The Fluorimetric Microdetermination of Glyoxylic Acid in Blood, Urine and Bacterial Extracts

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1. A spectrophotofluorimetric method for the determination of glyoxylic acid in biological materials is described. 2. The method is based on the reaction between glyoxylic acid and resorcinol in acid solution, a fluorescent complex being obtained on the subsequent addition of alkali. 3. The reaction was found to be sensitive and highly specific, the minimum detectable amount of glyoxylic acid being 1.35×10^{-8} mole. 4. The urinary excretion of glyoxylic acid by ten normal adults ranged from 1.4 to 4.7 mg./24 hr. Small but measurable amounts of glyoxylic acid were found in cell-free extracts of *Pseudomonas oxalaticus* OX1 grown on oxalic acid as a source of carbon. No glyoxylic acid was detected in human serum.

Existing methods for the determination of glyoxylic acid include the iodometric or potentiometric titration of the bisulphite addition product (Clift & Cook, 1932; McFadden & Howes, 1960). Several colorimetric procedures have been described, for example, the isolation of glyoxylic acid 2,4-dinitrophenylhydrazone (which yields a coloured solution on the addition of strong alkali) (Friedemann & Haugen, 1943), the formation of 1,5-diphenylformazancarboxylic acid by the mild oxidation of glyoxylic acid phenylhydrazone with potassium ferricyanide (Kramer, Klein & Baseline, 1959), the formation of glyoxylic acid semicarbazone (Olson, 1959) and the reaction of glyoxylic acid with methylamine and *o*-aminobenzaldehyde (Albrecht, Scher & Vogel, 1962). A fluorimetric procedure for the microdetermination of α -oxo acids, including glyoxylic acid, with *o*-phenylenediamine was proposed by Spikner & Towne (1962).

These methods are unsatisfactory for the determination of glyoxylic acid in biological materials because of their low sensitivity or their inability to distinguish this acid from other carbonyl compounds unless separated from other interfering substances. The present method is based on the reaction between glyoxylic acid and resorcinol, first described by Hewitt & Pope (1896).

EXPERIMENTAL

Reagents. All reagents were prepared in water that had first been distilled in glass and then deionized by passage through a mixed-bed ion-exchange resin (Elgastat Deioniser; Elga Products Ltd., Lane End, Bucks.). Carbonate-veronal buffer was prepared as described by King & Delory (1940).

Potassium carbonate-bicarbonate buffer, pH 9.6, was prepared by mixing 200 ml. of 0.1 M- K_2CO_3 and 800 ml. of 0.1 M- $KHCO_3$. Sodium glyoxylate monohydrate was prepared according to the method described by Radin & Metzler (1955). The problems encountered in defining the criteria for purity of glyoxylic acid or its salts have been discussed by McFadden & Howes (1960). The purity of the sodium glyoxylate prepared in this Laboratory and of several commercial preparations of glyoxylic acid was determined by potentiometric titration (McFadden & Howes, 1960) and by the colorimetric procedure described by Kramer *et al.* (1959). The purity of the sodium glyoxylate preparation was found to be 98.5%, whereas the average purity of the commercial glyoxylic acid preparations was 96.5%. The lower values obtained with glyoxylic acid appeared to be due, at least in part, to the hygroscopic nature of the acid. Sodium glyoxylate has the advantage of being non-hygroscopic.

Apparatus. Fluorescence was measured in an Aminco-Bowman spectrophotofluorimeter (American Instrument Co. Ltd., U.S.A.) by using 10 mm. fused quartz cells, and the following instrumental parameters were used unless stated otherwise: slit arrangement, no. 3; meter multiplier switch, position 1; sensitivity-control reading, 50; activation wavelength, 490 m μ ; fluorescence wavelength, 530 m μ . Extinction was measured at 490 m μ in a model SP.600 spectrophotometer (Unicam Instruments Ltd.). Exelo glass-stoppered graduated test tubes were used for most of the operations, the tubes being checked for accuracy before use.

Preparation of serum and urine samples. Samples of serum or urine (2 ml.) were mixed with 4 ml. of N-HCl in a 10 ml. stoppered tube. The tubes were heated in a boiling-water bath for 2 min., then centrifuged at 1200g for 10 min. For serum, 3 ml. of supernatant fluid was taken for analysis. For urine, 1.5 ml. of supernatant fluid was mixed with 1.5 ml. of N-HCl.

Preparation of bacterial extract. *Pseudomonas oxalaticus* OX1, grown on a nutrient-agar slope, was kindly supplied by Dr J. R. Quayle, Department of Biochemistry, Univer-

sity of Sheffield. For the present experiment the organism was grown on a liquid medium containing potassium oxalate (Jayasuriya, 1955). Samples were analysed immediately after inoculation and after incubating at 37° for 48 hr. The packed-cell volume obtained after culture and centrifugation was 1 ml. The whole sample was suspended in water (1 ml.) and an extract was prepared by disrupting the bacteria in a Mullard-MSE ultrasonic cell disintegrator (19 kcy./sec. at 75 w for 5 min. at 0°). The whole sample was adjusted to pH 1.0 with N-HCl and centrifuged at 1500g at 0° for 20 min. The cell-free extract (1 ml.) was deproteinized by mixing with 3% (w/v) metaphosphoric acid (1 ml.) and centrifuged at 1200g for 10 min. (Dekker & Maitra, 1962). The supernatant fluid was decanted into a 25 ml. glass-stoppered tube and the volume was made up to 3 ml. with water.

Preparation and stability of standard solutions. A standard solution of sodium glyoxylate (67.5 μM) was prepared in N-HCl. This solution, which contained 5 μg . of glyoxylic acid/ml., was stable for 7 days at 4°. A series of six tubes was prepared containing 0–10 μg . of glyoxylic acid in multiples of 2 μg ., the volumes being made up to 3 ml. with N-HCl.

Analytical procedure. A sample (3 ml.) of deproteinized serum, urine, bacterial extracts or standards was mixed with 0.1 ml. of 5% (w/v) resorcinol and 1.5 ml. of conc. HCl in a 10 ml. stoppered tube and the mixture was heated on a boiling-water bath for 5 min. After cooling, isobutanol (5 ml.) was added and the tube was shaken for 2 min. and centrifuged. The isobutanol phase was transferred to a 25 ml. stoppered tube, 10 ml. of 25% (w/v) K_2CO_3 was added and the tube was shaken again for 2 min. After centrifuging, the aqueous phase was transferred to another 25 ml. stoppered tube, 1 ml. of 10% (w/v) ascorbic acid was added and

the volume was made up to 25 ml. with carbonate-bicarbonate buffer. After mixing, the solution was allowed to stand for 30 min. and the fluorescence intensity or extinction was measured. (The extinction may be measured if the amount of glyoxylic acid is greater than 5 μg ./sample.) Fluorescence and colour intensity were stable for at least 24 hr.

RESULTS

Emission and absorption spectra. The glyoxylic acid-resorcinol complex was colourless and without fluorescence in acid solution, but it exhibited a greenish-yellow fluorescence in alkaline solution. The fluorescence-excitation and emission spectra are shown in Fig. 1. Maximum excitation and emission were observed at 490 and 530 $m\mu$ respectively. Maximum extinction was identical with the excitation maximum, i.e. 490 $m\mu$ (ϵ 1.63 $\times$ 10³). A linear relationship was observed between the amount of glyoxylic acid in the test solution and the intensity of fluorescence up to 10 μg ./sample (Figs. 2 and 3). The relation between extinction and concentration of glyoxylic acid was linear within the range 0–225 μM .

Optimum concentration of resorcinol. The coloured complex was developed with 10 μg . of glyoxylic acid and concentrations of resorcinol reagent varying from 1 to 50% (w/v). The maximum emission of fluorescence was observed with 5% (w/v) resorcinol (Table 1). Maximum fluorescence did not coincide

Fig. 1. Fluorescence-excitation and emission spectra of the glyoxylic acid-resorcinol complex. ○, Excitation spectrum; ●, emission spectrum.

Fig. 2. Relation between fluorescence intensity and the amount of glyoxylic acid at high concentrations (sensitivity-control reading set at zero). The abscissa indicates μg . of glyoxylic acid in the sample taken for analysis.

Fig. 3. Relation between fluorescence intensity, extinction and the amount of glyoxylic acid (sensitivity-control reading set at 50). The abscissa indicates $\mu\text{g.}$ of glyoxylic acid in the sample taken for analysis. ●, Fluorescence intensity; ▲, extinction (E_{490}).

Table 1. *Effect of resorcinol concentration on the fluorescence intensity and extinction of the glyoxylic acid-resorcinol complex*

Resorcinol solution (0.1 ml. volumes) in the concentrations listed in column 1 was added to 10 $\mu\text{g.}$ samples of glyoxylic acid in 3 ml. of solution and the colour was developed in the usual way.

Concn. of resorcinol (g./100 ml.)	Fluorescence intensity	Extinction (E_{490})
1	37	0.11
5	79	0.26
10	74	0.28
20	71	0.32
30	68	0.30
40	65	0.28
50	61	0.26

with maximum extinction, probably because of fluorescence-quenching at higher concentrations of resorcinol.

Optimum concentration of hydrochloric acid. The coloured complex was developed with 10 $\mu\text{g.}$ of glyoxylic acid in 3 ml. of solution and various volumes of concentrated hydrochloric acid. Maximum fluorescence was obtained when the ratio of concentrated hydrochloric acid to test solution was 1:2 (v/v) (Table 2).

Effect of duration of heating. The coloured complex was developed with various times of heating in a

Table 2. *Effect of hydrochloric acid concentration on the conjugation of glyoxylic acid and resorcinol*

Conc. HCl in the volumes listed in column 1 was added to 10 $\mu\text{g.}$ samples of glyoxylic acid in 3 ml. of solution, the final concentration of HCl being listed in column 2. The colour was then developed in the usual way.

Vol. of conc. HCl (ml.)	Concn. of HCl (% w/v)	Fluorescence intensity	Extinction (E_{490})
0.5	5.9	32	0.47
1.0	11.8	41	0.95
1.5	17.7	41	1.12
2.0	23.6	40	1.20
2.5	29.5	39	1.25
3.0	35.4	38	1.30

Table 3. *Effect of duration of heating on the conjugation of glyoxylic acid and resorcinol*

The colour was developed in the usual way with 10 $\mu\text{g.}$ samples of glyoxylic acid in 3 ml. of solution, but the duration of heating was varied as indicated in column 1.

Duration of heating (min.)	Fluorescence intensity	Extinction (E_{490})
1.0	17	0.22
2.5	34	0.73
5.0	41	0.98
10.0	38	0.98
15.0	36	0.96
20.0	36	0.90

Table 4. *Effect of different alkali reagents on the fluorescence intensity and extinction of the glyoxylic acid-resorcinol complex*

The glyoxylic acid-resorcinol mixture was prepared in the usual way with 10 $\mu\text{g.}$ of glyoxylic acid. After heating, the solution was made alkaline by the addition of 10 ml. of 2N solutions of the substances listed in column 1.

Reagent	Fluorescence intensity	Extinction (E_{490})
Sodium hydroxide	7.2	0.08
Ammonia solution	32.8	0.11
Sodium carbonate	70.5	0.25
Potassium carbonate	75.0	0.26

boiling-water bath. Maximum fluorescence was obtained after heating for 5 min. (Table 3).

Effect of different alkalis. A comparison of the fluorescence intensity of the glyoxylic acid-resorcinol complex was made with different alkali reagents. Equal volumes of alkali (10 ml.) of equal concentration (2N) were added to the glyoxylic acid-resorcinol mixture and the colour was de-

Fig. 4. Relation between the fluorescence intensity of the reagent 'blank' and the concentration of ascorbic acid.

veloped as described above. Potassium carbonate was found to be the reagent of choice (Table 4).

Colour stability. The coloured complex was unstable in the absence of an electron donor such as ascorbic acid, the colour changing from light yellow to dark brown with an appreciable loss of fluorescence. In contrast, the fluorescence intensity and extinction of the reagent 'blank' increased progressively with time. The fluorescence of the reagent 'blank' was diminished by the addition of ascorbic acid, the fluorescence decreasing asymptotically with increasing concentration of ascorbic acid (Fig. 4). The fluorescence and extinction of the glyoxylic acid-resorcinol complex were constant for at least 24 hr. when the concentration of the ascorbic acid reagent was 0.55 M (10%, w/v) or higher.

Effect of pH. The effect of pH on the fluorescence of the coloured complex was investigated by replacing the carbonate-bicarbonate buffer by a series of carbonate-veronal buffers (pH 7–11). Glyoxylic acid (20 μ g.) in N-hydrochloric acid (2 ml.) was conjugated with resorcinol in the usual way, after which the reaction mixture was adjusted to pH 7.0 by the addition of potassium carbonate (25%, w/v). The required pH was obtained by diluting the reaction mixture to 15 ml. with the appropriate carbonate-veronal buffer. The colour was then developed as described in the Experimental section under 'Analytical procedure'. Maximum fluorescence was observed between pH 9 and 10 (Fig. 5). Similar results were obtained with a series of potassium carbonate-bicarbonate buffers, but fluorescence emission was slightly higher with this reagent than with carbonate-veronal buffer.

Reproducibility and specificity. Replicate determinations were performed with 10 μ g. samples of glyoxylic acid in water. The standard deviation of 16 determinations of glyoxylic acid was 0.28 μ g. (s.e.m. 0.07 μ g.). The fluorescence intensity of a number of naturally occurring compounds, structurally or metabolically related to glyoxylic acid, was

Fig. 5. Relation between the fluorescence intensity of the glyoxylic acid-resorcinol complex and the pH of the fluorescent solution.

Table 5. Glyoxylic acid content of human serum and urine, and cell-free extracts of *P. oxalaticus*

Description	No. of samples	Glyoxylic acid
Serum	10	Not detectable
Urine	10	1.4–4.7 mg./24 hr.
Cell-free extract:		
(a) Before culture	2	Not detectable
(b) After culture	2	5.0–6.0 μ g.

investigated with approximately equimolar quantities of each compound. Glyoxal was the only compound that produced a detectable fluorescence, and the molar extinction coefficient ($\epsilon 1.16 \times 10^2$) was less than one-tenth that of glyoxylic acid ($\epsilon 1.63 \times 10^3$). The other compounds investigated were sodium pyruvate, α -oxoglutaric acid, α -oxobutyric acid, oxaloacetic acid, formaldehyde, sodium formate, formic acid, oxalic acid, glycollic acid, citric acid, malic acid, lactic acid, succinic acid, tartaric acid, malonic acid, acetic acid, salicylic acid and chloral hydrate.

Glyoxylic acid content of serum, urine and cell-free extracts of *P. oxalaticus*. The results of a number of analyses of human serum and urine, and of bacterial extracts of *P. oxalaticus*, are summarized in Table 5. No glyoxylic acid was detected in serum. The recovery of glyoxylic acid (5 and 10 μ g.) added to serum (2 ml.) varied between 90 and 94%. The recovery of glyoxylic acid (10 μ g.) from urine was 92–96%.

DISCUSSION

Since biological materials contain many compounds having structural similarities to glyoxylic acid it is essential that any method for estimating this acid should be highly specific. Specificity may

be achieved by a preliminary separation of glyoxylic acid from other interfering substances, e.g. by the use of an anion-exchange resin (Zelitch, 1958) or by extraction with suitable solvents (Buckle, 1963), but such procedures are time-consuming. A high degree of specificity was achieved in the present method by utilizing the reaction between glyoxylic acid and resorcinol; this reaction also has the advantage of high sensitivity. With the present procedure, the minimum detectable amount of glyoxylic acid was approx. $1.0\text{ }\mu\text{g}$. (1.35×10^{-8} mole).

Hewitt & Pope (1897) suggested that the product formed by the reaction of glyoxylic acid with resorcinol is a lactone of 2,2',4,4'-tetrahydroxydiphenylacetic acid (I). Experimental evidence in

support of this structure has been presented by Vièles & Badré (1947).

The fact that the reaction product was colourless in acid solution and showed a decreased fluorescence in strongly alkaline solutions is in agreement with the general properties of dihydroxybenzenes; these compounds fluoresce when in the un-ionized state. Thus Williams (1959) observed that ionization of the first hydroxyl group of resorcinol occurs between pH 10 and 11, the intensity of fluorescence diminishing as the pH of the solution is increased above pH 10.

The choice of deproteinizing agent was found to be important. Dekker & Maitra (1962) reported poor recoveries of glyoxylic acid after deproteinization with trichloroacetic acid or perchloric acid or by boiling. Recovery of glyoxylic acid was almost complete, however, after deproteinization with 3% (w/v) metaphosphoric acid, and this reagent was used in the present study for the deproteinization of cell-free extracts of *P. oxalaticus*. We confirmed that deproteinization by boiling resulted in incomplete recovery of glyoxylic acid, but almost complete recovery was obtained if the solution was acidified with hydrochloric acid before boiling. This procedure was used for the deproteinization of serum and urine, and it was found to be equally suitable for the treatment of cell-free extracts of *P. oxalaticus*. Deproteinization by ultrafiltration through Visking membranes was found to be unsatisfactory, since these membranes acquire a negative charge in water (Gilbert & Swallow, 1950) and this retards the diffusion of anions. Further, accumulation of H^+ ions within the membrane may

occur (Markham & Smith, 1952), with resultant partial hydrolysis of glyoxylic acid.

Extraction of the glyoxylic acid-resorcinol complex with isobutanol was found to be essential for serum and urine samples to separate the complex from interfering substances that combine with the excess of resorcinol present, forming pinkish-brown products. Approx. 98% of the glyoxylic acid-resorcinol complex was recovered by one extraction with this solvent. Although extraction with isobutanol was employed for *P. oxalaticus* preparations, this step is not essential since identical results were obtained when the extraction was omitted.

Since glyoxylic acid is a relatively toxic substance in animals (Barnes & Lerner, 1943), its concentration in the blood would be expected to be very low in normal human subjects. Several workers have failed to detect glyoxylic acid in human blood (Biserte & Dassonville, 1956; van der Horst, 1960; Buckle, 1963). However, its presence on paper chromatograms has been reported by Kaser (1961). No glyoxylic acid was detected in serum by using the procedure recommended in the present paper. The use of larger samples of serum (10ml.) also yielded negative results. It is concluded that if glyoxylic acid is present in normal human serum the concentration is probably less than $10\text{ }\mu\text{g}/100\text{ml}$. Liang (1962) determined the glyoxylic acid content of a number of tissues in the rat and found detectable amounts only in the kidneys and liver ($40\text{ }\mu\text{g}$. and $10\text{ }\mu\text{g}/100\text{g}$. fresh wt. of tissue respectively).

The presence of glyoxylic acid in human urine was demonstrated by van der Horst (1960) and Henning & Ammon (1957). Hockaday, Clayton, Frederick & Smith (1964), using a new isotope-dilution method, found that the excretion of glyoxylic acid by normal adults ranged from 2.2 to 6.0mg./day. This agrees with the present findings (Table 5).

Quayle, Keech & Taylor (1961), using the 2,4-dinitrophenylhydrazine method, reported that only traces of glyoxylate were formed when *P. oxalaticus* was grown in the presence of oxalic acid as a source of carbon. Small but measurable amounts of glyoxylic acid were observed in the present study (Table 5).

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