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INHIBITION OF GLYCOLLATE OXIDASE AS A RATIONAL WAY OF DESIGNING A HERBICIDE*

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Abstract—Inhibitors of glycollate oxidase (E.C. 1.1.3.1.) might be useful herbicides. An attempt was therefore made to improve on the known aldehyde bisulphite addition compounds as inhibitors. Unfortunately, it appears that, with the exception of the formaldehyde bisulphite addition compound, all these structures inhibit by reacting with enzymatically produced glyoxylate to give glyoxylate bisulphite. A wide range of other potential inhibitors was then tested on the enzyme. Some aminomethanesulphonates are good inhibitors, but they again are unstable, and probably work by conversion to the formaldehyde bisulphite addition compound. None of the inhibitors show useful activity on a general screen against higher plants and fungi.

INTRODUCTION

GLYCOLLATE oxidase (glycollate: oxygen oxidoreductase, E.C. 1.1.3.1.) is found widely in plants, though there is considerable discussion about its importance and function.[1-5] Mammals do have the ability to oxidize glycollate,[6-8] but it does not seem to be an important metabolite. Zelitch[1,9,10] has shown that aldehyde bisulphite addition compounds are powerful inhibitors of glycollate oxidase. We therefore studied the inhibition of the enzyme by these compounds with the object of improving them by molecular modification. We report here our findings on the mechanism of this inhibition, together with information on some new inhibitors of glycollate oxidase.

RESULTS

Inhibition by Aldehyde Bisulphite Addition Compounds

Inhibition by the formaldehyde bisulphite addition compound. Using Warburg manometry, Zelitch[9] studied the inhibition of glycollate oxidase by the formaldehyde addition compound, and he found that the inhibition was competitive in nature, and he quotes a K_i of 1·7–1·9 μM. Using an oxygen electrode, which allowed us to see the initial part of the reaction,

* Part I in the series "Rational Design of Pesticides".

[1] I. ZELITCH, *J. Biol. Chem.* **234**, 3077 (1959).
[2] N. E. TOLBERT, *Nat. Acad. Sci.—Nat. Res. Council Publication* **1145**, 648 (1963).
[3] I. ZELITCH, *J. Biol. Chem.* **240**, 1869 (1965).
[4] C. P. WHITTINGHAM, J. COOMBS and A. F. H. MARKER, in *Biochemistry of Chloroplasts* (edited by T. W. GOODWIN), Vol. II, p. 155, Academic Press, New York (1967).
[5] T. KISAKI and N. E. TOLBERT, *Plant Physiol.* **44**, 242 (1969).
[6] E. KUN, J. M. DECHARY and H. C. PITOT, *J. Biol. Chem.* **210**, 269 (1954).
[7] M. NAKANO, Y. USHIJIMA, M. SAGA, Y. TSUTSUMI and H. ASAMI, *Biochim. Biophys. Acta* **167**, 9 (1968).
[8] Y. USHIJIMA and M. NAKANO, *Biochim. Biophys. Acta* **178**, 429 (1969).
[9] I. ZELITCH, *J. Biol. Chem.* **224**, 251 (1957).
[10] I. ZELITCH, *J. Biol. Chem.* **233**, 1299 (1958).

TABLE 1. PURIFICATION OF GLYCOLLATE OXIDASE*

Fraction	Specific activity†	Purification	Yield
Original brei	0·024	1	100
$(NH_4)_2SO_4$	0·060	2·5	25
DEAE wash	0·646‡	27	21·5

* Results of a typical experiment. All enzyme fractions were desalted, except as noted in footnote.‡

† μmoles Glycollate oxidised/min/mg protein.

‡ The enzyme solution used for assays was the DEAE wash fraction totally precipitated with $(NH_4)_2SO_4$ and dissolved in Na phosphate, 0·025M, pH 7·0; a typical value for specific activity was 0·43 (protein measured by 260/280 nm method).

we found that the rate of the inhibited reaction was linear with time (until substrate depletion occurred), and we agree with the K_i value that Zelitch quotes, and with his conclusion that the nature of the inhibition is one of simple competition.

Inhibition by aldehyde bisulphite addition compounds other than formaldehyde bisulphite. With compounds other than formaldehyde bisulphite, we did not obtain initially linear rates of inhibition from which to measure K_i values. Instead we measured the concentration of inhibitor necessary to give 50 per cent inhibition of a standard enzyme preparation (Table 2). It is obvious from this table that the enzyme was not significantly affected by the type of group to which the $-CHOH.SO_3Na$ was attached, since all compounds exerted a similar level of inhibition. The data also suggest, but do not prove, that the most powerful inhibitors were those having two hydroxysulphonate groups in the molecule.

These observations would be explained if the inhibitory action was due to the formation of the glyoxylate bisulphite addition compound, which would therefore be acting as a universal inhibitory product of all aldehyde bisulphite compounds tested.

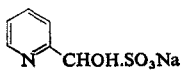
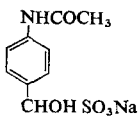
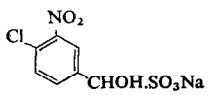
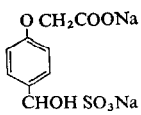
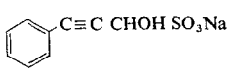
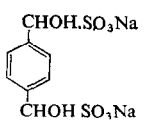
Chemical reaction between glyoxylate and aldehyde bisulphite addition compounds. It is relatively simple to show by spectrophotometry that glyoxylate reacts non-enzymatically with aldehyde addition compounds to yield the parent aldehyde. The pyridine-2-aldehyde addition compound has a different spectrum from pyridine-2-aldehyde itself, and excess glyoxylate added to the addition compound caused a change in the spectrum (in less than 2 min) to give one resembling pyridine-2-aldehyde. There was no change in spectrum when the pyridine-2-aldehyde addition compound was incubated with enzyme in the absence of glycollate, but when glycollate was added a rapid spectral change to that of the free aldehyde occurred.

This conversion was not unique to the pyridine-2-aldehyde, since the bisulphite addition compound of $PhC{\equiv}C.CHO$ was tested under similar conditions, and it was found that the addition of glyoxylate caused a spectral change consistent with the formation of the free parent aldehyde.

These experiments support the view that the inhibition of glycollate oxidase by aldehyde bisulphite addition compounds is due to their chemical interaction with enzymatically produced glyoxylate to give the glyoxylate bisulphite addition compound.

Kinetics of inhibition by glyoxylate bisulphite. Previous work on the inhibition of glycollate oxidase by aldehyde bisulphite addition compounds has usually been done with a

TABLE 2. INHIBITION OF GLYCOLLATE OXIDASE BY ALDEHYDE BISULPHITE ADDITION COMPOUNDS

Structure	Concn. (μM) giving 50 per cent inhibition*
$NaHSO_3$	2·0 ± 0
COONa | $CHOH.SO_3Na$	1·85 ± 0·15
$CCl_3.CHOH.SO_3Na$	2·0 ± 0
$CH_2.CHOH.SO_3Na$ / CH_2 \ $CH_2.CHOH.SO_3Na$	0·83 ± 0·07
	1·20 ± 0·05
	0·83 ± 0
	0·94 ± 0·06
	2·0 ± 0·5
	2·4 ± 0·1
	0·46 ± 0·06

* Mean of results from two independently weighed solutions ± difference from mean; the level of inhibition was measured when the electrode trace was linear.

Warburg manometer. We used an oxygen electrode, which enabled us to see the initial part of the time course. Figure 1 shows the normal time course of the reaction, which is initially linear, but then declines as the substrate is used up. Addition of more glycollate increases the rate again. Figure 1 also shows the effect of adding the pyridine-2-aldehyde bisulphite

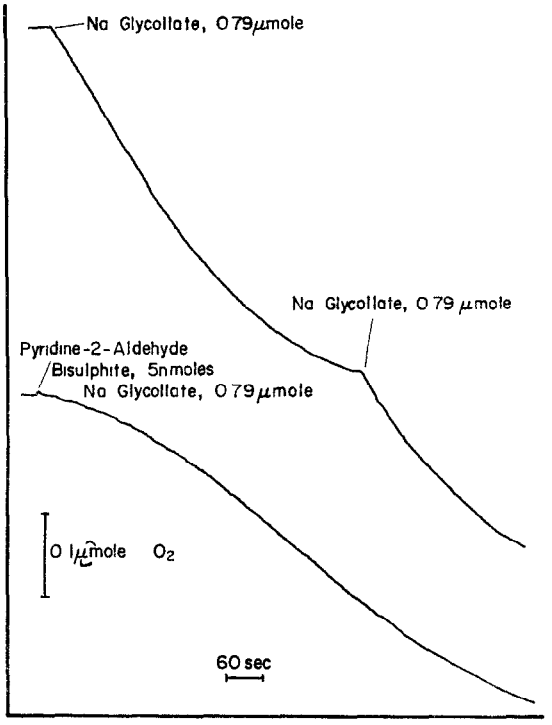


FIG. 1. INHIBITION OF GLYCOLLATE OXIDASE BY THE PYRIDINE-2-ALDEHYDE BISULPHITE ADDITION COMPOUND.
Conditions: standard oxygen electrode assay.

addition compound. The initial inhibition is strong, but then declines with time until substrate depletion reduces the rate. Figure 2 shows that glyoxylate bisulphite itself produces a similar degree of inhibition and a similarly shaped curve. This type of kinetic behaviour was found with all the aldehyde bisulphite addition compounds tested, with the exception of that of formaldehyde, giving further evidence that a common mechanism, such as the formation of glyoxylate bisulphite, is operating. We also agree with Zelitch's[9] view that bisulphite itself is inhibitory by virtue of its conversion of glyoxylate bisulphite, since we found that addition of sodium metabisulphite, which is immediately converted by water to 2 moles of bisulphite, gave similar traces to glyoxylate bisulphite of equivalent molarity.

What remains unexplained is the shape of the curve produced by the aldehyde bisulphite inhibitors. Since the curves for the inhibitors are similar to the curve for glyoxylate bisulphite itself, there is probably an extremely rapid conversion of at least part of the inhibitor to glyoxylate bisulphite as the enzyme forms glyoxylate. Note that the enzyme reaction will rapidly produce a large excess of glyoxylate. The inhibitors are present at a concentration of about 2 μM or less, yet an uptake of oxygen of 0·1 μmole corresponds to the conversion of 0·2 μmoles of glycollate, which, in the assay volume of 3·0 ml, gives a glyoxylate concentration of 67 μM (0·2 $\times$ 1000/3 = 67). With this excess of glyoxylate the equilibrium between glyoxylate and bisulphite (derived from the aldehyde bisulphite inhibitor) will be towards the glyoxylate bisulphite side, and as the enzyme reaction progresses this tendency will increase. Why then does the inhibition decrease with time? It appears that glyoxylate

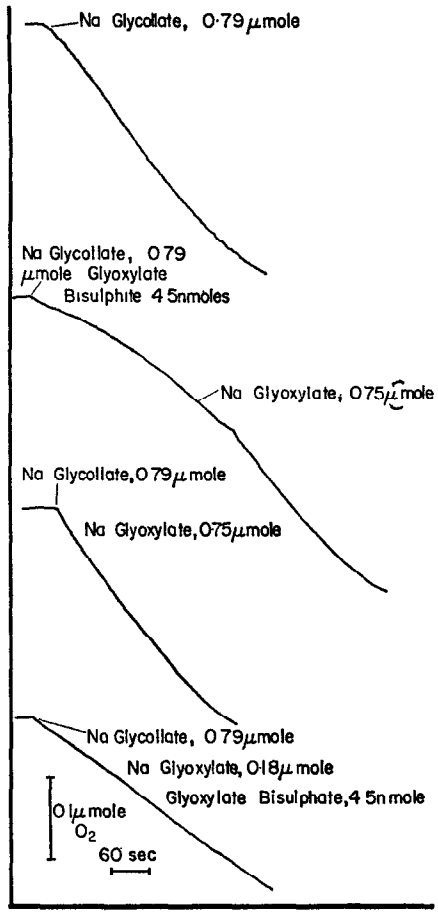


FIG. 2. EFFECT OF GLYOXYLATE ON THE INHIBITION OF GLYCOLLATE OXIDASE BY THE GLYOXYLATE BISULPHITE ADDITION COMPOUND.

Conditions: standard oxygen electrode assay.

itself relieves the inhibition caused by glyoxylate bisulphite. Figure 2 shows that addition of glyoxylate will stimulate the uptake of oxygen when added some time after the inhibitor, or at the same time as the inhibitor. The control shows that glyoxylate does not affect the uptake of oxygen in the absence of inhibitors.

Search for a New Inhibitor of Glycollate Oxidase

As a result of this work, we were left with only two inhibitors of glycollate oxidase, the formaldehyde bisulphite and glyoxylate bisulphite addition compounds. We therefore looked for new inhibitors, based on the information available on the binding of compounds to the enzyme.[9,11,12] Of the 43 potential inhibitors tested (Table 3), only 7 showed strong inhibition of glycollate oxidase. Of these we did not investigate the active sulphinates since we

[11] I. ZELITC and S. OCHOA, *J. Biol. Chem.* **201**, 707 (1953).
[12] K. E. RICHARDSON and N. E. TOLBERT, *J. Biol. Chem.* **236**, 1280 (1961).

TABLE 3. COMPOUNDS TESTED AS INHIBITORS OF GLYCOLLATE OXIDASE, OTHER THAN ALDEHYDE BISULPHITES

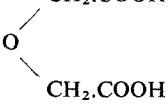
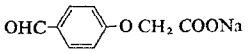
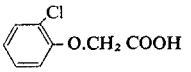
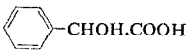
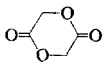
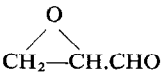
Structure	Conc. (μM) giving 50 per cent inhibition*	Comments
A. Compounds related to glycollate		
1. O-substituted glycollates		
$CH_3.O.CH_2.COOH$	None at 333	
$CH_3.CH_2\ O.CH_2.COOH$	None at 333	
$(CH_3)_2CH.O.CH_2.COOH$	None at 333	
	None at 333	
	None at 333	
	None at 333	
2. C_1-substituted glycollates		
$CH_2.OH.CO.CH_2OH$	None at 333	
$CH_2.OH.CO.NH.NH_2$	None at 333	
3. C_2-substituted glycollates		
$COOH.CHOH.COOH$	None at 333	
$CCl_3.CHOH.COOH$	None at 333	
$CH_2Cl.CCl_2.CHOH.COOH$	None at 333	
	None at 333	
4. Miscellaneous compounds related to glycollate		
$CH_2SH.COOH$	None at 333	Takes up oxygen slowly in assay system.
$CH_2OH.CO.OCH_2.CH_3$	None at 333	Takes in oxygen slowly in assay system
	None at 333	
	None at 333	
B. Compounds related to glyoxylate		
$CH_3.CO.COOH$	None at 333	
$NH_2.CO.COOH$	None at 333	

TABLE 3 (*cont.*)

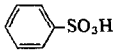
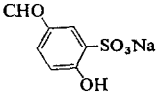
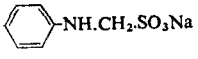
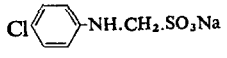
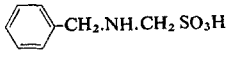
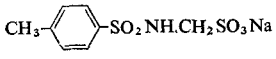
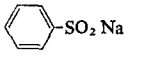
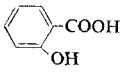
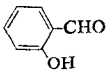
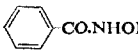
Structure	Conc. (μM) giving 50 per cent inhibition*	Comments
C. Sulphonic acids		
1. Non amino sulphonates		
$CH_3.SO_3H$	None at 333	
	None at 333	
	None at 333	
2. Amino sulphonates		
$NH_2.SO_3H$	None at 333	
$NH_2.CH_2.SO_3H$	32	
$CH_3.NH.CH_2.SO_3H$	2·8	
$CH_3.CH_2.NH.CH_2.SO_3H$	3	
	> 333	333 μM gave 25 per cent inhibition
	> 333	333 μM gave 36 per cent inhibition
	3·7	
$CH_3.CO.NH.CH_2.SO_3Na$	> 333	333 μM gave 16 per cent inhibition
$NH_2.CO.NH.CH_2.SO_3K$	None at 333	
	> 333	333 μM gave 25 per cent inhibition
$NH_2.CH_2.CH_2.SO_3H$	None at 333	
D. Sulphinic acids		
1. Non amino sulphinates		
$CH_2.OH.SO_2Na$	19	
	None at 333	
2. Amino sulphinates		
$NH_2.CH_2.SO_2Na$	8	
	333	

TABLE 3 (*cont.*)

Structure	Conc. (μM) giving 50 per cent inhibition*	Comments
$(CH_3\ NH.CH_2.SO_2)_2Zn$	2	2 μM Zn^{2+} has no effect on enzyme
E. Miscellaneous compounds		
$CH_3\ CCl_2.COONa$	None at 333	
	None at 333	
	None at 333	
	None at 333	
$CH_2OH.CH_2.NH.NH_2$	None at 333	
$NH_2.NH.CO.OCH_2CH_3$	None at 333	

* With substrate conc. equal to $1K_m$ (262 μM sodium glycollate).

felt it likely that they might be oxidised, possibly after breakdown, but we did look at the aminosulphonates in some detail.

Inhibition by Aminosulphonates

We think that the aminosulphonates inhibit glycollate oxidase by conversion to a bisulphite addition compound. The evidence is as follows:

Chemical stability and activity. Table 3 shows that the order of activity in inhibiting glycollate oxidase is

$$CH_3.NH.CH_2.SO_3Na \simeq CH_3.CH_2.NH.CH_2.SO_3Na > C_6H_5.CH_2.NH.CH_2.SO_3Na > NH_2.CH_2.SO_3Na > p\text{-}IIC_6H_4.NH.CH_2.SO_3Na > C_6H_5.NH.CH_2.SO_3Na.$$

Hydrolysis might be expected to occur by initial protonation of the nitrogen. If this is correct electron donating groups will give relatively unstable compounds, and thus appear as better inhibitors, while electron withdrawing groups will give stable compounds and poor inhibitors. It can be seen from Table 3 that this is the case.

Chemical evidence of breakdown. Preliminary work showed that the spectrum of $Ph.CH_2.NH.CH_2.SO_3.Na$ in sodium phosphate buffer at pH 7·0 altered in a few minutes to one resembling that of benzylamine. Under the same conditions the spectrum of $Ph.NH.CH_2.SO_3Na$ only altered very slightly towards that of aniline. This shows that $Ph.CH_2.NH.CH_2.SO_3Na$ is less stable than $Ph.NH.CH_2SO_3.Na$. Table 3 shows that the first compound is a powerful inhibitor of glycollate oxidase (50 per cent inhibition at 3·7 μM), while the latter compound only gives 25 per cent inhibition at 333 μM. These results are consistent with the inhibition being caused by the breakdown of the compounds.

The hydrolysis rate of $CH_3.CH_2.NH.CH_2.SO_3H$ was tested using a conductometric method and the half-life was 4–6 min when measured in sodium phosphate buffer at pH 7·0, showing that this compound, which is a powerful inhibitor of glycollate oxidase (50 per cent inhibition at 3 μM), is unstable.

DISCUSSION

With the exception of the formaldehyde compound, aldehyde bisulphite addition compounds appear to react with enzymatically produced glyoxylate to form glyoxylate bisulphite. Thus: (i) all compounds tested including glyoxylate bisulphite itself had a similar level of inhibitory activity, apart from compounds based on dialdehydes which appeared to be more inhibitory; (ii) spectrophotometric evidence with aromatic aldehyde compounds suggests breakdown by glyoxylate; and (iii) the kinetic behaviour of the enzyme was similar with all aldehyde bisulphite addition compounds, except that of formaldehyde.

It could be argued that all aldehyde bisulphite compounds (except the formaldehyde derivative) should give exactly the same inhibition as glyoxylate bisulphite. Table 2 shows that the 50 per cent inhibition levels did vary, but this could be explained by there being different equilibria in the reaction between glyoxylate and the aldehyde bisulphite compound.

The kinetic behaviour of the enzyme with glyoxylate bisulphite can be explained by supposing the glyoxylate competes with the glyoxylate bisulphite for a site on the enzyme surface which is not used by glycollate. If the site were used by glycollate, one would expect glyoxylate to cause inhibition of glycollate oxidation in the absence of glyoxylate bisulphite, and this does not occur under our conditions.

Aminomethanesulphonates are also excellent inhibitors of glycollate oxidase, but they also seem to act by breaking down to give an inhibitor, although glyoxylate is not required. Thus: (i) the best inhibitor of the enzyme are those compounds which might be expected to readily undergo protonation on the nitrogen atom, with subsequent breakdown; and (ii) spectral and conductometric evidence suggests that the compounds are unstable.

On hydrolysis the aminomethanesulphonates will form an amine plus the formaldehyde bisulphite addition compound. Whether the glyoxylate bisulphite addition compound forms as well in the presence of glycollate oxidase is not known.

Our object was not to investigate mechanisms of inhibition of glycollate oxidase but to rationally design a herbicide or growth regulator by synthesizing inhibitors of the enzyme. Unfortunately, none of the compounds mentioned above showed any interesting activity when broadly screened against a variety of higher plants and fungi.* This could be because the enzyme was not effectively inhibited in the intact plant, and/or because it is not a vital enzyme. Our work suggests that all effective inhibitors work by conversion to simpler molecules, probably the bisulphite addition compounds of formaldehyde and glyoxylate.

EXPERIMENTAL

Plant Material. Peas (*Pisum sativum* L.) were grown in the light in soil in a heated greenhouse, and the shoots used approximately 2–3 weeks after planting the seed.

Enzyme Assay. The oxygen electrode method of Breidenbach *et al*[14] was used with the following assay mixture: 300 μmoles Na phosphate, pH 7·0, 10 μg catalase (Sigma) (to ensure destruction of peroxide),

* While this study was in progress we found that glycollate oxidase had been suggested as a target enzyme for fungal antisporulants.[13]

[13] R. J. Lukens and J. G. Horsfall, *Phytopathology* **58,** 1671 (1968).

[14] R. W. Breidenbach and H. Beevers, *Biochem. Biophys. Res. Commun.* **27,** 462 (1967).

0·02 μmoles FMN in 1 μmole Na acetate buffer, pH 5·0, 0·2–0·4 ml enzyme, 26·2 μl of $3·0 \times 10^{-2}$M Na glycollate (BDH) giving a final concentration of 262 μM,* and water to 3·0 ml.

The assay was done out at 25° (rather than 30°) and at pH 7·0 rather than the pH optimum of 8·3[11] in an attempt to imitate the natural conditions of the enzyme.

Since the rate of glyoxylate oxidation by the preparation was insignificant when added at the same molar concentration as glycollate (e.g. approximately $1K_m$) the stoichiometry of the reaction was taken to be

$$\begin{matrix}CH_2OH \\ | \\ COOH\end{matrix} + O_2 \longrightarrow \begin{matrix}CHO \\ | \\ COOH\end{matrix} + H_2O_2 \text{ (glycollate oxidase)}$$

$$H_2O_2 \longrightarrow H_2O + \tfrac{1}{2}O_2 \text{ (catalase)}$$

i.e. the uptake of 0·5 mole of O_2 corresponds to the oxidation of 1 mole of glycollate.

It was assumed from standard tables that the solubility of oxygen in the assay medium was 0·26 μmole ml^{-1}.

Results are expressed in enzyme units (1 unit is the amount of enzyme catalyzing the conversion of 1 μmole substrate/min under the conditions stated). The linearity of response of the electrode to oxygen concentration was tested with NADH[15] and found to be satisfactory.

Enzyme purification. All operations were done at 0–4°. 200 g fr. wt. of pea shoots were washed with cold distilled water, and macerated in a blender with 400 ml 0·01M Na phosphate buffer, pH 7·0. The brei was strained through muslin and centrifuged at 23,000 *g* (at maximum radius) for 10 min and the pellet discarded. The supernatant was adjusted to pH 5·3 with 10 per cent HOAc[16] and the volume brought to 500 ml with water. 70 g $(NH_4)_2SO_4$ was added to the supernatant and the resulting precipitate was discarded. 40 g $(NH_4)_2SO_4$ was then added to the supernatant and the resulting precipitate was suspended in about 40 ml of 0·005 M Na phosphate buffer, pH 7·0. The suspension was centrifuged and the pellet discarded. The supernatant (45 ml) was passed through a 40 × 2 cm column of Sephadex G-25 equilibrated with 0·005M Na phosphate buffer, ph 7·0. The eluant (45 ml) was diluted to 80 ml with this buffer, and the solution stirred batchwise with 40 g of wet DEAE gel pre-equilibrated with this buffer. The DEAE was then eluted with 3 × 40 ml 0·025M Na phosphate pH 7·0, by more stirring, centrifuged and the supernatant collected and brought to 70 per cent saturation with $(NH_4)_2SO_4$. The resulting pellet was dissolved in about 20 ml 0·025M Na phosphate pH 7·0 and stored at −15° till required for assay purposes. Typical results are shown in Table 1.

Protein. The protein was measured by the Folin–Ciocalteu method[17] using bovine serum albumin as a reference material, with the exception that the enzyme fraction used for assays was measured by the 260/280 nm method[17] since $(NH_4)_2SO_4$ was present.

Measurement of inhibition. The ideal is to measure the K_i for each compound, but this was not possible for reasons given above. Therefore the concentration of inhibitor needed to give 50 per cent inhibition was determined with 1 K_m of glycollate present. Each determination was repeated on an independently weighed solution of the inhibitor.

Synthesis of inhibitors. Chemical syntheses were supervised by Mr. P. J. Brooker, and will be reported in a separate publication.

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* This is the K_m concentration (mean of 8 determinations).

[15] J. B. CHAPPELL, *Biochem. J.* **90,** 225 (1964).
[16] N. A. FRIGERIO and H. A. HARBURY, *J. Biol. Chem.* **231,** 135 (1958)
[17] E. LAYNE, in *Methods in Enzymology* (edited by S. P. COLOWICK and N. O. KAPLAN), Vol. III, p. 447, Academic Press, New York (1957).