grouping, to the diol. Hydroxypyruvic acid in aqueous solution might therefore be expected to have a potential ability to react in either of the two forms

$CH_2(OH)\cdot CO\cdot CO_2H$ and $CH_2(OH)\cdot C(OH)_2\cdot CO_2H$.

Experimental. Spectra were measured with a Grubb Parsons S. 3A double-beam spectrometer with a sodium chloride prism. Samples were examined as capillary films or as thin mulls in paraffin and in fluorinated paraffin oil.

SUMMARY

1. The infrared-absorption spectra of pyruvic acid, hydroxypyruvic acid monohydrate and their monohydrated lithium salts are reported.

2. Pyruvic acid can be regarded as a normal α-keto acid except for features in its spectrum suggesting intramolecular bonding.

3. Lithium pyruvate monohydrate has a quite different spectrum, no ketonic carbonyl absorption being detectable but CO_2^- stretching bands are well marked, as well as bands characteristic of strongly bonded hydroxyl groups. The spectrum is consistent with the formulation $CH_3\cdot C(OH)_2\cdot CO_2^-Li^+$ and not with $CH_3\cdot CO\cdot CO_2^-Li^+,H_2O$.

4. The spectra of the hydrates of hydroxypyruvic acid and of its lithium salt are parallel with the above, and indicate that whereas the free acid retains its ketonic character in the solid state and the hydration is present as water of crystallization, the lithium salt shows no ketonic carbonyl absorption and its spectrum is consistent only with the structure $CH_2(OH)\cdot C(OH)_2\cdot CO_2^-Li^+$.

We are extremely grateful to Professor F. Dickens for making these interesting materials available to us for study.

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The Determination of Hydroxypyruvate and Glycolaldehyde

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In order to investigate the biological occurrence and function of hydroxypyruvate, suitably sensitive methods for its determination are essential. Hitherto the only qualitative colorimetric reaction described for this substance has been the purple colour with alkaline ferric salts (Fenton & Jones, 1900), but this proved unsuitable for quantitative purposes and similar colours are given by other hydroxyl compounds. The lack of a stable solid preparation of the acid or its salts, for use as an analytical standard, has been overcome by the preparation of the monohydrates of lithium hydroxypyruvate and hydroxypyruvic acid (Dickens & Williamson, 1958). The analytically pure recrystallized lithium salt, $CH_2(OH)\cdot C(OH)_2\cdot CO_2Li$, provides the most suitable standard. By the use of this material a satisfactory colorimetric assay has been developed, based upon the grass-green colour produced on heating hydroxypyruvate solutions with naphtharesorcinol in 23 N-sulphuric acid (Dickens & Williamson, 1956*a*). Glycolaldehyde gives the same colour, but may be distinguished by its non-absorption on a mixed-bed resin, as is described below.

A specific enzymic reaction of hydroxypyruvate, which like the above reaction clearly distinguishes it from pyruvate, has been described by Stafford, Magaldi & Vennesland (1954). In the presence of a hydroxypyruvate reductase extracted from parsley leaves, reduction of hydroxypyruvate by reduced diphosphopyridine nucleotide (DPNH) can be followed photometrically. This stoicheiometric reaction has been used to check the purity of our preparations of hydroxypyruvate (Dickens & Williamson, 1958), and its application to determinations in biological material is described below.

In solutions containing both pyruvate and hydroxypyruvate, the total keto acid present may be determined by the oxidation of DPNH in the presence of L-lactic dehydrogenase of muscle, since this enzyme reacts equally well with both substrates (Meister, 1952).

A combination of these various methods is therefore capable of wide application to the analysis of various biological systems.

EXPERIMENTAL

Colorimetric assay of hydroxypyruvate and glycolaldehyde

Solutions. Naphtharesorcinol (L. Light and Co. Ltd.) was recrystallized from 20 vol. of benzene, with a little charcoal added. A solution (5 mg./ml.) in ethanol was freshly prepared before use, and usually had a very faint pink colour.

Sulphuric acid solution (27 N) was prepared by cautiously adding 388 ml. of analytical-grade H_2SO_4 (sp.gr. 1·84) to 147 ml. of water.

As standards, mM-solutions of recrystallized lithium hydroxypyruvate (Dickens & Williamson, 1958) and glycolaldehyde (L. Light and Co. Ltd.) were prepared just before use.

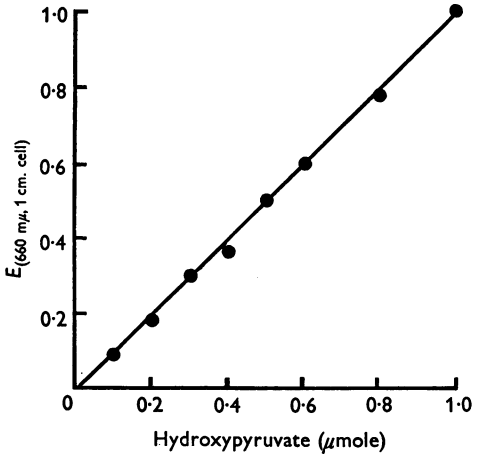

Fig. 1. Calibration curve at 660 mμ of naphtharesorcinol reaction for hydroxypyruvate. The curve for glycolaldehyde is superimposable. The amounts shown are μmoles of lithium hydroxypyruvate in the standard test.

Table 1. *Analysis of mixtures containing hydroxypyruvate and glycolaldehyde*

The quantities are μmoles/5 ml. of solution, weighed as lithium hydroxypyruvate monohydrate and glycolaldehyde respectively. Glycolaldehyde was determined by colorimetric estimation of the portion not adsorbed on mixed-bed resin and hydroxypyruvate by difference, colorimetry on the mixture being used for total μmoles of both substances present (see text).

Amount taken (μmoles)		Found (μmoles)	
Hydroxypyruvate	Glycolaldehyde	Hydroxypyruvate	Glycolaldehyde
2·5	1·0	2·6	0·9
2·5	2·5	2·6	2·6
2·0	2·0	1·9	2·1
1·0	1·0	1·0	0·9

Procedure. The solutions containing hydroxypyruvate (1 ml. containing 0·1–1·0 μmole) in glass-stoppered test tubes are mixed with 0·1 ml. of naphtharesorcinol reagent. A reagent blank (1 ml. of water) and suitable standards (1 ml.) are also similarly treated. The H_2SO_4 reagent (6 ml.) is slowly added to each tube and the contents are well mixed by shaking the stoppered tubes, which are then immersed in a boiling-water bath for 20 min. They are cooled in running water and the density at 660 mμ is read in a spectrophotometer (1 cm. cells). Unless the colour is a clear green the estimation should be rejected, as the presence of interfering substances (see below) is indicated.

With biological material, deproteinization with trichloroacetic acid (3%, w/v) is an essential preliminary, the acidic filtrate being used directly without neutralization. The method cannot be applied to solutions containing much free carbohydrate.

A typical calibration curve is shown in Fig. 1. That obtained for glycolaldehyde, in the same molar amounts, is superimposable on this graph.

Determination of glycolaldehyde and hydroxypyruvate when both are present. The formation of a green colour immediately on mixing with the H_2SO_4 reagent in the above test, before heating, indicates the probable presence of free glycolaldehyde. The final colour developed after heating for 20 min. then gives the total amount (μmoles) of hydroxypyruvate and glycolaldehyde present.

In order to determine the relative amounts of each substance, the hydroxypyruvate is adsorbed by shaking the solution with the mixed-bed ion-exchange resin Bio-Deminrolit G (The Permutit Co. Ltd.) previously found (Dickens & Williamson, 1956*b*) to give maximum recovery of neutral reducing sugars. This has the further advantage that the resulting deionized solutions are excellent for use in paper chromatography (cf. Dickens & Williamson, 1958).

Procedure. The acidic solution (*A*) containing hydroxypyruvate and glycolaldehyde (0·5–10 μmoles of each, in 5 ml.) is shaken in a stoppered test tube with 2 g. of Bio-Deminrolit G until the pH of the liquid, tested with indicator paper, reaches approx. 4. The resin is removed by filtration (filtrate = solution *B*) with slight air pressure on a small sintered-glass disk (porosity 1, 1 cm. diam. sealed into a 10 cm. glass tube). The naphtharesorcinol reaction is carried out on solutions *A* and *B*, as already described.

In practice, whereas the hydroxypyruvic acid is nearly quantitatively absorbed by the resin (99% of 50, 98% of 10 and 95% of 2·5 μmoles), the recovery of glycolaldehyde is consistently between 76 and 83%, mean 80%. Consequently the values found for glycolaldehyde by this method require to be multiplied by a factor of 1·25. Table 1 shows the resulting analysis of the mixed substances.

Notes on the colorimetric method

Absorption spectra of the colorimetric solutions. The absorption spectrum given by hydroxypyruvate is shown in Fig. 2. That given by glycolaldehyde is superimposable, within the experimental limits, suggesting that the same chromogen is concerned. The well-defined absorption maximum is at 660 mμ.

Strength of sulphuric acid used (differential analysis of hydroxypyruvate and glycerate). The strength of acid used affects both the specificity of the method

and the colour developed. In the test described above, the final solution is 23 N in H_2SO_4. When nearly concentrated (35 N) sulphuric acid is used instead, with the minimum practical volumes of aqueous solution and of naphtharesorcinol reagent, only a faint yellow–green is given by hydroxypyruvate. These are approximately the conditions, however, of the Rapoport (1936) method for estimation of glyceric acid, which gives a clear prussian-blue colour suitable for colorimetric estimation. (The specificity of the Rapoport reaction is discussed by Feigl, 1954.)

In 23 N-H_2SO_4, however, with naphtharesorcinol under the standard conditions already described, glyceric acid (10 μmoles) gives zero density at 660 mμ, no trace of blue or green colour being formed. Hence, by simultaneous determinations of the colour given with naphtharesorcinol in nearly concentrated and in 23 N-H_2SO_4, the differential analysis of glycerate and hydroxypyruvate is possible. This principle has been successfully used to follow the enzymic transformation of hydroxypyruvate into glycerate by means of cytochrome b_2 of yeast (Dickens & Williamson, 1956*a*). A period of heating at 100° for 1 hr. is suitable for the estimation of glyceric acid, whereas 20 min. is sufficient for hydroxypyruvate.

The effect of the normality of H_2SO_4 in the heated reaction mixture (containing hydroxypyruvate and naphtharesorcinol) on the subsequent light absorption at 660 mμ is shown in Fig. 3. The optimum is 23 N, final concentration.

It is worth noting that the concentration of the H_2SO_4 reagent (27 N) happens to be the same as that used in the cysteine–carbazole reaction of Dische & Borenfreund (1951) for ketopentoses, and will serve for both estimations.

Period of heating. The rate of colour development at 100° is such that hydroxypyruvate yields maximum colour development in 15 min. and this is unchanged after 20 min., the standard period adopted. After heating for 3 min., only 10 % of this maximum occurs with hydroxypyruvate, whereas 50 % of the maximum is given in this short period by glycolaldehyde. The difference can be increased by using a 70° colour development (Fig. 4), but is still not really adequate for the accurate differential determination of the two substances.

Specificity. A variety of colours is given by a number of substances when these are tested by the standard procedure (Table 2). In the presence of those substances giving strong colours, the method cannot be applied. Unless purification can be achieved, the enzymic-assay method must be used in such cases. Fortunately, the only substance tested other than hydroxypyruvate and glycolaldehyde, which was found to give a clear-green colour, was dihydroxyfumaric (dihydroxymaleic)

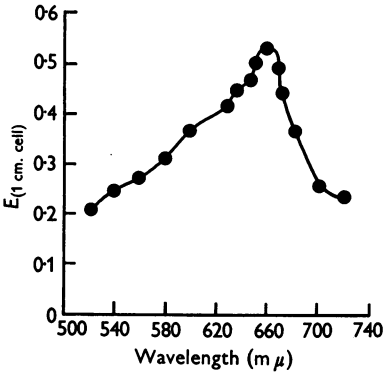

Fig. 2. Absorption spectrum of naphtharesorcinol reaction for hydroxypyruvate. That for glycolaldehyde is superimposable. The amount of either substance taken in the standard test is 0·5 μmole. Peak absorption is at 660 mμ.

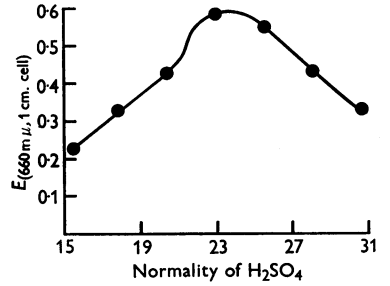

Fig. 3. Effect of normality of sulphuric acid in the final reaction mixture on naphtharesorcinol test for hydroxypyruvate. Lithium hydroxypyruvate (0·5 μmole) was used in conditions as in the standard test, except for the varying concentrations of sulphuric acid.

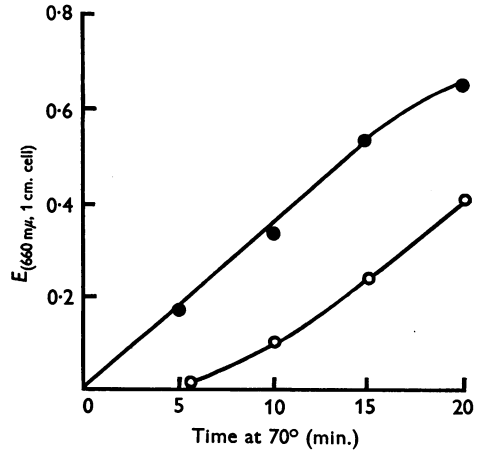

Fig. 4. Rate of colour development at 70°. Conditions were as in the standard naphtharesorcinol test, except for temperature (70° instead of 100°) and periods of heating as shown. ○, Hydroxypyruvate (0·5 μmole); ●, glycolaldehyde (0·5 μmole).

Table 2. *Specificity of the naphtharesorcinol reaction*

All the compounds were tested under standard conditions of the method as described. Sources of material: A, authors' preparation; B, British Drug Houses Ltd., Poole, Dorset; R, Roche Products Ltd., 15 Manchester Square, London, W. 1; Sch, Schering A.G., Berlin; Si, Sigma Chemical Company, St Louis, Mo., U.S.A. The phosphohydroxypyruvic acid was a gift from Dr C. E. Ballou provided through the kindness of Dr D. D. Davies.

Compound	Amount (μmoles)	Final colour	$D_{660}^{1\,cm.}$	Source
Xylose	1	Cherry-red	0·310	R
Xylulose	1	Faint pink	0·110	A
Ribose	1	Strong violet	0·400	R
Ribulose	1	Brownish red	0·300	A
Erythrose	2	Grey–green	0·190	A
Erythrulose	2	Grey–green	0·195	A
Glyceric acid	2	None	0·000	A
Glycollic acid	2	None	0·000	B
Glyceraldehyde	2	Strong grey	0·980	Sch
Fructose	2	Brown with greenish fluorescence	0·780	B
Glucose	2	Brown with greenish fluorescence	0·740	B
Glucuronic acid	2	Brownish red	0·570	Si
Ascorbic acid	1	Faint pink	0·075	B
Pyruvic acid	10	Faint yellow	0·012	R
Glyoxylic acid	2	Orange–brown	0·150	A
Formaldehyde	1	Olive–brown	0·125	B
Dihydroxymaleic (dihydroxyfumaric) acid	0·5	Green	0·290	A
Glycolaldehyde	0·5	Strong green	0·610	A
Hydroxypyruvic acid	0·5	Strong green	0·600	A
Phosphohydroxypyruvic acid	0·5	Strong green	0·580	Gift

acid. It is probable that on heating with H_2SO_4 of this strength both acids are decarboxylated to give glycolaldehyde.

Glyoxylic acid shows a well-defined maximum absorption at 480 mμ in the standard test (1 μmole gives a density of 0·8 at 480 mμ compared with only 0·1 at 660 mμ). Consequently either glyoxylic or hydroxypyruvic acid may be estimated by comparison of the densities at these two wavelengths, provided that the other substance is not present in large excess.

Enzymic assay of hydroxypyruvate

The colorimetric method is quick and convenient, but is applicable only when the amounts of interfering substances present are inconsiderable. If this is not so, and also where high specificity is needed, the photometric estimation of reoxidation of DPNH by means of the hydroxypyruvate reductase of Stafford *et al.* (1954) is preferable. This enzyme causes the reduction of hydroxypyruvate to glycerate:

$$CH_2(OH)\cdot C(OH)_2\cdot CO_2^- + DPNH + H^+ \rightarrow CH_2(OH)\cdot CH(OH)\cdot CO_2^- + DPN^+ + H_2O.$$

The reaction is virtually irreversible except at alkaline pH with high glycerate concentration. Although Sallach (1956) has recently used this reaction to measure changes in hydroxypyruvate in the L-serine–L-alanine transamination system, no detailed description of the assay of hydroxypyruvate by this useful method appears to have been published hitherto.

Materials. Diphosphopyridine nucleotide (enzymically reduced form, 85% DPNH), was purchased (Boehringer und Soehne, Mannheim). A 0·5% solution (10 ml.) is adjusted to pH 8 with dilute NaOH and stored at −15°.

Phosphate buffer, 0·1 M, pH 7·2, is prepared by mixing 3 vol. of 0·1 M-KH_2PO_4 with 7 vol. of 0·1 M-K_2HPO_4.

Hydroxypyruvate reductase (cf. Stafford *et al.* 1954). Parsley leaves are washed, frozen at −15° overnight and minced in a domestic mincer. The juice is expressed (40 ml. from 100 g. of mince), and centrifuged. To the decanted clear fluid (30 ml.) saturated $(NH_4)_2SO_4$ soln. (45 ml.) is added. After several hours at 0° the precipitate is collected and the precipitation with $(NH_4)_2SO_4$ is repeated. Finally, the precipitate is dissolved in 12 ml. of 0·01 M-phosphate, pH 7·4. Stored at −15°, the solution remains active for months.

Method. Solutions for analysis are first deproteinized, when necessary, by trichloroacetic or perchloric acid, followed by cautious neutralization with dilute alkali.

Into a 1 cm. silica cell are measured 1 ml. of phosphate buffer, 0·1 ml. of DPNH, the test solution (or hydroxypyruvate, 0·05–0·4 μmole, as standard), and water to make 3 ml. total volume. Two other cells are simultaneously set up, one a control with all reagents except hydroxypyruvate and the other a blank with water only (3 ml.). The control and hydroxypyruvate densities are read against the blank at 340 mμ in a Unicam SP. 600 spectrophotometer. Then to each of the three cells hydroxypyruvate reductase (0·02 ml.) is added at zero time. Readings are taken at minute intervals until the rate of decrease of density becomes very slow and equal in the cells with and without hydroxypyruvate (7–10 min.). The total density decrease of the control is subtracted from that of the hydroxypyruvate cell. This value × 0·478 gives the μmoles of hydroxypyruvate present in the cell; this factor is calculated for an extinction coefficient of $6·3 \times 10^6$ cm.^{2}/mole for DPNH.

Table 3. *Determination of hydroxypyruvate by enzymic oxidation of diphosphopyridine nucleotide with hydroxypyruvate reductase*

Amounts are μmoles of hydroxypyruvate, weighed as the lithium salt monohydrate, contained in 3·02 ml. in 1 cm. spectrophotometer cells. For details see text.

Amount taken (μmole)	Found (μmole)	Recovery (%)
0·10	0·095	95·5
0·20	0·195	98
0·30	0·308	102·5
0·40	0·385	97
	Mean	98

Notes on the enzymic method

Specificity. Pyruvate is reduced at less than 2 % of the rate of an equimolar amount of hydroxypyruvate by this preparation of the enzyme. Since both pyruvate and hydroxypyruvate rapidly oxidize DPNH in the presence of lactic dehydrogenase of muscle, which is obtainable commercially (Boehringer und Soehne, Mannheim), a differential analysis of pyruvate and hydroxypyruvate in mixtures of the two substances is readily performed by a comparison of the density decrease obtained with these two enzymes either separately or consecutively in the same solution.

Recovery of hydroxypyruvate. When recrystallized lithium hyproxypyruvate monohydrate (Dickens & Williamson, 1958) was used as a standard, the recovery was quantitative within the experimental limits of ± 5 % (Table 3).

SUMMARY

1. A colorimetric method for the determination of hydroxypyruvate and glycolaldehyde is described. The intensity of the green colour obtained on heating the solution with naphtharesorcinol in 23 N-H_2SO_4, measured at 660 mμ, is proportional to the amount of these substances present under the defined conditions of the test.

2. When both substances are present together the estimation is repeated after adsorption of hydroxypyruvic acid on Bio-Deminrolit G.

3. The specificity of the test has been studied, and certain interfering substances are noted. Modifications permitting the estimation of glyceric acid and of glyoxylic acid are indicated.

4. The use of hydroxypyruvate reductase from parsley leaves and lactic dehydrogenase of muscle for enzymic assay of hydroxypyruvate is described. Whereas the former enzyme reacts only with hydroxypyruvate, the latter reduces pyruvate also and the two enzymes may therefore be used in conjunction for quantitative analysis of mixtures of these two keto acids.

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The Degradation of Cartilage Chondroitin Sulphate by the Chondroitinase of *Proteus vulgaris*

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Dodgson, Lloyd & Spencer (1957) have shown a strain of *Proteus vulgaris* (National Collection of Type Cultures, no. 4636) to be a particularly potent source of two enzymes, chondroitinase and chondrosulphatase, which are collectively capable of degrading cartilage chondroitin sulphate with release of reducing substances and sulphuric acid respectively. Subsequent work (Dodgson & Lloyd, 1957*a*, *b*) showed that whereas chondroitinase action could proceed independently of the associated chondrosulphatase, the latter enzyme was inactive towards polymer chondroitin sulphate. However, the enzyme was capable of liberating sulphuric acid from the sulphated oligosaccharide fragments which were obtained by degrading cartilage chondroitin sulphate with testicular