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The Determination of Lactic Acid in Microgram Quantities

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The conversion of lactic acid to acetaldehyde by hot concentrated sulphuric acid was first reported by Denigès (1909) and its use as a basis for the determination of lactic acid was described by Mendel & Goldscheider (1925). Eegriwe (1933) described a reaction between *p*-hydroxydiphenyl and acetaldehyde to yield a violet colour and this colour reaction was first adopted as a sensitive means of estimating lactic acid by Miller & Muntz (1938).

The method most widely employed for determining lactic acid on a microscale is that of Barker & Summerson (1941). In the course of work in this laboratory requiring the estimation of very small amounts of lactic acid, usually in the presence of pyruvate, reproducible results could not be achieved with this technique; further, the final optical densities were not directly proportional to the amounts of lactic acid present.

This paper contains the results of our investigations whereby several modifications of Barker & Summerson's procedure were made. With these changes, 1–8 μg. of lactic acid could be determined with an accuracy of $\pm 2\%$. When interfering pyruvic acid is present, the procedure involves a dilution so that the method in this case is applicable to 2–10 μg. lactic acid.

METHODS

Reagents

p-Hydroxydiphenyl (1·5 g.) is dissolved in 10 ml. of 5% (w/v) NaOH and diluted to 100 ml. with water.

Standard lactic acid solution: 0·2133 g. of pure, dry lithium lactate (Hillig, 1937), was dissolved in about 100 ml. of water, 1 ml. of concentrated H_2SO_4 (A.R.) added and the solution made up to 1 l. with water.

Recommended method

The protein-free solution (2 ml.) containing 10–80 μg. of lactic acid, is pipetted into a 150 × 25 mm. test tube which contains 1 ml. of 20% (w/v) $CuSO_4$, $5H_2O$ and the final volume is made up to 10 ml. with water. Approximately 1 g. of solid $Ca(OH)_2$ is added and, after thoroughly mixing and allowing to stand for 30 min. or more, the solution is centrifuged.

If the original 2 ml. of protein-free solution contains 100–600 μg. of pyruvic acid, 6 ml. of the supernatant solution from the first treatment with copper-lime is pipetted into a second tube containing 0·6 ml. of 20% (w/v) $CuSO_4$, $5H_2O$ and 0·6 g. of solid $Ca(OH)_2$ added. This procedure is then repeated a third time with 3 ml. of supernatant solution, 0·3 ml. of 20% $CuSO_4$, $5H_2O$ and 0·3 g. of $Ca(OH)_2$, whereby the amount of pyruvic acid present is reduced to a minimum and the colour interference due to it becomes a small constant value which may be deducted from the final optical density.

1 ml. of the supernatant solution from the third copper-lime treatment is transferred to another 150 × 25 mm. test tube, held in the arm of a mechanical shaker with its lower end immersed in an ice-water mixture. $CuSO_4$, $5H_2O$ (0·05 ml. of 12% (w/v) solution) is added followed by 6 ml. of conc. H_2SO_4 (A.R.) dropwise from a burette with vigorous shaking, the tap of the burette being lubricated with the concentrated acid. After complete addition, the contents are poured and allowed to drain into a small ground-glass stoppered Pyrex tube which is then heated for 30 min. in a water bath maintained at $60 \pm 1°$. The tube is allowed to

cool to 10–15°, the stopper removed, 0·1 ml. of the *p*-hydroxydiphenyl reagent added and the precipitated *p*-hydroxydiphenyl thoroughly dispersed in the H_2SO_4. Incubation of the tube for 20 min. at 28–30° to ensure maximum colour development is then followed by 90 sec. in a boiling-water bath to destroy excess *p*-hydroxydiphenyl. After this treatment the tube is immediately cooled in ice-water and the optical density of the resulting violet-coloured solution is determined in circular tubes of 1 cm. diameter with the Unicam diffraction-grating spectrophotometer at a wavelength peak of 560 mμ. against a reagent blank prepared by taking distilled water through the whole of the above procedure.

The calibration curve obtained by this method ($E = \mu g.$ lactic acid × 0·059) for 0–8 μg. of lactic acid is a straight line.

DEVELOPMENT OF METHOD

The effect of varying the experimental conditions at different stages of the method is described in Table 1. The absorption curve of the acetaldehyde-*p*-hydroxydiphenyl complex shows a sharp maximum at 560 mμ. as illustrated by the following optical densities obtained from 6 μg. of lactic acid in the conditions described on p. 289 at wavelengths between 520 and 600 mμ.: 520, 0·210; 540, 0·295; 550, 0·330; 560, 0·345; 570, 0·330; 580, 0·305; 600, 0·185.

The calibration curve, which is strictly reproducible, indicates that the coloured complex obeys the Lambert-Beer Law in the concentration range of 1–8 μg. of lactic acid. Above 8 μg. the optical density does not increase in direct proportionality with the amount of lactic acid but falls off slightly. If coloured solutions, obtained from larger quantities of lactic acid (up to 10 μg.), are diluted with H_2SO_4: water (6:1, v/v) the optical densities of the diluted solutions are directly proportional to the amounts of lactic acid originally present.

Specificity of the method

The work for which the method has been used required the determination of lactic acid in the presence of acetoin, butane-2:3-diol, diacetyl and pyruvate. None of the first three of these compounds gives any colour when the method is applied to solutions containing 60 μg. of it and quantitative recoveries of lactic acid are always obtained in the presence of such amounts. However, pyruvic acid, which is commonly present with lactic acid in media from enzymic studies, seriously interferes with the reproducibility of the method unless steps are taken to remove it. This is illustrated by the results collected in Table 2 for pyruvic acid samples without copper-lime treatment.

One copper-lime treatment does not yield reproducible results, although it clearly removes a considerable amount of pyruvic acid. The repetition of the copper-lime procedure seemed a likely way of completely eliminating the interference due to pyruvate and the effect of three successive treatments is also illustrated by the results in Table 2. The third treatment reduces the optical density of the interfering pyruvic acid to a small constant value. Using this technique, recoveries of lactic acid ranging from 98 to 102% may be obtained in mixtures containing 2–10 μg. of lactic acid and 10–60 μg. of pyruvic acid/ml. of final solution;

Table 1. *Effects of varying experimental conditions*

('Colour' means optical density at 560 mμ.)

Stage	Condition varied	Recommended procedure	Effect of other conditions
Oxidation	Heating at 100° in open tube	—	The longer the heating, the less colour obtained
Oxidation	Stoppered and unstoppered tubes	Stoppered tubes	Unstoppered tubes give about 15% less colour
Oxidation	Time of heating at 60°	30 min.	24–65 min. gives maximum colour; <24 min., less colour
Oxidation	Concn. of Cu^{2+} added	0·05 ml. of 12% (w/v) $CuSO_4$, $5H_2O$	12–32%, same colour; <12%, less colour
Incubation with *p*-hydroxydiphenyl reagent	Time at 28–30°	20 min.	20–50 min., same colour; <20 or >50 min., less colour
Destruction of excess *p*-hydroxydiphenyl	Time of heating at 100°	90 sec.	30–120 sec., identical colour

Table 2. *The effect of successive copper-lime treatments on the colour obtained with pyruvic acid samples*

((I) 9 ml. of standard solutions (containing 200, 300, 400, 500 and 600 μg. of pyruvic acid) + 1 ml. of 20% $CuSO_4$, $5H_2O$ + 1 g. $Ca(OH)_2$. (II) 6 ml. of supernatant solution from (I) + 0·6 ml. 20% $CuSO_4$, $5H_2O$ + 0·6 g. $Ca(OH)_2$. (III) 3 ml. of supernatant solution from (II) + 0·3 ml. 20% $CuSO_4$, $5H_2O$ + 0·3 g. $Ca(OH)_2$. 1 ml. of supernatant solution taken for analysis in each case.)

Pyruvic acid concn. (μg./ml. in final solution)	Optical densities			
	Without treatment	(I) After one copper-lime treatment	(II) After two copper-lime treatments	(III) After three copper-lime treatments
20	0·200	0·070	0·020	0·017
30	0·275	0·105	0·030	0·020
40	0·38	0·110	0·050	0·023
50	0·43	0·125	0·050	0·022
60	0·52	0·130	0·055	0·020

Table 3. *Recoveries of lactic acid from mixtures of pyruvic and lactic acids*

Final solution				
Lactic acid (μg./ml.)	Pyruvic acid (μg./ml.)	Optical density of mixture (Obs.)	Optical density (Corr.*)	Recovery of lactic acid (%)
2	60	0·140	0·120	101
4	60	0·265	0·245	102
6	60	0·368	0·348	98
8	60	0·490	0·470	99
6	20	0·385	0·365	103
6	30	0·375	0·355	100
6	40	0·380	0·360	101
6	50	0·368	0·348	98
6	60	0·375	0·355	100

* Observed value *minus* 0·02 which is the average optical density produced by remaining pyruvic acid (20–60 μg./ml. final solution).

these recoveries are corrected for the small constant optical density due to interfering pyruvic acid after three copper-lime treatments. The results are shown in Table 3.

Similar recoveries are obtained when known quantities of lactic acid are added to tissue and enzyme extracts and deproteinization carried out with 10% (w/v) trichloroacetic acid.

DISCUSSION

The conditions under which the oxidation of lactic acid to acetaldehyde is carried out, in the first stage of the method of estimation, are of critical importance. When this oxidation is carried out in open tubes, the acetaldehyde recoverable as the final coloured complex varies greatly with the time of heating employed. These findings are contrary to those of Barker & Summerson (1941) who state that identical results are obtained with heating periods of 3–10 min. Mendel & Goldscheider (1925), whose method of lactic acid estimation used 1:2-dimethoxybenzene instead of *p*-hydroxydiphenyl to give a red compound, also claimed that heating in open tubes for 4–8 min. during the sulphuric acid oxidation stage produced no differences in the optical density of the resulting coloured complex. However, Miller & Muntz (1938) did recommend the use of stoppered tubes, but gave no reason for this.

Our results confirm those of Barker & Summerson regarding the influence of copper on the lactic acid oxidation but we have found it necessary to use a larger amount (0·05 ml. of 12% $CuSO_4$, $5H_2O$ instead of 0·05 ml. of a 4% solution) to achieve the full increase in sensitivity. This modification may be required owing to the greater recoveries of lactic acid as acetaldehyde using stoppered tubes.

The modification of method necessary to estimate lactic acid accurately in the presence of pyruvic acid was suggested by the work of Van Slyke (1917). Miller & Muntz (1938) have suggested heating the sulphuric acid oxidation mixture for 15 min. instead of 5 min. in order to remove any interfering pyruvate.

SUMMARY

1. Lactic acid has been determined in the range 0–8 μg./ml. with an accuracy of ± 2%.

2. The method, which can be employed over a 2–10 μg./ml. range in the presence of 10–60 μg./ml. of pyruvic acid, is based on that of Barker & Summerson (1941), but certain modifications are incorporated to achieve reproducibility.

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