where $V_{\max.}$ is the velocity in the absence of inhibitor and all the enzyme is combined with the substrate, I the inhibitor concentration, S the substrate concentration, K_m the Michaelis constant, K_i the dissociation constant of the enzyme-inhibitor complex and K_i the dissociation constant of enzyme-substrate-inhibitor complex to enzyme-substrate complex and inhibitor.

This is the type of equation used by Lineweaver & Burke (1934). That of Hunter & Downs (1945) may be derived from it and is

$$I \frac{\alpha}{1-\alpha} = K_i \left(1 + \frac{S}{K_m} \right) / 1 + \frac{K_i S}{K_i K_m}, \quad (2)$$

where α , the fractional activity, is the ratio of the velocities of the reaction in presence and absence of the inhibitor at a given substrate concentration S .

If in these equations K_i is infinite (which implies that the enzyme-substrate-inhibitor complex cannot exist), the well-known equations for competitive inhibition are obtained, whilst if $K_i = K_i$ the equations for non-competitive inhibition result, as may be readily verified. The situation where the substrate and the inhibitor each reduce but do not abolish the affinity of the enzyme for the other is represented by taking values of K_i between K_i and infinity. This case may be called 'intermediate' inhibition.

In the present study of the effects of inhibitors, the form of equation used by Hunter & Downs has advantages over that of Lineweaver & Burke. First, the Hunter & Downs method requires the experimental determination of the ratio of the velocities of the inhibited to the uninhibited reaction at various concentrations of inhibitor. The two velocities can be conveniently measured simultaneously in a spectrophotometer and unavoidable variations in the experimental conditions affect each velocity similarly and so tend to cancel out. Secondly, all data from experiments with different concentrations of inhibitor can be used to define a single line from which deductions can be made about the type of inhibition.

In Fig. 1 plots of the Hunter & Downs type are shown for $K_i = \infty$, $K_i = K_i$ and for two intermediate values of K_i , K_i and K_m being constant in all four cases. Competitive inhibition ($K_i = \infty$) gives a single straight line cutting the $I\alpha/(1-\alpha)$ axis at K_i and the S axis at $-K_m$. Non-competitive inhibition ($K_i = K_i$) is represented by another straight line which is parallel to the S axis and cuts the $I\alpha/(1-\alpha)$ axis at K_i . For an intermediate value of K_i , the plot of $I\alpha/(1-\alpha)$ against S is not linear but is a curve cutting the vertical axis at K_i and the

Fig. 1. Plot of $I\alpha/(1-\alpha)$ against S in different types of inhibition. C , Competitive inhibition: single straight line which cuts $I\alpha/(1-\alpha)$ axis at K_i and S axis at $-K_m$. NC , Non-competitive inhibition: single straight line which is parallel to S axis and cuts $I\alpha/(1-\alpha)$ axis at K_i . IC , Intermediate case: all lines are curved and cut $I\alpha/(1-\alpha)$ axis at K_i and S axis at $-K_m$; each value of K_i/K_i produces one curve, those shown being for $K_i/K_i = \frac{1}{2}$ and $K_i/K_i = \frac{1}{8}$.

horizontal axis at $-K_m$. The degree of curvature is determined by the ratio of K_i to K_t .

Thus in the Hunter & Downs plot there are three possibilities. The graph may be a straight line parallel to the S axis. With this the inhibition is of the non-competitive type and the value of K_i is given by the point of intersection with the $I\alpha/(1-\alpha)$ axis. Secondly, the plot may be a straight line inclined to the S axis and cutting it at $S = -K_m$. Here the inhibition is competitive and K_i is given by the intersection of the line and the $I\alpha/(1-\alpha)$ axis. In the third case, the observed points lie on a curve which, however, will not deviate greatly from linearity over the relatively limited range of substrate used. If the best-fitting straight line is drawn through these points it will be again inclined to the S axis but will cut it at values of $-S$ appreciably greater than K_m ; in this case inhibition is of the intermediate type.

The point at which the best-fitting straight line cuts the $I\alpha/(1-\alpha)$ axis will not in general coincide with the intersection of the curve with this axis, which is at K_i . However, it can be shown that the tangent to the curve at the point where S equals K_m cuts the vertical axis at $K_i(1+3K_i/K_t)/(1+K_i/K_t)^2$. This expression can be shown to have a maximum value for a fixed K_t when K_i equals $3K_t$. This maximum value is therefore $9K_t/8$. The tangent to the curve at any point between S equals 0 and S equals K_m must therefore cut the ordinate between K_i and $9K_t/8$. It follows that the straight line best fitting a series of points on the curve, for values of S between 0 and K_m , must cut the ordinate between K_i and $9K_t/8$. If the concentration of substrate in a series of experiments does not exceed K_m then the intersection of the best-fitting straight line with the $I\alpha/(1-\alpha)$ axis will give a value which does not differ from the true value of K_i by more than 12.5%, and which will usually be much closer than that. This agreement demonstrates the approximate linearity of the curve between S equals 0 and S equals K_m and justifies the use of observations over this range for distinguishing between the various types of inhibition according to the method here suggested.

It is well known (Jowett & Quastel, 1933; Schubert, 1935) that methylglyoxal and GSH react non-enzymically to yield an adduct, probably according to the equation

It is not known whether glyoxalase I acts on the adduct or on free methylglyoxal and GSH. In the latter case the enzyme would have two substrates. A mathematical treatment applicable to the case of an enzyme acting on two substrates was therefore developed (Matheson, 1954). At any fixed concentration of the second substrate, the resulting

equations are equivalent to those already given provided it is assumed that combination of the enzyme with the second substrate does not affect its affinity for either the first substrate or the inhibitor. Whether the enzyme combines with the adduct or its free components, the same type of equation will be obtained to describe the dependence of the velocity on the concentration of the adduct or of the GSH as the case may be.

There is evidence, as will be seen below, that the equilibrium constant of the above reaction is of the order of 10^{-2} M. It can be calculated that within the range of concentrations of methylglyoxal and GSH used, only a small proportion of the methylglyoxal and of the glutathione exist in the combined form, and therefore, provided that the concentration of methylglyoxal is kept constant, the amounts of adduct and of free GSH are approximately proportional to the total GSH concentration. From this result and the conclusions reached in the preceding paragraph it may be inferred that the dependence of the velocity on the total GSH concentration will follow the same type of equation for constant methylglyoxal concentration whether the enzyme reacts with the adduct or independently with the two components.

It is convenient in the first instance to give the values of K_i and K_m in terms of the total GSH concentration. The real values of K_i and K_m should be expressed in terms of concentration of free GSH or of adduct, according to which theory is correct. It will be clear that for constant methylglyoxal concentration this figure will be proportional to the value deduced from the total GSH concentration. It will be seen later that it is probable that glyoxalase I acts on free GSH.

EXPERIMENTAL

Materials

Methylglyoxal. A commercial 30% (w/v) solution was diluted to about 10% and distilled twice under reduced pressure, the fraction boiling below 50° at 20 mm. Hg being collected. The distillate was standardized by the method of Friedemann (1927), which is accurate to $\pm 1\%$ (Woo & Chang, 1945). This solution, which also contained lactic acid, was stored at 0° and was stable over a period of several months.

Glutathione. The material used was a preparation of over 99% purity [The Distillers Co. (Biochemicals) Ltd.]. The solutions were prepared immediately before use and brought to pH 6.6 with NaOH.

Glutathione analogues. The preparation of these compounds is described in a previous paper (Kermack & Matheson, 1957). Neutral solutions of the required concentration were stored at 0° and fresh solutions were made every few days. Where the analogue contained a sulphhydryl group, a solution was prepared immediately before use.

Phosphate buffers. These were prepared by mixing stock M solutions of KH_2PO_4 and K_2HPO_4 in the required proportions and diluting as necessary.

Serum albumin. A crude commercial preparation of serum albumin (B.D.H.) was purified by fractionation with $(\text{NH}_4)_2\text{SO}_4$, the material precipitated between 50 and 100% saturation being taken. The concentration of albumin was determined by micro-Kjeldahl analysis after removal of $(\text{NH}_4)_2\text{SO}_4$ by thorough dialysis.

Glyoxalase I and II. The enzymes were prepared from yeast and from ox liver respectively by the methods of Racker (1951). The glyoxalase I preparation was taken to step 8 and the glyoxalase II preparation to step 4. Both enzymes were stored at -15° . Glyoxalase I was very stable in concentrated solution, no change in activity being found on storage for a year at -15° . When diluted, however, it was very unstable but could be stabilized by serum albumin (Racker, 1951). Solutions for kinetic studies were made by diluting the stock solution 1:500 with 0.1M potassium phosphate buffer, pH 7.4, containing 100 μg . of serum albumin/ml. Glyoxalase II was much less stable; even in concentrated solution it lost all its activity within a few weeks. The stock solution itself was used in manometric studies of the complete glyoxalase system.

Methods

Measurement of the activity of the complete glyoxalase system. These measurements were carried out in a standard Warburg manometric apparatus. Since the potential inhibitors used were analogues of GSH, which is in effect a substrate of glyoxalase I, it was desirable that this enzyme should be the rate-limiting factor. In practice this condition was never completely fulfilled, since sufficiently active preparations of glyoxalase II were not obtained. All the Warburg flasks contained NaHCO_3 , GSH, methylglyoxal, glyoxalase I and II and certain flasks also contained the analogue being studied. Half the NaHCO_3 and all the methylglyoxal were present in the side arm. The flasks were swept out with $\text{N}_2 + \text{CO}_2$ (95:5, v/v) for 10 min., then equilibrated in a bath at 25° for a further 10 min. The side-arm contents were added and the reaction was allowed to proceed for 5 min. before the taps were closed, to eliminate errors due to evolution of gas produced by chemical changes on mixing. Readings were taken on closing the taps and subsequently at 5 min. intervals. The velocity of the reaction was linear over the whole period of measurement.

Measurement of the activity of glyoxalase I. Racker's (1951) observation that *S*-lactylglutathione, which is formed by the action of glyoxalase I, has a high optical density at 240 $m\mu$, led him to develop a spectrophotometric method suitable for the measurement of glyoxalase I activity. The rate of reaction was measured by following the increase in optical density at 240 $m\mu$ by means of a Unicam SP. 500 quartz spectrophotometer. Into each of two 1 cm. quartz cells were pipetted phosphate buffer, pH 6.6 (final concn. $3.3 \times 10^{-2}M$), methylglyoxal and GSH. The analogue under study was added to one cell; to each cell water was added to give a total volume of 3.0 ml., after addition of enzyme. The cells were read against a blank containing water and buffer. After the initial increase in readings had stopped, 0.1 ml. of diluted glyoxalase I solution was added to each cell, the moment of mixing being taken as zero. The cells were replaced in the instru-

ment as rapidly as possible and readings were made in quick succession, the exact time of each reading being noted. The readings were plotted against time and the initial reading was obtained by extrapolation. During the first 5 or 10 min. the curves were practically linear at the enzyme and substrate concentrations used in experiments on inhibition, so the rate of reaction was found by dividing the change in optical density by the time. Room temp. was held at $20 \pm 1^\circ$: the temperature of the solutions in the cells did not change during the period of measurement.

RESULTS

Experiments with the complete glyoxalase system

A number of experiments were carried out in the Warburg apparatus to test the effect of analogues of glutathione on the complete glyoxalase system. No great importance can be attached to the quantitative aspect of these experiments, but several of the compounds proved to be inhibitory. The results for some of the analogues are shown in Fig. 2.

Experiments with glyoxalase I

Non-enzymic reactions. As a preliminary the non-enzymic reactions were studied spectrophotometrically. While a solution of methylglyoxal

Fig. 2. Effects of analogues of glutathione on the complete glyoxalase system in the Warburg apparatus. All flasks contained potassium bicarbonate, $4.0 \times 10^{-2}M$, methylglyoxal, $2.2 \times 10^{-2}M$, glutathione $1.62 \times 10^{-2}M$, glyoxalase I, 0.05 ml. of 1:100 stock solution, glyoxalase II, 0.3 ml. of stock solution. Flask 1 was an uninhibited control; flasks 2-4 contained *S*-methylglutathione, $6.5 \times 10^{-2}M$, *S*(*N*-ethylsuccinimido)glutathione, $6.5 \times 10^{-2}M$, and *S*-methylcysteine, $16 \times 10^{-2}M$, respectively. All figures are of final concentration after tipping in: the side arm contained half the bicarbonate and all the methylglyoxal. The pH of the flask contents was 7.6 and reaction was carried out at 25° ; the taps were closed 5 min. after tipping in. The final volume was 2 ml.

($2.16 \times 10^{-3} \text{ M}$) in phosphate buffer ($3.3 \times 10^{-2} \text{ M}$) at pH 6.6 did not change in optical density at $240 \text{ m}\mu$. over a period of 5 hr., a solution of GSH ($2.16 \times 10^{-3} \text{ M}$) slowly increased in absorption after an initial lag period of about 15 min. The increase was approximately linear, being about $2.5 \times 10^{-2}/\text{hr.}$ over the 5 hr. period. This slow increase may be due to oxidation of the sulphhydryl group to a disulphide. The fact that the intensity of the colour produced in the nitroprusside test for sulphhydryl groups fell, as the optical density rose, supports this view.

The behaviour of a mixture of glutathione and methylglyoxal (each $2.16 \times 10^{-3} \text{ M}$) in phosphate buffer was quite different from that of the separate components. On mixing, there was a relatively rapid increase in optical density (0.14) during the first 60–70 min., followed by an increase similar to that observed with GSH alone but at an even slower rate ($1.3 \times 10^{-2}/\text{hr.}$).

It is known that methylglyoxal and GSH react non-enzymically to form a labile addition compound (Jowett & Quastel, 1933; Schubert, 1935). The relative amount of free and of combined GSH in the presence of methylglyoxal was determined by Platt & Schroeder (1934) by rapid titration of free sulphhydryl groups. From their results it is possible to deduce that the dissociation constant K of the addition compound is probably of the order of 10^{-2} M , but the values of K calculated from their various experiments extend over a considerable range. This lack of constancy of K may be due to progressive dissociation of the addition compound during the titration.

A new estimate of K has been made by the following method. Various amounts of glutathione were mixed with a solution of methylglyoxal and the change in optical density accompanying the

reaction was measured. For each mole of complex formed, one mole of GSH and one mole of methylglyoxal would disappear. Thus if n be the number of moles of the complex formed, and m_1 , m_2 and m_3 be the molecular absorption coefficients of the complex, methylglyoxal and GSH respectively, the change in optical density will be proportional to $n(m_1 + m_2 + m_3)$, that is, to n . Now if the initial concentration of methylglyoxal (C) is kept constant and several GSH concentrations are used, the concentration of the complex formed (q) will depend on the concentration of GSH (S) according to the equation

$$\frac{(S - q)(C - q)}{q} = K. \quad (4)$$

From this, assuming some particular value of K , we can calculate the concentrations of the complex, q_1 , q_2 , q_3 , etc., for the concentrations of GSH, S_1 , S_2 , S_3 , etc. The values of q are, however, proportional to the corresponding values of n , that is, they should be proportional to the measured changes in optical density. Thus the required value of K will be that for which this condition is satisfied, and from Table 1 this value of K is $10^{-2.3} \text{ M}$. The advantage of this method is that it depends on physical measurements which do not interfere with the equilibrium (as titration of the free SH groups may do) and that independent knowledge of the absorption coefficient of the complex is not necessary. It is not claimed that this method is very accurate as the slow increase in optical density in a solution of GSH is an unavoidable complication. For the purpose of the present work only an approximate value of K was required.

Enzymic reactions. Action of the enzyme on the addition compound and on its components. Glyoxalase I may act on free GSH and free methylglyoxal or on their addition compound. The

Table 1. *Evaluation of the dissociation constant K of the methylglyoxal–GSH complex*

All cells contained buffer and water to 3.0 ml. final vol.; cells 2, 3 and 4 contained methylglyoxal, 2.16 mM, and varying amounts of GSH. Readings were made before addition of GSH, immediately afterwards, and at frequent intervals for the next 70 min., by which time the increase in optical density had practically stopped. The initial reading on the mixture of GSH and methylglyoxal was obtained by extrapolating the curves back to zero time.

	Cell		
	2	3	4
Concn. of GSH (mM)	1.08	2.16	4.32
Change in $\log I_0/I$	0.086	0.155	0.255
Ratio of changes in $\log I_0/I$	1.00	1.80	2.97
Calc. concn. of addition compound (mM), when			
$K = 10^{-2}$	0.18	0.34	0.59
$K = 10^{-3}$	0.65	1.11	1.58
$K = 10^{-2.3}$	0.29	0.53	0.88
Ratio of calc. concn. of addition compound (mM), when			
$K = 10^{-2}$	1.00	1.89	3.28
$K = 10^{-3}$	1.00	1.71	2.43
$K = 10^{-2.3}$	1.00	1.83	3.03

velocity of the reaction might therefore depend on whether the equilibrium concentration of the intermediate compound had been reached before the enzyme was added.

This possibility was studied by filling two cells with buffer and methylglyoxal. GSH was added to one cell and after the relatively rapid, non-enzymic, increase in optical density had ceased, a similar amount of GSH was added to the other cell. To each cell was immediately added a solution of the enzyme and the rate of increase in optical density was determined. The results are shown in Fig. 3. The initial rate was slightly faster in the cell to which the enzyme had been added immediately after the components were mixed. In this cell there was little or no addition compound at the beginning of the enzymic reaction, whereas in the other cell the concentration of the addition compound was much greater and yet the reaction went more slowly. This definitely suggests that the enzyme did not act on the addition compound but on the free GSH and methylglyoxal. The rather greater initial rate of reaction in the cell containing the free components

may be accounted for by the fact that the increment in optical density when the components change into the enzymically formed compound is greater than that associated with the change of the addition compound into the enzymically formed compound. The changes in optical density/mole can be shown to be about 5 % greater in the former than in the latter case. This is less than the difference in the initial reaction rates shown in Fig. 3, but other factors, such as the slightly higher concentrations of the free components where equilibrium has not been allowed to occur and the initial tendency for the chemical addition compound to form, probably account for the rest of the difference. In subsequent work, to ensure standard conditions time was allowed for equilibrium to be reached between the addition compound and the free components before the enzyme was added. It should be noted that the non-enzymic changes in optical density are small compared to enzymic ones at the same substrate concentrations.

Effect of substrate concentration on reaction velocity: determination of K_m . The effect on reaction velocity of varying the concentration of GSH was studied with different concentrations of methylglyoxal. The increase in optical density was plotted against time and the rate of reaction was found from the initial linear portion of the curve. When the reciprocals of velocity were plotted against those of GSH concentration, the points for a constant methylglyoxal concentration fell on a line which was straight except near the $1/V$ axis, where it curved to run horizontally. This corresponded to the fact that the velocity of the reaction attained a limiting value when the concentration of GSH reached that of methylglyoxal. Further increases in the GSH concentration did not produce any increases in the reaction velocity within the limits of experimental error.

Two such lines, each for a constant concentration of methylglyoxal, are shown in Fig. 4. It will be seen that the linear portions of the lines, when extrapolated, meet the $1/S$ axis at -1.82×10^3 and -1.58×10^3 , giving values of K_m of $0.55 \times 10^{-3} M$ and $0.63 \times 10^{-3} M$ for methylglyoxal concentrations of $1.08 \times 10^{-3} M$ and $2.16 \times 10^{-3} M$ respectively. The average of these estimates is approximately $0.6 \times 10^{-3} M$, and this is taken as the value of K_m calculated from S , the total GSH concentration.

It appears from the previous section that the enzyme acts on free GSH, and therefore it would seem theoretically preferable to calculate the concentration of free GSH (S') in each experiment from equation (4) with $10^{-2.3} M$, i.e. $5 \times 10^{-3} M$, as the value of K . The effect of this modification depends on the concentration of methylglyoxal (C). If S , the total GSH concentration, is less than C so

Fig. 3. Rate of the glyoxalase I reaction on free GSH and free methylglyoxal and on their addition compound. All cells contained phosphate buffer, $3.3 \times 10^{-2} M$ at pH 6.6, and water to a final volume of 3 ml. Cells 2 and 3 contained GSH, $1.08 \times 10^{-3} M$, and methylglyoxal, $2.16 \times 10^{-3} M$, concn. after addition of enzyme in each case. In cell 2 (●) methylglyoxal was added initially but in cell 3 (▲) it was not added till the change in optical density in cell 2, due to the formation of addition compound, had stopped. Glyoxalase I (0.1 ml. of 1:200 stock solution) was then added immediately to both cells, and the rate of reaction measured spectrophotometrically.

that q is much less than C , equation (4) may be written

$$\frac{C(S-q)}{q} = K, \quad (5)$$

or
$$\frac{S'}{S} = \frac{S-q}{S} = \frac{K}{K+C},$$

i.e.
$$\frac{\text{free GSH}}{\text{total GSH}} = \frac{K}{K+C}. \quad (6)$$

This expression is approximately equal to $\frac{1}{2}$ when C is $1.08 \times 10^{-3} \text{ M}$ and $\frac{2}{3}$ when C is $2.16 \times 10^{-3} \text{ M}$. If in Fig. 4 $1/V$ had been plotted against $1/S'$ instead of against $1/S$, the abscissae of all the observed points would have been increased by $\frac{1}{2}$ and $\frac{2}{3}$ in the respective cases. The straight lines best fitting the points where the glutathione concentration is not greater than that of methylglyoxal are shown as two broken lines in Fig. 4, which on extrapolation meet the substrate axis at $-1/S'$ equals $2.20 \times 10^3 \text{ M}^{-1}$ and $2.21 \times 10^3 \text{ M}^{-1}$ for methylglyoxal concentrations of $1.08 \times 10^{-3} \text{ M}$ and $2.16 \times 10^{-3} \text{ M}$ respectively. This close agreement lends further support to the view that the enzyme acts on free GSH. It further suggests that over the range of concentrations used, the underlying assumptions of the theory are valid and the methylglyoxal concentration does not affect the GSH-enzyme equilibrium. The Michaelis constant K_m for the equilibrium: enzyme + free GSH $\rightleftharpoons$ enzyme-GSH, has the value $0.46 \times 10^{-3} \text{ M}$.

Fig. 4. Plot of $1/V$ against $1/S$ for two methylglyoxal concentrations. The concentrations of methylglyoxal used were $1.08 \times 10^{-3} \text{ M}$ (●), and $2.16 \times 10^{-3} \text{ M}$ (■). At each methylglyoxal concentration the velocity, V , was measured at a series of glutathione concentrations, S . The solid lines are those which best fit the points where S is equal to or less than the methylglyoxal concentration. The broken lines are obtained if the calculated free GSH concn. are used instead of the total GSH concn.

Effects of analogues of glutathione on the glyoxalase I reaction. The initial velocity of the reaction in cells with and without the inhibitor was determined at a series of inhibitor and substrate concentrations. From the fractional activity α , which is the ratio of the velocities of the inhibited to the uninhibited reaction, $I\alpha/(1-\alpha)$ was calculated and then plotted against substrate concentration. The 'best' straight line was fitted to the experimental points by the method of least squares and extrapolated to meet the substrate axis, where possible.

As already indicated, these lines should cut the S axis at $S = -K_m$ if inhibition is competitive and at infinity if non-competitive. If they are cut at some other value of S then the inhibition is of an intermediate type.

The graphs of $I\alpha/(1-\alpha)$ against S for various analogues of glutathione and allied compounds are shown in Figs. 5-7. These experiments are summarized in Table 2. In this table K_i has been obtained as the value of $I\alpha/(1-\alpha)$ at which the line best fitting the experimental points cuts the ordinate axis. It has been shown in the Theoretical section that in intermediate-type inhibition the intercept differs from the true value of K_i by less than 12.5%, provided that the substrate concentrations are less than K_m .

Since the compounds tested had appreciable optical densities at $240 \text{ m}\mu$, a limit was set to the concentrations which could be used without affecting the accuracy of the spectrophotometer readings. As a result, for weak inhibitors (K_i greater than about 10^{-2}) only small degrees of inhibition could be produced; the value of $1-\alpha$ was therefore small and the error in $\alpha/(1-\alpha)$ relatively large. Thus with such weak inhibitors, points may show a considerable degree of scatter in comparison with those for inhibition by stronger ones.

Experiments were also carried out with L-cysteine and L-cysteinylglycine as inhibitors. With low analogue concentrations no inhibition was observed. As the concentration of the analogue approached that of methylglyoxal a large increase in optical density occurred before the addition of the enzyme, and the velocity of the subsequent enzymic reaction was diminished (Fig. 8). This suggests that these two compounds react non-enzymically with methylglyoxal and prevent it from taking part in the enzymic reaction.

DISCUSSION

The postulated mechanism of the glyoxalase reaction involves the formation of *S*-lactylglutathione by glyoxalase I. Evidence in support of this conclusion was first obtained by Racker (1951), who by using glyoxalase I free from glyoxalase II was able to prepare the intermediate compound and

showed that its formation was accompanied by a marked increase in absorption at 240 m μ . This characteristic light absorption disappeared when the compound was broken down by glyoxalase II. Thioesters in general show a characteristic absorption peak in this neighbourhood (Racker & Krimsky, 1952; Burton & Stadtman, 1953). Strecker (1954) showed that glyoxalase II can replace brain thioesterases in the hydrolysis of certain thioesters, in conformity with the view that glyoxalase II is a thioesterase. The identity of the thioester has been confirmed by Racker (1954) who has shown that *S*-lactylglutathione prepared synthetically (Wieland & Koppe, 1953) is hydrolysed by glyoxalase II at approximately the same rate as the enzymically prepared intermediate.

With regard to the kinetics of the reaction catalysed by glyoxalase I, it has been shown in the present work that when $1/V$ is plotted against $1/S$, the curve obtained is approximately linear for sufficiently small values of S but departs from

Fig. 6. Inhibition of glyoxalase I by γ -L-glutamyl-L- β -sulphoalanylglycine.

Fig. 5. Inhibition of glyoxalase I by *S*(*N*-ethylsuccinimido)-glutathione (●), and by *S*-methylglutathione (■): plot of $I_a/(1-a)$ against S ; a is determined as the ratio of inhibited velocity to uninhibited velocity with the same concentration of substrate S , over a period of 5 min.

Fig. 7. Inhibition of glyoxalase I by γ -DL-glutamyl-DL-alanylglycine (●), by γ -DL-glutamylglycylglycine (▲) and by *S*-methyl-L-cysteine (■).

Table 2. Action of GSH analogues on the glyoxalase I reaction

Values of K_i were obtained from experimental observations of the type illustrated in Figs. 5–7, by the method described in the Theoretical section.

Inhibitor	K_i (mM)	Apparent K_m (mM)	Type of inhibition
<i>S</i> (<i>N</i> -Ethylsuccinimido)glutathione	0.64	0.55	Competitive
<i>S</i> -Methylglutathione	2.3	0.67	Competitive
γ -DL-Glutamyl-DL-alanylglycine	15.1	8.3	Intermediate, largely non-competitive
γ -L-Glutamyl-L- β -sulphoalanylglycine	18.7	0.43	Competitive
γ -DL-Glutamylglycylglycine	40.9	∞	Non-competitive
<i>S</i> -Methyl-L-cysteine	22.5	9.3	Intermediate, largely non-competitive
<i>S</i> (<i>N</i> -Ethylsuccinimido)-L-cysteine	—	—	None
Glutamic acid	—	—	None
Glycine	—	—	None

linearity near the $1/V$ axis. This corresponds to the fact that the velocity of the reaction increases with increase of GSH concentration according to the Michaelis-Menten equation until the concentration of GSH approaches that of methylglyoxal. Once the concentration of the latter is exceeded, however, further increases in the GSH concentration have little effect on the reaction velocity. The converse does not hold since at any GSH concentration an increase in the methylglyoxal concentration increases the velocity. No explanation which will fit all the facts can be advanced at present. However, the experiments with enzyme inhibitors have been carried out at GSH concentrations well below that of methylglyoxal, that is, on the linear portion of the curves. Under these conditions it would seem justifiable to treat the enzyme in equilibrium with a fixed concentration of methylglyoxal as if it were a simple enzyme reacting with GSH at these low concentrations according to the usual Michaelis-Menten theory.

If, as appears probable from the considerations adduced in the Results section, it is the free GSH which combines with the enzyme, it is more appropriate to use the calculated concentrations of free GSH rather than those of total GSH in working

out the kinetics of the reaction. This change does not significantly alter the nature of the graphs. The chief result is to lower the values of K_m calculated from the two methylglyoxal concentrations and to bring them into very close agreement.

In the treatment of results from the experiments with inhibitors it has been considered best to employ total GSH concentration, as this involves fewer assumptions about the methylglyoxal-GSH equilibrium. In all these experiments the same concentration of methylglyoxal has been used so that in accordance with equation (6), the ratio of free to total GSH would remain constant. This implies that all the relevant graphs would merely be altered in scale as far as the abscissae are concerned and none of the conclusions would be affected.

The discussion in the theoretical section serves to underline the fact, generally recognized by workers in the field, that the enzyme kinetics of an inhibited reaction may not correspond to either a purely competitive or purely non-competitive type. Owing to the inevitable experimental error it is often difficult to decide whether the observed departure from one or other of these two types is significant or not. The test we have applied is to plot the value of $I\alpha/(1-\alpha)$ against S , the substrate concentration, where S is less than K_m . If the best-fitting straight line through the observed points cuts the S axis at or close to the point where S equals $-K_m$, the inhibition is presumably competitive. Non-competitive inhibition is suggested if this line is parallel to the S axis and intermediate lines suggest intermediate types of inhibition.

For purely competitive or purely non-competitive inhibition the value of K_i is given by the intersection of the line and the $I\alpha/(1-\alpha)$ axis. For intermediate-type inhibition the true value of K_i will, in general, be less than the value of $I\alpha/(1-\alpha)$ at this intersection, but as was shown in the Theoretical section the difference will be less than $K_i/8$. The value of $I\alpha/(1-\alpha)$ at the point of intersection is therefore a reasonable approximation to K_i .

In considering the action of the various compounds used in the inhibition experiments, we find that the most active of the tripeptides listed in Table 2 is $S(N$ -ethylsuccinimido)glutathione, followed by S -methylglutathione. Both these compounds act in a purely competitive manner as also does γ -L-glutamyl-L- β -sulphoalanylglycine. γ -DL-Glutamyl-DL-alanylglycine has a fairly high K_i value and therefore would be classed as a weak inhibitor, but it should be remembered that the synthetic compound is a mixture of four stereoisomeric forms. If only one of these forms, the LL, were active the value of K_i would only be a quarter of that found, so that this stereoisomeric form would be almost comparable in activity with S -methylglutathione, though its inhibition is of a

Fig. 8. Action of cysteine on methylglyoxal: inhibition of glyoxalase I. All cells contained buffer and water to bring volume to 3.0 ml. after addition of the enzyme. In addition, cells 2 (●), 3 (▲) and 4 (■) contained methylglyoxal, $2.16 \times 10^{-3} M$, and GSH, $0.216 \times 10^{-3} M$, and cells 3 and 4 contained cysteine, $2.16 \times 10^{-3} M$ and $4.32 \times 10^{-3} M$ respectively. These concentrations are those attained after the addition of the enzyme. The cells were equilibrated for 10 min. before cysteine was added. Measurements began immediately this addition was made, by using cell 1 as blank and extrapolating to zero time to find the initial reading. After 20 min. an almost steady reading had been reached in all cells. To each was added 0.1 ml. of glyoxalase I (stock solution diluted 1:500) and the subsequent enzymic reactions were followed.

mixed type. γ -DL-Glutamylglycylglycine had a very low activity of the non-competitive type. Here it is possible that only one of the two stereoisomers is active, but, even if allowances were made for this, the compound has only low inhibitory power. It would, of course, have been preferable to have synthesized the pure optical isomers and used them in these inhibition experiments but the time available did not permit this extension of the work.

Of the group of analogues which are not tripeptides only *S*-methyl-L-cysteine was definitely inhibitory and showed an intermediate, largely non-competitive, type of inhibition. It is rather curious that the *N*-ethylmaleimide adduct of GSH is more effective than *S*-methylglutathione, and *S*-methylcysteine is inhibitory and the *N*-ethylmaleimide adduct of cysteine is not.

Thus the compounds which are tripeptides differing from GSH only in the $-\text{CH}_2\text{SH}$ side chain are the most effective inhibitors. Those compounds which are not tripeptides are either weak inhibitors or are without effect.

The behaviour of two of the analogues, not possessing the tripeptide structure, calls for further comment: these are L-cysteine and L-cysteinylglycine. In the presence of methylglyoxal both compounds undergo a reaction which is accompanied by an increase in optical density at 240 m μ . It is believed that the formation of a thiazolidine ring occurs with the elimination of water (Schubert, 1935, 1936).

In the range of concentrations used, methylglyoxal was not completely combined unless a considerable excess of aminothiols was present, for increasing the concentration of the latter beyond that of methylglyoxal produced a further increase in the optical density in the absence of enzyme. The removal of methylglyoxal inhibited the enzymic reaction with GSH, and when two molecules of aminothiols were present for each molecule of methylglyoxal, the enzymic reaction ceased.

Racker (1951) suggested that oxidized glutathione could be estimated by the glyoxalase reaction in the Warburg apparatus, after its reduction with excess cysteine. It is clear that this method will only be satisfactory provided that sufficient methylglyoxal remains, after addition of the cysteine, to allow the reaction to proceed at maximum velocity.

Fodor, Miller & Waelsch (1952, 1953) have reported that estimations of GSH by the glyoxalase system in the Warburg apparatus are inaccurate in the presence of cysteinylglycine. They believed that this was due to competitive inhibition. It appears likely that the inhibition was due to combination of the peptide with methylglyoxal rather than to direct competition between cysteinylglycine and GSH.

SUMMARY

1. Some glutathione analogues were tested on the complete glyoxalase system (glyoxalase I plus glyoxalase II) and shown to be inhibitory. No quantitative deductions could be made because of the complexity of the system.

2. The non-enzymic reaction between glutathione and methylglyoxal in aqueous solution involved a small increase in optical density which could be used to follow the reaction. The equilibrium constant was shown to be approximately $10^{-2.3}$ M.

3. The variation in the velocity of the enzymic reaction with glutathione concentration was studied at two different concentrations of methylglyoxal. The results suggest that the enzyme combines with free GSH and not with the GSH combined with methylglyoxal in the non-enzymic reaction, and that the Michaelis constant for the equilibrium is 0.46×10^{-3} M.

4. The effect of analogues of glutathione on the glyoxalase I reaction was studied and some were found to be inhibitory. The stability constants of the enzyme-inhibitor complexes (K_i) were calculated and the nature of the inhibition was investigated.

5. The relationship between the structure of the analogues and the degree and type of inhibition they produced is discussed in the light of the mathematical theory of enzyme inhibition briefly outlined.

6. A non-enzymic reaction of L-cysteine and of L-cysteinylglycine with methylglyoxal caused a large increase in optical density. It differed from the reaction of glutathione and methylglyoxal and probably involved the formation of a thiazolidine ring.

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The Conversion of Dimefox into an Anticholinesterase by Rat Liver *in vitro*

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The organic phosphorus insecticides schradan (bisdimethylaminophosphonous anhydride) and parathion (*OO*-diethyl-*O*-*p*-nitrophenyl phosphorothioate) have been shown to be converted by mammals *in vivo*, and by liver tissue *in vitro*, into powerful anticholinesterases (see, e.g. Gardiner & Kilby, 1950*a, b*; Dubois, Doull & Coon, 1950; Diggle & Gage, 1951; Aldridge & Barnes, 1952; Casida, Allen & Stahmann, 1952, 1954; Gage, 1953; Metcalf & March, 1953; Davison, 1954, 1955).

Dimefox (bisdimethylaminofluorophosphine oxide), another insecticide, was claimed by Aldridge & Barnes (1952) to be converted into a more active inhibitor *in vivo*. High concentrations were required to inhibit rabbit-erythrocyte cholinesterase *in vitro* as compared with those calculated to occur *in vivo* after injection of dimefox. They reported that about 7×10^{-2} M dimefox caused 50% inhibition of rat-brain cholinesterase, and that rat-liver slices converted it into a more active but unstable anticholinesterase. Okinaka, Doull, Coon & Dubois (1954), however, found that 4×10^{-5} M dimefox *in vitro* caused 50% inhibition of rat-brain cholinesterase in 15 min., and that rat-liver slices reduced the anticholinesterase activity aerobically or anaerobically.

This paper presents further studies on the mode of action and metabolism of dimefox in mammals. The work was undertaken as part of a programme of investigation of the toxicology of dimefox, the systemic insecticidal properties of which are utilized to combat insect infestation on hops, sugar beet and other crops.

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MATERIALS AND METHODS

Dimefox, b.p. 205° at 760 mm., 96° at 18 mm., was prepared by fractional distillation of Hanane (Fisons Pest Control Ltd.) and was 98.6% pure by chemical assay. Diphosphopyridine nucleotide (DPN) (95/100%), adenosine triphosphate (ATP) and cytochrome *c* were obtained from L. Light and Co. Ltd. Triphosphopyridine nucleotide (TPN) was provided by Dr W. N. Aldridge and contained at least 70% of TPN (assayed with isocitric dehydrogenase) and no detectable DPN.

Glaxo-Wistar albino rats were killed by decapitation. Males were used unless otherwise stated.

Liver preparations. Liver slices about 0.5 mm. thick were cut and trimmed by hand to about 5 mm. square. They were washed twice in 0.9% (w/v) NaCl solution at room temp. before use.

For the preparation of homogenates, washed slices of liver were damp-dried on filter paper, chopped with scissors, weighed and homogenized in 2 ml. of ice-cold medium (isotonic sucrose or NaCl) in an ice-cold tube of the Potter-Elvehjem type. More liquid was then added to give a final volume of 4 ml./g. fresh weight of liver.

Cell-free suspensions were prepared by centrifuging the whole homogenate at 600 *g* for 5 min. at room temp., and withdrawing the supernatant. Chilled glassware was used throughout. Such suspensions had a cholinesterase activity, measured as described below, of about 5 μ moles of acetylcholine/ml./hr.

Further fractionation of homogenates in 0.25 M sucrose was carried out at 0° by the method of Schneider (1948). Cells and nuclei were removed by centrifuging at 600 *g* for 10 min., mitochondria by a further 10 min. at 8500 *g*, and microsomes by a further 60 min. at 18000 *g*. Each sediment was resuspended without washing in 0.25 M sucrose to the original volume of whole homogenate.

Boiled liver extract was made by dropping fresh chopped rat liver into boiling water (2 ml./g.) and boiling for 2 min.