1956

Micro-estimation and determination of lactic acid in biological fluids.

Halligan, Edward Griffith

Monterey, California: U.S. Naval Postgraduate School

MICRO-ESTIMATION AND DETERMINATION OF LACTIC ACID IN BIOLOGICAL FLUIDS

Edward Griffith Halligan

MICRO-ESTIMATION AND DETERMINATION OF LACTIC ACID IN BIOLOGICAL FLUIDS (with an appendix on related acids)

* * *

Edward G. Halligan

MICRO-ESTIMATION AND DETERMINATION OF LACTIC ACID IN BIOLOGICAL FLUIDS

(with an appendix on related acids)

by

Edward Griffith Halligan

Lieutenant Colonel, Chemical Corps, United States Army

Submitted in partial fulfillment
of the requirements
for the degree of
BACHELOR OF SCIENCE
IN
PHYSICS

United States Naval Postgraduate School
Monterey, California

1 9 5 6

This work is accepted as fulfilling

the thesis requirements for the degree of

BACHELOR OF SCIENCE

IN

PHYSICS

from the

United States Naval Postgraduate School

PREFACE

This thesis constitutes a search of the literature and a collection of the methods currently in vogue for the micro-estimation and determination of lactic acid in biological fluids, together with a history of their development.

It should be of use and interest to the biologist or biochemist who is faced with determination of lactic acid in some unknown substance as it presents the approach to the problem by the best authorities on the subject.

In addition are appended abstracts from papers written on the determination of other acids, which often are found in company with lactic acid in biological fluids. These abstracts should be of similar interest to the investigator and might provide material for the development of future papers of this type on allied subjects.

The author wishes to express his appreciation to Professor Roderick K. Clayton of the United States Naval Postgraduate School, who proposed this topic and under whose guidance it was written. Also thanks are extended to Stanford University for the interlibrary loan of Biochemistry Journals, unavailable in the USN Postgraduate School Library and to the Director of the Hopkins Marine Station of Stanford University whose cooperation in the use of the facilities of the Station is appreciated.

TABLE OF CONTENTS

LIST OF ILLUSTRATIONS

CHAPTER I

INTRODUCTION

Lactic acid is a substance of major significance throughout the orders of life, from microbe to man. Its relation to the carbohydrate metabolism has been very extensively studied. It is not surprising, therefore, that many methods have been proposed for its quantitative estimation.

The classical method for the determination of lactic acid involves its separation, usually by ether extraction combined with other means, from interfering substances, its conversion into zinc lactate by boiling with zinc oxide or carbonate, and the weighing of the lactate. The purity of the latter can then be determined by analysis. This method was used in the famous studies of lactic acid formation in muscle by Fletcher and Hopkins (1) and was applied to blood by Wolf (2). Because the purity of the salt can be proven, this procedure still remains a standard method. It is, however, time consuming because of the prolonged extraction required to remove lactic acid from other biological material, and not suited to determination of small amounts.

In consequence other methods have been devised which are more rapid and suited to micro analysis, although not capable of the same rigid analytical proof of specificity as the zinc lactate procedure. Such methods depend upon decomposition of the lactic acid and estimation of one of the products, acetaldehyde, CO_2, or CO.

When heated with potassium permanganate or manganese dioxide suspension, lactic acid is oxidized to acetaldehyde and carbon dioxide:

$$CH_3CH(OH)COOH + \tfrac{1}{2}O_2 \rightarrow CH_3CHO + CO_2 + H_2O$$

Boas (3) and Jerusalem (4) distilled the aldehyde into alkaline 0.1 N iodine and estimated the aldehyde from the amount of iodine reduced.

von Fürth and Charnass (5), finding the direct iodimetric titration inaccurate, distilled into bisulfite and used the Ripper (6) titration of the amount of $NaHSO_3$ bound by the aldehyde to determine the latter.

In a gasometric determination of lactic acid Baumberger and Field (7) measured the CO_2 formed by the permanganate oxidation, carried out in blood filtrate at room termperature in the Van Slyke-Neill gas apparatus. Hastings and Avery developed the same procedure independently and applied it to the Folin-Wu blood filtrate.

The determination of CO_2 has a theoretical advantage over determination of the acetaldehyde. If excess permanganate is allowed to act on the aldehyde it is oxidized further and the result of the analysis is too low. Addition of permanganate in all modifications of the von Furth-Charnass method is cautious in order to avoid error from this source. The CO_2 formed, however, can not be further oxidized.

Another decomposition reaction is undergone when lactic acid is heated with strong sulfuric acid at 100°. It yields again acetaldehyde but the other product is CO instead of CO_2 (8).

$$CH_3CH(OH)COOH \rightarrow CH_3CHO + CO + H_2O$$

For determination of the acetaldehyde yielded by this reaction, both the Clausen titration and several colorimetric methods have been used. Ryffel (9) used the color reaction with Schiff's reagent (rosaniline hydrochloride decolorized with sulfur dioxide), Harrop (10) the color formed with guiacol, and Mendel and Goldschneider (11) the color formed with veratrol.

One of the most precise methods is that of Friedemann, Cotonio and Shaffer (12). Lactic acid, according to this method, is oxidized by acid $KMnO_4$ in the presence of $MnSO_4$. The resulting acetaldehyde is aerated out of the solution, absorbed in bisulfite, and determined by the Clausen titration (13). The chief advantages of the method over

the older oxidation methods of von Fürth and Charnas and Clausen are

(1) speed

(2) considerably increased yield of acetaldehyde

(3) smaller fluctuations between individual determinations.

CHAPTER II

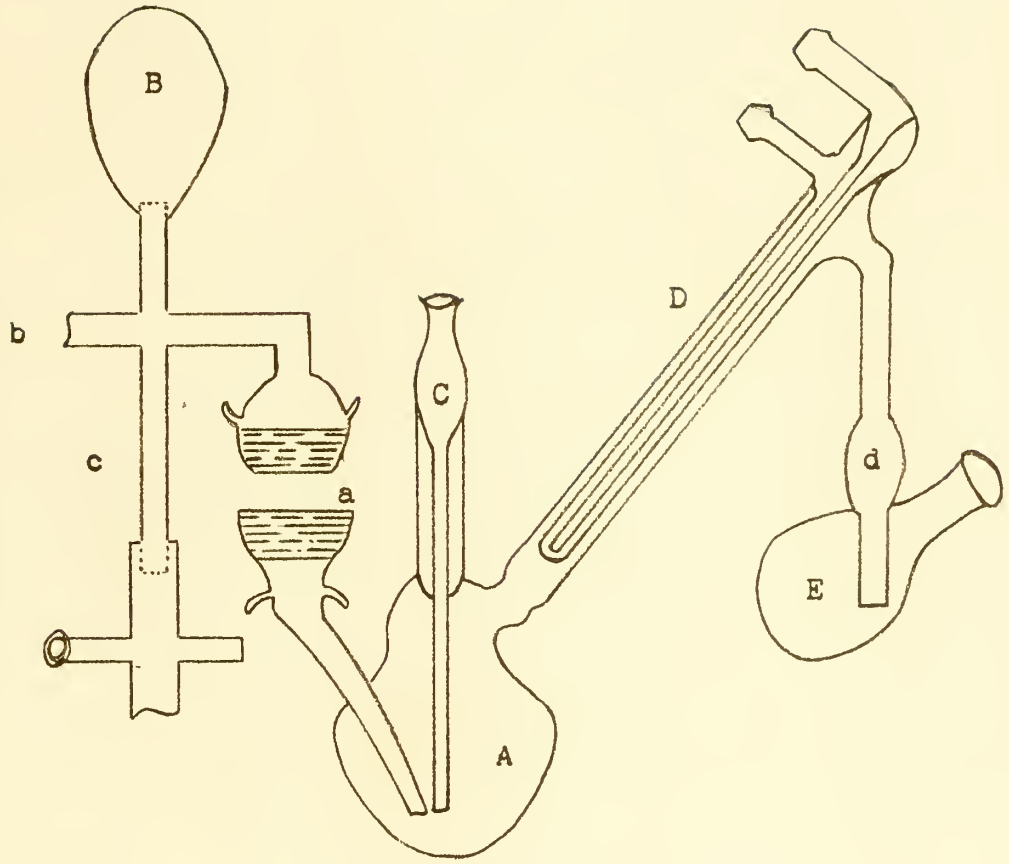
TITRIMETRIC METHOD FOR MICROGRAM QUANTITIES USING PERMANGANATE AND IODINE

The method of McCready, Mitchell, and Kirk (14) is a microgram modification of the titrimetric methods of Friedemann, Cotonio, and Shaffer (12) and Friedemann and Graeser (15). The method was tested in the range 1 to 25 γ of lactic acid and was found to yield average recoveries of better than 98% with a variation which was usually less than ± 2%, an occasional value being greater. This is not less accurate than the method from which it was modified, even though it involved a reduction in scale of two to three orders of magnitude.

The lactic acid in the sample is oxidized in a boiling solution by the slow addition of potassium permanganate to the sample containing manganous sulfate, and acidified with phosphoric acid. The oxidation produces acetaldehyde which is aerated and distilled past a reflux condenser which returns most of the water and certain interfering volatile products to the boiling solution. The acetaldehyde is collected in a sodium bisulfite solution. The excess bisulfite is titrated to a starch-iodine end point with iodine solution. The acetaldehyde-bisulfite compound is then dissociated by addition of sodium bicarbonate solution, and the liberated bisulfite is titrated with standard iodine solution to the same starch-iodine end point. Physiological solutions require deproteinization and removal of sugars which interfere. This is accomplished by a zinc hydroxide precipitation of protein followed by precipitation of sugars with copper sulfate solution and calcium hydroxide suspension, the two precipitates being removed simultaneously.

APPARATUS

The distillation apparatus is shown in Fig. 1. The reaction vessel (A) is equipped with a capillary inlet from a funnel (C) through which



Lactic Acid Apparatus

Actual Size

Figure 1

the reagents are added, an air inlet attached through a ball-and-socket ground joint to a source of air pressure, an outlet to the atmosphere, and a rubber bulb (B). The capacity of the reaction vessel (A) is about 8 ml. The outlet from the reaction vessel (A) carries a small reflux condenser and a delivery tube to the titration chamber (E). A small bulb (d) serves as a trap to prevent sucking back of the reagent from (E) during distillation.

REAGENTS

1. Potassium permanganate solution, apx .04 M.

2. Catalyst solution, prepared by dissolving 10 g of manganous sulfate and 2.5 ml of 85% o-phosphoric acid and diluting it to 100 ml.

3. Sodium bicarbonate solution, saturated at room temperature.

4. Sodium bisulfite solution, about .1 M, made fresh each week.

5. Iodine-potassium iodide solution, apx .05 M in iodine, made with the smallest practical amount of potassium iodide to dissolve the iodine. No standardization is necessary.

6. Iodine-potassium iodide solution, standard, apx .01 N. This is restandardized daily by titrating a measured quantity of standard thiosulfate solution with the capillary buret in the receiver of the distillation apparatus so as to duplicate the end point appearance.

7. Sodium thiosulfate solution, standard, apx .01 N. This is standardized against a standard potassium iodate solution, with a 10 ml buret.

8. Potassium iodate solution, standard, .01 N, made by dissolving the required quantity of pure iodate, accurately weighed and made to volume.

9. Starch indicator solution, .5%, made fresh every second day.

10. Standard lithium lactate solution, made by dissolving .0375 g

of pure dry salt and diluting to 100 ml. This will be equivalent to .35γ of lactic acid per 1λ of solution.

11. Zinc sulfate solution, containing 10 g of $ZnSO_4 \cdot 5H_2O$ in 100 ml.

12. Sodium hydroxide solution, .5 N.

13. Copper sulfate solution, containing 10 g of $CuSO_4 \cdot 5H_2O$ in 100 ml.

14. Calcium hydroxide suspension, made by shaking 5 g of pure calcium hydroxide with 100 ml of distilled water.

PROCEDURE

A sample, such as blood filtrate freed of sugars or zinc lactate solution, is added with the capillary pipet to the distillation apparatus, followed by the addition of 100λ of catalyst solution. The solutions are rinsed into the reaction vessel with 2 or 3 ml of distilled water, to fill the vessel about half full. A little distilled water is added to the receiver to cover just the end of the delivery tube. Low-pressure air (compressed) which has been washed through strong sodium hydroxide solution is then admitted through tube (b) to give an aeration rate of about 1 bubble per second in the receiver. The pressure of the air may be controlled nicely with a pressure regulator of the type described by Pregl for carbon and hydrogen combustion. The distillation vessel is then heated with a small flame from a micro-burner, to give a boiling rate such that only the lower inch of the reflux condenser is used in condensing steam. When the boiling and aeration are adjusted satisfactorily, about 50λ of sodium bisulfite solution is added to the receiver. Then, about 90λ of .04 M potassium permanganate is run into the capillary cup (C) and added to the solution over a period of about 20 seconds. The distillation and aeration are continued for about 10 minutes, after which the flame is removed and the aeration continued to prevent sucking back of the receiver solution.

To the contents of the receiver is added 50λ of .5% starch solution

to serve as indicator. With a pipet, apx .05 M iodine solution is added carefully until a definite blue color is produced throughout the solution. Aeration is then stopped, and the rubber bulb is used to stir the receiver solution by pumping it up and down in the delivery tube and trap. With a capillary pipet, .01 N sodium thiosulfate is added carefully until the blue color is just dissipated, the solution being stirred continuously. The tip of the capillary buret charged with .01 N iodine solution is then inserted, and the solution is again titrated to a definite, faint blue. During this and the subsequent titration, good reflection from above and a non-reflecting white background are essential. Unglazed paper or cotton is suitable for a proper background. After the first end point is obtained, which represents the exact destruction of the excess bisulfite, the buret is read. To the solution is then added 100λ of saturated sodium bicarbonate solution to decompose the aldehyde-bisulfite complex. The bisulfite released is then titrated to the same end point as at first. The difference in the buret readings gives the lactic acid in terms of standard iodine solution, one mole of lactic acid requiring two equivalents of iodine to oxidize the bisulfite corresponding to the acetaldehyde formed in the oxidation.

A blank determination must be made for each set of reagents. The blank titration is to be subtracted from that of the determination. Blanks were found by the authors of the method to be about .6λ of .01 N iodine solution.

to serve as indicator. With a pipet, apx .05 M iodine solution is added carefully until a definite blue color is produced throughout the solution. Aeration is then stopped, and the rubber bulb is used to stir the receiver solution by pumping it up and down in the delivery tube and trap. With a capillary pipet, .01 N sodium thiosulfate is added carefully until the blue color is just dissipated, the solution being stirred continuously. The tip of the capillary buret charged with .01 N iodine solution is then inserted, and the solution is again titrated to a definite, faint blue. During this and the subsequent titration, good reflection from above and a non-reflecting white background are essential. Unglazed paper or cotton is suitable for a proper background. After the first end point is obtained, which represents the exact destruction of the excess bisulfite, the buret is read. To the solution is then added 100λ of saturated sodium bicarbonate solution to decompose the aldehyde-bisulfite complex. The bisulfite released is then titrated to the same end point as at first. The difference in the buret readings gives the lactic acid in terms of standard iodine solution, one mole of lactic acid requiring two equivalents of iodine to oxidize the bisulfite corresponding to the acetaldehyde formed in the oxidation.

A blank determination must be made for each set of reagents. The blank titration is to be subtracted from that of the determination. Blanks were found by the authors of the method to be about .6λ of .01 N iodine solution.

CHAPTER III

QUALITATIVE DETECTION WITH SULFURIC ACID AND p-HYDROXYDIPHENYL COLOR TEST (16)

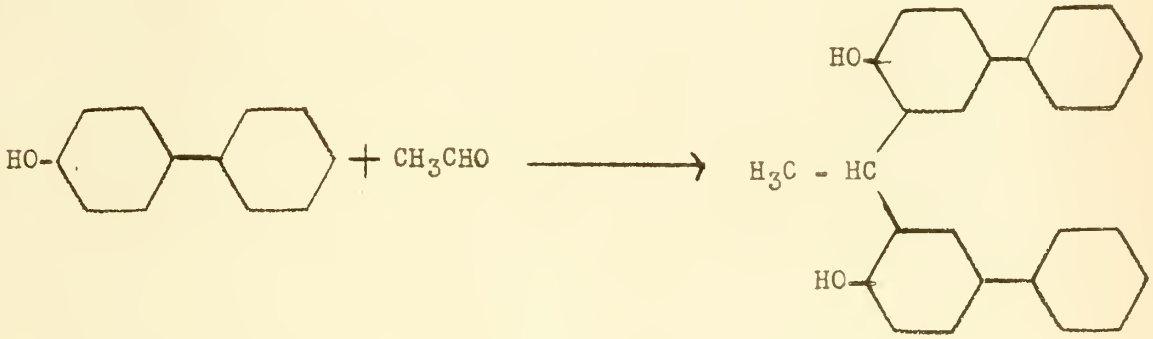
When lactic acid is gently warmed with concentrated sulfuric acid, it decomposes at first into acetaldehyde and formic acid:

$$CH_3CH(OH)COOH \longrightarrow CH_3CHO + HCOOH$$

The formic acid is dehydrated by the concentrated sulfuric acid:

$$HCOOH \longrightarrow H_2O + CO$$

The acetaldehyde reacts with p-hydroxydiphenyl, probably by condensation at the position ortho to the OH-group, and forms di-p-hydroxydiphenyl ethane:



In sulfuric acid solution, di-p-hydroxydiphenyl ethane is oxidized to a violet material of unknown constitution. Therefore, the present test involves an aldehyde-phenol reaction in which concentrated sulfuric acid functions as condensing and oxidizing agent.

A bad feature of this test is that α-hydroxybutyric acid and pyruvic acid give the same color reaction as lactic acid. Also, metaldehyde, paraldehyde, aldol, and propionaldehyde react similarly to acetaldehyde with p-hydroxydiphenyl and sulfuric acid to give deep violet products. With formaldehyde, the color is blue-green, with butyraldehyde red, and with heptyl aldehyde orange.

PROCEDURE

A drop of the test solution and 1 ml concentrated sulfuric acid

are heated for two minutes in a dry test tube in a water bath at 85°. After cooling under the tap to 28°, a pinch of solid p-hydroxydiphenyl is added, the mixture is swirled several times, and left to stand for 10 to 30 minutes. The violet color appears gradually and deepens after some time. A blank test for comparison is advisable when small quantities of lactic acid are suspected.

Limit of Identification: 1.5 γ lactic acid

Specificity: 1 part lactic acid in 100,000 parts of solution

Reagents: 1) Sulfuric acid, 96%

2) p-Hydroxydiphenyl (solid)

FLUORESCENCE TEST (16)

The acetaldehyde formed on heating lactic acid with concentrated sulfuric acid can also be identified by the blue fluorescence with o-hydroxydiphenyl. Formaldehyde, metaldehyde, paraldehyde, aldol, and the next higher homologs of the aldehyde series behave analogously to acetaldehyde. Pyruvic acid does not interfere. The chemical basis of the fluorescing reaction is not known.

PROCEDURE

A drop of the test solution is mixed with a crystal of o-hydroxydiphenyl and .5 to 1 ml concentrated sulfuric acid in a dry test tube and heated for two minutes at 85° C in a water bath. It is then examined for a blue fluorescence while holding the test tube against black paper.

Limit of Identification: 1 γ lactic acid

Specificity: 1 part lactic acid in 50,000 parts of solution

Reagents: 1) Sulfuric acid, 96%

2) o-Hydroxydiphenyl (solid)

CHAPTER IV

QUANTITATIVE DETECTION WITH SULFURIC ACID AND p-HYDROXYDIPHENYL

The method of Miller and Muntz (17) converts the Eegriwe test (16) into a method for the quantitative estimation of ultramicroquantities of lactic acid.

This method has an optimum sensitivity between 2 and 10 μ g, and may be employed to determine quantities as small as .1 μ g. The method has certain advantages, in that:

(1) It is almost completely specific for lactic acid.

(2) It does not give a reaction with carbohydrates, and thereby obviates the procedures for removal of carbohydrate.

(3) It is extremely simple to perform.

REAGENTS

1) Sulfuric acid, Sp Gr 1.84

2) p-Hydroxydiphenyl

This compound must be purified as follows: Dissolve about 30 gms in a minimum volume of warm, pure acetone. Add water until a solid begins to separate and chill to 0°. Remove the crystals by filtration; redissolve them in acetone and repeat the crystallization twice. The third crop of crystals is dried rather completely by a current of clean air, and then dissolved in a minimum volume of hot absolute acetone (about 55°). The solution is chilled to 0°; the crystals are filtered off and dried for 12 hours at 80° in a perfectly clean place.

PROCEDURE

Exactly .2 cc of the sample is measured into a glass-stoppered, Pyrex test tube (15 x 120 mm). The tube is placed in ice water while exactly 1.5 cc of the concentrated sulfuric acid are added slowly. The

ground glass portion of the tube is lubricated conveniently with the small amount of acid that remains in the tip of the 1.5 cc delivery pipette. The tube is stoppered lightly, and its contents are mixed by gentle shaking. The tube is then placed in a copper rack and heated in a boiling water bath for 5 minutes. It is cooled immediately in deep ice water for 10 to 15 minutes and then removed from the bath and gently rotated for a few seconds on its long axis at an angle of about 25° to the horizontal. It is placed in the upright position, allowed to drain for a few seconds, and then unstoppered. 8 Mg of very finely pulverized p-hydroxydiphenyl (weighed to within .2 mg) are added through a small funnel whose partially constricted stem almost touches the solution in the tube. This technique prevents undesirable scattering of the powder on the walls of the test tube. The tube is restoppered firmly and its contents mixed by gentle, but thorough agitation. The mixture should not be splashed on the walls of the tube.

The tube is kept at room temperature for one hour to develop maximum color. It is then placed in a boiling water bath for exactly 90 seconds and cooled immediately to room temperature. After this final heating the solution should have a clear, blue-violet color. The color is stable for several hours at room temperature.

Miller and Muntz (17) maintain that p-hydroxydiphenyl is used in the solid form since they were unable to find a solvent which would not interfere with the color test. However, Koenemann (18) describes a means of adding p-hydroxydiphenyl in solution, with a slight modification of the method.

Since the reagent is weakly acidic, it will dissolve in a solution of NaOH. The addition to the reaction mixture of a small amount of sodium hydroxide necessary, is sufficient to neutralize only a negligible

portion of the concentrated sulfuric acid used for oxidation of the lactic acid to acetaldehyde. The intensity of color developed is slightly decreased by this procedure, but is relatively independent of the amount of reagent used. Varying the volume from .01 up to .025 cc produces no detectable difference in the final color.

In the Koenemann modification, analysis proceeds according to the following two exceptions to the method of Miller and Muntz (17).

(1) In place of the addition of dry p-hydroxydiphenyl, add .02 cc of the reagent (in NaOH solution) with a Krogh micropipet. The reagent at first adheres to the glass but can be completely suspended in the solution by shaking for a few minutes.

(2) After one hour, heat in boiling water for 30 to 40 seconds (instead of 90 seconds) agitating constantly.

Koenemann (18) states that carbohydrates, if present in concentration greater than 200 mg per 100 cc of sample, yield yellow oxidation products which interfere with the determination. To avoid this, the procedure of Peters and Van Slyke (19) may be followed for the precipitation of protein and the subsequent removal of carbohydrate by the use of copper and lime. This method is also satisfactory for the removal of 3% gum acacia.

Solutions may be read in a photoelectric colorimeter.

CALIBRATION

The colorimeter should be calibrated by making readings on a series of standard zinc lactate solutions (.5 to 10γ per .2 cc) carried through the entire procedure. The weakest standard solutions, containing .5γ per .2 cc, deteriorate in a few days. Stronger solutions, containing 8γ per .2 cc, show a change after a month but none after 6 days.

To make the standard solutions, dissolve 165 mg of $Zn(C_3H_5O_3)_2 \cdot 3H_2O$

in 500 cc of water. This gives a concentration (expressed as lactic acid) of 40γ per .2 cc. Dilute this as needed to give standards in the range of 2γ to 10γ per .2 cc. Store in a cold room.

MODIFICATION FOR SPECIFIC DETERMINATION OF LACTIC ACID IN THE PRESENCE OF PYRUVIC ACID

The method given above allows 5 minutes for sulfuric acid to oxidize the lactic acid to aceteldehyde. With this procedure, pyruvic acid has been found to yield a color equivalent to 20% of that given by an equal weight of lactic acid. Longer periods of heating with sulfuric acid diminish the color given by pyruvic acid, and after 15 minutes heating, the color reaction with pyruvic acid disappears completely. With the longer heating period, the final color given by lactic acid is only about 8% less than that obtained in the shorter period with the regular procedure. Thus, if necessary, lactic acid may be sharply differentiated from pyruvic acid by increasing the period of heating with sulfuric acid to 15 minutes. The other details of the procedure remain unaltered. If the modified method is employed, a new calibration should be obtained with standard lactate solutions.

SPECIFICITY

The method has been tested on a number of organic acids which are related structurally to lactic acid, or which may be associated with lactic acid in biological reactions. The results are given in the following Table, which shows that the method has a very high degree of specificity for lactic acid as compared with other compounds.

TABLE I

COMPARISON OF COLOR REACTION GIVEN BY LACTIC ACID AND RELATED COMPOUNDS

COMPOUNDS GIVING BLANK TEST	
Malic acid	
Fumaric acid	
Malonic acid	
Succinic acid	
Citric acid	
Tartaric acid	
Glycerophosphoric acid	
Acetic acid	
Glycolic acid	
Oxalic acid	
Formic acid	
REACTIVE COMPOUNDS	
β-Rhamnose	Very slight trace of color
1,3-Dihydroxybutyric lactone	Very slight trace of color
Pyruvic acid (5 min period of heating with H_2SO_4)	Color equal to that given by 1 μg lactic acid
Pyruvic acid (15 min period of heating with H_2SO_4)	Blank test

(In each test 6 μg of the compounds listed above were used).

CHAPTER V

ESTIMATION BY OXIDATION WITH CERIC SULFATE

While investigating the magnitude of the error in pyruvic acid estimations caused by the presence of lactic acid in tissue extracts, Gordon and Quastel (20) found that at relatively high temperatures e.g. 50°, lactic acid is oxidized quantitatively by ceric sulfate to acetaldehyde. Analysis showed that the reaction obeyed the equation:

$$CH_3CH(OH)COOH + 2\ Ce^{++++} \longrightarrow CH_3CHO + CO_2 + 2H^{+} + 2Ce^{+++}$$

Lactic acid could be estimated with accuracy by allowing it to react with an excess of standard ceric sulfate solution at 50° for a suitable time and titrating the excess Ce^{++++} with standard ferrous ammonium sulfate solution. This procedure is of little value when applied to biological material, owing to the large errors introduced by the presence of other substances attacked by ceric sulfate under the conditions employed. Estimation, however, of the acetaldehyde produced by the oxidation of lactic acid showed that its amount was not materially affected by the presence, in the lactic acid solution, of substances commonly encountered in tissue extracts, so long as excess ceric sulfate was used.

The method adopted by Gordon and Quastel (20) for the estimation of lactic acid consists in the oxidation of the substance by ceric sulfate to acetaldehyde, followed by estimation of the latter by absorption in sodium bisulfite solution as in the well known method of Friedemann et al (12). They found that the presence of glucose, fructose, starch and a variety of other substances does not interfere with the lactic acid estimation so long as these are not present in abnormally large concentrations. Hence it is unnecessary to remove these substances from tissue extracts before making lactic acid estimations.

PROCEDURE

Five ml of the fluid, of which the content of lactic acid is to be estimated, are placed in the main vessel of a Schrödter flask. In the dropping funnel are placed 5 ml of 10% ceric sulfate in 1.0 N H_2SO_4 solution. Five ml of 1% sodium bisulfite solution are placed in the absorption tube of the apparatus. The apparatus is placed in a water bath, or thermostat, kept at 50°. It is so arranged that the absorption tube is well above the water level in the thermostat, the temperature of the contents of the tube being kept as near that of the room as possible. The dropping funnel is now connected to a nitrogen cylinder, the tap of the funnel is opened, and a slow stream of nitrogen (3 or 4 bubles a sec) is passed through the apparatus. Sixty to ninety minutes are ample for the completion of the reaction and for the complete sweeping out of the acetaldehyde from the reaction vessel into the bisulfite solution, using quantities of lactic acid varying from .4 to 4 mg. If nitrogen is passed through the flask too quickly, there is incomplete absorption of acetaldehyde by the bisulfite.

If it is found that the ceric sulfate in the reaction flask becomes nearly or completely reduced, during the course of the experiment, it will be necessary to start again using a more concentrated solution of ceric sulfate. It is essential that at the end of the experiment there should still be an excess of ceric sulfate present in the reaction flask.

At the end of the allotted time, the contents of the absorption tube are washed into a beaker. The free bisulfite is titrated with .1 N iodine solution and the bound bisulfite, after treatment with $NaHCO_3$, with .02 N iodine solution, the procedure being precisely the same as that in the method of Friedemann et al (12). The apparatus used in the latter method can obviously be used in place of the Schrödter flask.

The error of the method appears not to exceed ± 5% when using quantities of lactic acid varying from .15 to 1.5 ml of .032 M solution (i.e. .4 - 4 mg lactic acid).

The estimation can be carried out with accuracy in the presence of trichloroacetic acid. The presence of urea or of nutrient peptone broth as used in bacteriological work introduces no error.

Winnick (21) suggests a lactic acid method which is simpler and more sensitive than distillation and aeration procedures, if the Conway unit is substituted for the aeration apparatus of Gordon and Quastel (20).

The following are the chief modifications in the latter method.

(1) Single lactic acid determinations are performed on 1 ml blood samples or on approximately .5 g of tissue.

(2) Zinc hydroxide is used instead of trichloroacetic acid to precipitate blood proteins.

(3) Blood filtrates are freed of sugar by treatment with cupric hydroxide prior to analysis.

(4) Determinations are usually conducted at room temperature, instead of 50°.

(5) The acetaldehyde which results from the oxidation of lactic acid passes by simple gaseous diffusion from the outer chamber of the Conway unit into bisulfite solution in the central chamber, thus obviating the need for a current of gas.

The diffusion is complete in about 5 hours at 25° or 2 hours at 50°. The bound bisulfite is determined iodometrically.

The method of Winnick (21) was further modified by Conway (22), who carried out the oxidation in stoppered test tubes. After 30 minutes incubation at 37°, he transferred 1 ml samples of the reaction mixture

to diffusion vessels where the acetaldehyde was trapped in bisulfite placed in the center chamber.

Long (23) describes a modification of the method of Gordon and Quastel (20) in which he substitutes aeration in a specially constructed apparatus for the bubbling of nitrogen through a Schrödter flask. One of the advantages claimed for this method is that a series of determinations can now be carried out simultaneously. This has proved very useful when conducting tolerance tests, and by this method both pyruvic and lactic acids may be determined on the same blood sample.

Acetaldehyde, formed from lactic acid by oxidation with ceric sulfate, is itself slowly oxidized by excess Ce^{++++} at 50-60°. This secondary oxidation can be prevented by removal of the acetaldehyde from the reaction mixture in a current of air.

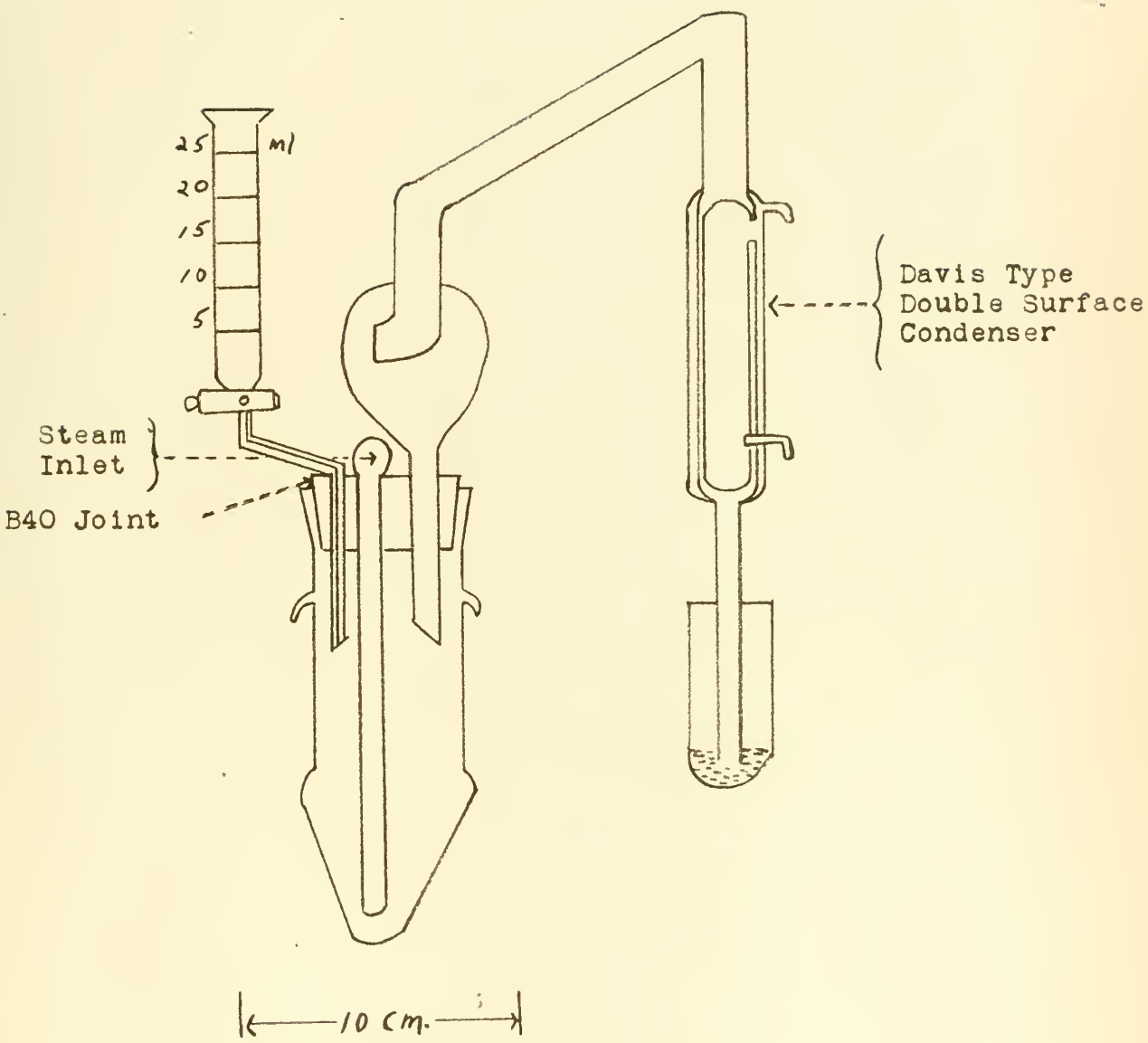
The latest modification of the ceric sulfate method is the steam distillation method of Elsden and Gibson (24). They designed a special apparatus in which it is possible to add ceric sulfate dropwise while the steam is passing. (Figure 2)

REAGENTS for Elsden and Gibson (24) modification

1) .05 N ceric sulfate in N sulfuric acid; stock ceric sulfate is standardized with ferrous ammonium sulfate.
2) 10 N sulfuric acid
3) .5% sodium bisulfite solution
4) .1 N, .01 N and .005 N iodine solution

PROCEDURE

The sample containing lactic acid, which preferably should not exceed 5 ml, is measured into the reaction flask and sufficient of the 10 N sulfuric acid added to make the concentration of this acid 1.0 N. The reaction flask is then attached to the apparatus. The receiving



Apparatus for the Estimation of Lactic Acid by the Steam Distillation Method

Figure 2

tube, which contains 2 ml of the 5% sodium bisulfite, is attached in such a way that the tip of the condenser dips below the surface of the bisulfite solution. The micro-burner, heating the reaction flask, is now turned on and the flame adjusted so that the solution just boils. The steam is then turned on and the steam flow adjusted so that 15 - 20 ml/min of the distillate is collected. This rapid distillation rate is essential and necessitates the use of an efficient double surface condenser. The .05 N ceric sulfate is then run dropwise into the reaction flask at such a rate that each drop is decolorized before the next drop goes in. When a permanent yellow color is obtained, indicating that an excess of ceric sulfate is present, further ceric sulfate up to a total of 5 ml is rapidly added. The only critical feature of the estimation is the dropwise addition of ceric sulfate. When 15 ml of distillate have been collected, the receiver is lowered and distillation is continued until 20 ml have been collected. The steam is then discontinued, the micro-burner turned off and the receiver placed in an ice-water bath to cool. While it is cooling, the reaction flask is disconnected and thoroughly washed out with distilled water. The still head is also carefully washed to remove traces of ceric sulfate which, if left, could bring about the premature oxidation of part of the lactic acid in subsequent samples to be analyzed.

The acetaldehyde is then estimated in the usual way, using solid sodium bicarbonate to destroy the aldehyde-bisulfite compound. It is important to cool the mixture thoroughly before the final titration, as described by Friedemann and Graeser (15). The temperature should be 4 - 5°.

Under conditions of this method, glucose gives rise to carbonyl compounds and it is essential to treat the test solution with copper-

lime reagent.

A suitable reagent for this purpose consists of 1 ml of 20% $CuSO_4 \cdot 5H_2O$ per 10 ml of test solution and a spatula full of solid $Ca(OH)_2$.

Trichloroacetic acid cannot be used, since under the conditions of the estimation it is oxidized with formation of such large volumes of gas that frothing is uncontrollable. The various methods for the precipitation of proteins which involve the use of tungstic acid are satisfactory. Perchloric acid, according to Neuberg, Strauss and Lipkin (25), is very satisfactory where an acid precipitation is required. If perchloric acid treatment is followed by treatment with copper-lime reagent, the copper solution should be followed by 2 N KOH until a slight trace of cupric hydroxide is formed, at which point the lime is added. In this way, the perchloric acid is removed as the insoluble potassium salt. The removal of perchloric acid is not essential, however.

Protein hydrolysates, such as are used in bacteriological media, cause some interference, but no carbonyl compounds are produced from ethanol, citric acid or malic acid.

Observations on the reaction between ceric sulfate and lactic acid confirm and amplify those published by Long (23). Significant amounts of the acetaldehyde produced are oxidized to acetic acid if the former is not rapidly removed from the reaction mixture. The rate of destruction of acetaldehyde is related to the concentration of ceric sulfate in the reaction mixture and to the temperature. Consequently, to obtain maximum recovery of acetaldehyde, the concentration of ceric sulfate should be such as to insure only a slight excess at the end of the reaction. The rate of aeration should be the maximum possible.

Elsden and Gibson (24) feel that the concentration of ceric sulfate

recommended by Long (23) is somewhat too high and also find it difficult to understand how a quantitative conversion of lactic acid to acetaldehyde can be obtained by the procedure of Winnick (21).

They prefer the steam distillation method over the aeration method for the following reasons:

(1) The blanks are negligible except when using .00257 N iodine; but even here they are small and are equivalent to about .1 micro mole of lactic acid.

(2) The method is extremely rapid, one distillation taking little more than a minute to perform.

(3) The end point is sharper because the final volume in which the titration is carried out is only 30 ml and this is particularly important when small amounts of lactic acid are to be estimated.

The advantages of the ceric sulfate over the permanganate methods described by Friedemann et al (12), (15), (26) are speed and the small volume in which the titration is carried out.

CHAPTER VI

COLORIMETRIC DETERMINATION WITH p-HYDROXYDIPHENYL AND CUPRIC ION

Of the various colorimetric methods which were available for the determination of small amounts of lactic acid prior to 1941, only those of Mendel and Goldscheider (11) and Miller and Muntz (17) are sufficiently sensitive to be of possible value in this connection.

Both of these last methods depend primarily upon the reaction of Denigès (8), by which lactic acid is converted quantitatively into acetaldehyde on being heated with concentrated sulfuric acid. Mendel and Goldscheider determine the acetaldehyde colorimetrically by virtue of its reaction with veratrole (o-dimethoxybenzene), while Miller and Muntz utilize the reaction between acetaldehyde and p-hydroxydiphenyl which was first described by Eegriwe (16).

The reaction between p-hydroxydiphenyl and acetaldehyde is fundamentally far more sensitive than is the veratrole reaction, but it was found that this reaction in concentrated sulfuric acid is markedly influenced by the presence of certain inorganic ions, notably those of iron, copper, and cerium. Other ions such as those of lead, mercury, nickel, and cobalt are without effect.

While the presence of neither ferrous ion nor ferric ion alone is of analytical value, a suitable mixture of the two results in a final color which is from three to five times as intense as when the reaction is carried out in the ordinary was as described by Miller and Muntz (17), and the specificity of the reaction is also increased. The disadvantage of iron as a promoter of the color reaction lies in the fact that variations in the ratio between ferrous and ferric iron markedly influence the color development, and an excessive amount of ferric ion produces a green color with the reagent alone.

The effect of copper is quite different from that of iron. The additon of cuprous ion is not necessary, since added cupric ion alone enhances color development. As the cupric ion concentration is increased color enhancement increases until it becomes almost as great as that obtained with the optimal mixture of ferrous and ferric iron. Higher concentrations of cupric ion have no effect further, nor does the ion produce a color with reagent alone. Cerium behaves similarly to copper but offers no advantages over the latter.

The method of Barker and Summerson (27) utilizes the above idea. The various steps in their procedure include:

(1) Treatment of the protein-free sample by the copper-lime procedure of Van Slyke (28) to remove interfering material.

(2) Conversion of lactic acid to acetaldehyde by heating in concentrated sulfuric acid in the proportion recommended by Mendel and Goldscheider (11).

(3) Color development by treatment in the concentrated acid solution with p-hydroxydiphenyl in the presence of added cupric ions. The p-hydroxydiphenyl is added as an aqueous alkaline solution in accordance with the Keonemann (18) modification of the Miller and Muntz (17) method.

The sensitivity of the procedure is such that color development is ordinarily carried out on a portion of the sample which contains not more than 5 to 10 γ of lactic acid. This represents 1 cc of a 1:50 or 1:100 dilution of the usual sample. Since the final volume of colored solution is about 7 cc, it would appear possible to apply this method to much smaller amounts of lactic acid than are specified above by the suitable use of smaller volumes and microphotoelectric colorimetry.

REAGENTS

(1) 20% solution of $CuSO_4 \cdot 5H_2O$

(2) 4% solution of same

(3) Solid Calcium Hydroxide, c.p., powdered

(4) Sulfuric acid, conc, sp gr 1.84

(5) 1.5% solution of p-hydroxydiphenyl in .5% NaOH

PROCEDURE

The solution undergoing analysis must be free from protein, which may be removed by any of the common procedures in which trichloroacetic acid, tungstic acid, zinc hydroxide, or cadmium hydroxide is employed. The protein-free fluid is treated with 20% copper sulfate solution and solid calcium hydroxide in the proportion recommended by Van Slyke (28), to remove glucose and any other interfering substances.

It has been found by Russell (29) that, using sulfuric acid from different sources, wide variations may be obtained in the amounts of color in this reaction.

This effect has been traced to small quantities of nitrate or nitrite ions existing in the sulfuric acid. Consequently, brands of acid which are stated by the manufacturer to have a low content of these ions must be selected, and each lot must be tested before use if satisfactory results are to be obtained in this method for determining lactic acid.

The effect of nitrate and nitrite ions on color development in this method would interfere with the application of the reaction to fluids which contain these ions, e.g., media or extracts from certain bacteria or fungi.

For the color development, transfer 1 cc of the supernatant liquid from the copper-lime treatment, containing between 2 and 10 γ of lactic

acid, to a fairly wide test-tube (18 to 23 mm inside diameter) and add .05 cc of 4% copper sulfate solution. Run in from a burette exactly 6 cc of concentrated sulfuric acid, mixing the contents of the tube while the acid is being added. It is not necessary to keep the mixture cold. With adequate mixing, the temperature of the solution does not rise above 70 - 80°. After the acid has been added, place the tube upright in a boiling water bath and allow it to remain for five minutes. Remove and place in cold water to cool to below 20°.

When the contents of the tube are sufficiently cool (but not before) add from a pipet exactly .1 cc of the alkaline solution of p-hydroxy-diphenyl. Disperse the precipitated reagent as quickly and uniformly as possible through the solution, and place the tube in a beaker of water at 30°. Allow to stand for 30 minutes (longer does no harm), then place the tube in boiling water for 90 seconds, remove, and cool in cold water to room temperature.

It is good practice to redisperse the precipitated reagent through the solution by gentle shaking at least once during the 30 minute incubation period. The 90 second heating in boiling water dissolves excess reagent, leaving a clear solution.

Transfer the colored solution to a suitable container and read, in a photoelectric colorimeter, using a filter which has a peak transmission at about 560 mμ . Because of the composite nature of the color (i.e. appreciable light absorption in various portions of the spectrum), it is important that a filter with a spectral band not over 50 to 60 mμ wide be used. Both the Rubicon No. 565 and the Klett No. 56 filters fulfil this condition.

For the initial setting of the colorimeter, either sulfuric acid alone or the reagent blank may be used. The latter procedure corrects

the unknown reading for the value of the reagent blank, while the former does not.

Hullin and Noble (30) suggest several modifications of Barker and Summerson's procedure. With these changes, 1 - 8 micrograms of lactic acid could be determined with an accuracy of ± 2%. When interfering pyruvic acid is present, the procedure involves a dilution so that the method in this case is applicable to 2 - 10 micrograms of lactic acid.

CHAPTER VII

COLORIMETRIC DETERMINATION IN MICROGRAM QUANTITIES

REAGENTS (30)

(1) p-Hydroxydiphenyl (1.5 g) is dissolved in 10 ml of 5% NaOH and diluted to 100 ml with water.

(2) Standard lactic acid solution:

.2133 g of pure dry lithium lactate was dissolved in about 100 ml of water, 1 ml of conc H_2SO_4 added and the solution made up to 1 liter with water.

PROCEDURE

The protein-free solution (2 ml) containing 10 - 80 micrograms of lactic acid, is pipetted into a 150 x 25 mm test tube which contains 1 ml of 20% $CuSO_4 \cdot 5H_2O$ 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 minutes or more, the solution is centrifuged.

If the original 2 ml of protein-free solution contains 100 - 600 micrograms of pyruvic acid, 6 ml of the supernatant solution from the first treatment with copper-lime is pipetted into a second tube containing .6 ml of 20% $CuSO_4 \cdot 5H_2O$ and .6 g of solid $Ca(OH)_2$ added. This procedure is then repeated a third time with 3 ml of supernatant solution, .3 ml of 20% $CuSO_4 \cdot 5H_2O$ and .3 g of $Ca(OH)_2$, whereby the amount of pyruvic acid present is reduced to a minimum and the color 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 x 25 mm test tube, held in the arm of a mechanical shaker with its lower end immersed in an ice-water mix-

ture. $CuSO_4 \cdot 5H_2O$ (.05 ml of 1% solution) is added, followed by 6 ml of concentrated H_2SO_4 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 minutes in a water bath maintained at $60 \pm 1^{\circ}$. The tube is allowed to cool to 10 - 15°, the stopper removed, .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 minutes at 28 - 30° to insure maximum color development is then followed by 90 seconds 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-colored solution is determined in circular tubes of 1 cm diameter with the Unicam diffraction grating spectrophotometer at a wavelength peak of 500 milli-microns against a reagent blank prepared by taking distilled water through the whole of the above procedure.

The calibration curve obtained by this method (E = μg of lactic acid x .059) for 0 - 8 μg of lactic acid is a straight line.

The absorption curve of the acetaldehyde-p-hydroxydiphenyl complex shows a maximum at 560 mμ as illustrated by the following optical densities obtained from 6 μg of lactic acid in the conditions described above at wavelengths between 520 and 600 mμ;

Wavelengths (mμ)	Optical density
520	.210
540	.295
550	.330
560	.345
570	.330
580	.305
600	.185

The calibration curve, which is strictly reproducible, indicates that the colored 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 colored solutions, obtained from larger quantities of lactic acid (up to 10 μg), are diluted with H_2SO_4:H_2O (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

This method has been used for the determination of lactic acid in the presence of acetoin, butane-2,diol, diacetyl, and pyruvate. None of the first three of these compounds gives any color 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 enzyme studies, seriously interferes with the reproducibility of the method unless steps are taken to remove it.

One copper-lime treatment does not yield reproducible results, although it clearly removes a considerable amount of pyruvic acid. If the copper-lime treatment is repeated, 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.

Similar recoveries are obtained when known quantities of lactic acid are added to tissues and enzyme extracts and deproteinization carried out with 10% trichloroacetic acid.

The conditions under which the oxidation of lactic acid to acet-

aldehyde 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 colored complex varies greatly with the time of heating employed. These findings are contrary to those of Barker and Summerson (27) who state that identical results are obtained with heating periods of 3 - 10 minutes. Mendel and Goldscheider (11), whose method of lactic acid estimation used veratrole (instead of p-hydroxydiphenyl) to give a red compound, also claimed that heating in open tubes for 4 - 8 minutes during the sulfuric acid oxidation stage produced no differences in the optical density of the resulting colored complex. However Miller and Muntz (17) did recommend the use of stoppered tubes.

The results of Hullin and Noble (30) confirm those of Barker and Summerson (27) regarding the influence of copper on the lactic acid oxidation but it is found necessary to use a larger amount (.05 ml of 12% $CuSO_4 \cdot 5H_2O$ instead of .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 (28). Miller and Muntz (17) have suggested heating the sulfuric acid oxidation mixture for 15 minutes instead of 5 minutes in order to remove any interfering pyruvate.

quantitative determination.

The reduction of pyruvic acid to lactic acid was accomplished in 1863 by Wislicenus (62) and Debus (63), but it was not until 1923 that this reaction was employed for quantitative determination. Lieben (56) boiled pyruvic acid with Zn and HCl under a reflux for some hours and determined the resulting lactic acid by the Fürth-Charnass procedure with recovery of 96 to 109%. Laufberger (64) and Krishna and Sreenivasaya (65) found the Lieben method unsuited to the determination of small amounts of pyruvic acid. The latter workers, by modifying the reduction procedure (employing Zn-Cu couple and H_2SO_4) and by using the Friedemann-Cotonio-Shaffer (12) method for lactic acid determination obtained consistent recoveries of 80% over a wide range of concentrations. Kendall and Friedemann (66) further modified the Lieben procedure and obtained recoveries of 94%.

Of the many methods used for the determination of pyruvic acid, the most sensitive are those which are based upon the reaction of this acid with the nitrophenylhydrazines. These methods are not specific for pyruvic acid, but they are highly specific for keto acids as a group.

The principles underlying these methods were first described in 1913 by Dakin and Dudley (67). These authors noted that 4-nitrophenylhyrdazinopropionic acid and the 4-nitrophenylhydrazones of pyruvic, glyoxylic and phenylglyoxylic acids could be separated from other hydrazones either by treatment with a 10% solution of sodium carbonate or by their differential solubility in alcohol. The salts of the hydrazones, if present in sufficient quantity, could be reprecipitated by acidification of the alkaline solution. Dakin and Dudley (67) further noted that the "merest trace" of the hydrazones of glyoxal, methylgly-

APPENDIX A

DETERMINATION OF PYRUVIC ACID - A BRIEF HISTORY

Since the discovery of pyruvic acid by Berzelius (31) a number of methods for its identification and determination have been reported. Useful color reactions are described by Simon (32), Quastel (33), Posternak (34), Alvarez (35), and Gerzarolli-Thurnlackh (36). Anderson, Peterson, and Fred (37) state that a color reaction is obtained with Uffelman's reagent. The phenylhyrdazone of pyruvic acid first described by Fischer (38) and Fischer and Jourdan (39) has been used in various ways for quantitative determination by de Jong (40), MacLean (41), Simon and Piaux (42), and Hahn, Fischbach, and Haarmann (43). The p-nitrophenylhydrazone, a less soluble derivative, first described by Hyde (44), has been used extensively by Neuberg and coworkers. 2,4-dinitrophenylhydrazine has been used by Neuberg and Kobel (45). The employment of this for quantitative separation and estimation of acetaldehyde, methylglyoxal, and pyruvic acid is described by Simon and Neuberg (46). The semicarbazone and its Ca and Zn salts have been used in isolation studies by Kostytschew and Soldatenkov (47). Methods based on partial or complete oxidation by a variety of oxidizing agents are described by Beilstein and Wiegand (48), Quastel (33), Fernbach and Schoen (49), Holleman (50), Levene and Meyer (51), Bleyer and Braun (52), and Mazé and Ruot (53). Criticisms of some of these methods are offered by Denis (54), Evans and Witzemann (55), Lieben (56), and Hatcher and Hill (57). Wieland (58), using the reaction noted by Neuberg (59), describes the conditions under which NaOI quantitatively converts pyruvic acid to iodoform. Warburg, Kubowitz, and Christian (60) decarboxylate pyruvic acid with yeast carboxylase and determine the CO_2 manometrically. Cook (61) utilizes the bisulfite-combining capacity of pyruvic acid for

quantitative determination.

The reduction of pyruvic acid to lactic acid was accomplished in 1863 by Wislicenus (62) and Debus (63), but it was not until 1923 that this reaction was employed for quantitative determination. Lieben (56) boiled pyruvic acid with Zn and HCl under a reflux for some hours and determined the resulting lactic acid by the Fürth-Charnass procedure with recovery of 96 to 109%. Laufberger (64) and Krishna and Sreenivasaya (65) found the Lieben method unsuited to the determination of small amounts of pyruvic acid. The latter workers, by modifying the reduction procedure (employing Zn-Cu couple and H_2SO_4) and by using the Friedemann-Cotonio-Shaffer (12) method for lactic acid determination obtained consistent recoveries of 80% over a wide range of concentrations. Kendall and Friedemann (66) further modified the Lieben procedure and obtained recoveries of 94%.

Of the many methods used for the determination of pyruvic acid, the most sensitive are those which are based upon the reaction of this acid with the nitrophenylhydrazines. These methods are not specific for pyruvic acid, but they are highly specific for keto acids as a group.

The principles underlying these methods were first described in 1913 by Dakin and Dudley (67). These authors noted that 4-nitrophenylhyrdazinopropionic acid and the 4-nitrophenylhydrazones of pyruvic, glyoxylic and phenylglyoxylic acids could be separated from other hydrazones either by treatment with a 10% solution of sodium carbonate or by their differential solubility in alcohol. The salts of the hydrazones, if present in sufficient quantity, could be reprecipitated by acidification of the alkaline solution. Dakin and Dudley (67) further noted that the "merest trace" of the hydrazones of glyoxal, methylgly-

oxal, and phenylglyoxal in sodium hydroxide solution, best with NaOH in alcohol, gave blue or purple colors which faded to red or brown; on the other hand, all of the acid hydrazones, including that of phenylglyoxylic acid, gave an intense red coloration with NaOH solution or with NaOH in alcohol. These principles have been applied by Neuberg (45), (68), (69), Barrenscheen and Dregus (70), Case (71), Peters and Thompson (72), Pi-Suñer and Farrán (73), Lu (74), Larsson and Liljedahl (75), and Bueding and Wortis (76).

Lu (74) has applied the procedure to the determination of pyruvic acid in small samples of blood. This has been modified somewhat by Bueding and Wortis (76) and others (77), (78). In these methods the trichloroacetic acid filtrate is allowed to react 10 minutes with 1 mg of 2,4-dinitrophenylhyrdazine in 2 N HCl, after which the solution is extracted three times with small volumes of ethyl acetate. The combined extracts are then reextracted three times with small volumes of 10% solution of Na_2CO_3. The combined alkaline extracts are extracted once more with ethyl acetate, after which they are made strongly alkaline with NaOH and the intensity of the resulting red color is determined in a photoelectric colorimeter.

The latest work in this field is that of Dische, Weil and Landsberg (79) who describe new color reactions of α-ketoacids, and α-keto- and hydroxyaldehydes with α-methylindole and HCl. The application of these reactions for the detection and quantitative determination of these substances is discussed.

APPENDIX B

CHROMATOGRAPHIC METHODS FOR ESTIMATION OF ACIDS

Paper chromatography offers a solution to the problem of analysis of mixtures of lower fatty acids.

One of the first to develop a chromatographic method was Elsden (80) in 1946. His silica-gel partition chromatogram, with bromocresol green as indicator, is found to resolve a chloroform solution of the acids, formic, acetic, propionic, n-butyric and n-valeric. The R values of the fatty acids vary with the concentration. The procedure has been adapted for the identification and quantitative estimation of acetic, propionic and butyric acids in a mixture containing all three acids. Formic acid cannot be estimated by this method and must be removed from the solution taken for analysis. Elution of acetic acid is accompanied by the leaching out of the indicator in amounts sufficient to obscure the end-point in the final titration. Acetic acid therefore is determined by difference.

Elsden's method satisfied a long felt need, but it has certain disadvantages. Moyle, Baldwin and Scarisbrick (81) found that the use of buffered columns obviated most of the disadvantages of the Elsden method, and gave extremely accurate results. However, it is tedious, and this is an important limiting factor in routine analyses. Particularly is this so when more than three acids are to be determined, and hence two or more columns must be used.

The method of Peterson and Johnson (82) also employs silica-gel columns, but is likewise tedious because more than one column must be used to effect the separation of the acids and many titrations are necessary.

Subsequently, paper chromatography was applied to the separation

and quantitative determination of relatively non-volatile organic acids (Lugg and Overell) (83)(84) and to the separation of aromatic monocarboxylic acids (Lederer) (85). The volatility of the lower fatty acids, however, limits the direct application of paper-chromatography for their separation and identification. Fink and Fink (86) overcame this difficulty by chromatographing the acids as their hydroxamate derivatives and spraying the developed chromatogram with ferric chloride. The derivatives were then visible as purple spots on a yellow background, but accurate quantitative determination was not possible.

Reid and Lederer (87) devised a paper partition-chromatographic procedure by which lower fatty acids from C_2 to C_7 can be separated, identified and estimated in one procedure and with sufficient accuracy for most biological purposes. Since their work was completed, Brown and Hall (88) and Brown (89) published a very similar method for the separation and identification of lower fatty acids, but the findings of the two groups of investigators differ in certain aspects.

Lactic and formic acids cannot be separated from acetic by the techniques of either of these groups. These two acids, when present in significant amounts, must be removed by preliminary distillation by a method such as that of Friedemann (90).

The chromatographic technique of Reid and Lederer (87) is the ascending development for 20-24 hours, as described by Williams and Kirby (91). The solvent is butanol, saturated with aqueous 1.5 N ammonia. The acids are run on the chromatogram in the form of their ammonium salts. Unlike Brown and Hall (88), Reid and Lederer (87) found that the use of sodium salts is unsatisfactory because, under most conditions, the spot given by the sodium ion partly or completely obliterates that given by the acetate.

After development and air-drying, the paper is sprayed with bromocresol purple in ethanol containing formaldehyde. The inclusion of formaldehyde in this reagent increases the subsequent spot definition.

Formaldehyde reacts with the ammonium ion to form the weak base hexamethylenetetramine. The free acids, after exposure of the chromatogram to ammonia, appear as regular yellow spots on a purple background.

Bromocresol purple is more sensitive to low concentrations of the acids than bromocresol green and bromothymol blue and gives spots which are more sharply defined and usually more stable.

Of the methods of quantitative analysis investigated, the only one found suitable was the calculation of acid content from spot area, the latter being related linearly to the logarithm of the former. The use of this technique was suggested by Fisher, Parsons and Morrison (92) for the quantitative determination of amino-acids and sugars. Bryant and Overell (93) have successfully used the relationship in the estimation of relatively non-volatile organic acids and their observations have been confirmed with work on lower fatty acids.

Wieland and Fischer (94) describe a paper chromatography of α - keto acids in which a fresh solution of o-phenylenediamine (.05%) in 10% aqueous CCl_3COOH is sprayed on filter paper and dried for 2 minutes at 100° to a moist yellow condition. α-Keto acids are dissolved in either of two solvents: PWB, 5:7:10 volume ratio of propionic acid, water, and butanol, or WDB, 3:4:6 ratio of water, α-(dimethylamino) isobutyronitrile, and butanol, so that the keto acid concentration in either is 1%. The R_f values obtained by chromatography of such solutions on the paper described (yellow green fluorescence at the locations of the keto acid) are .29 for pyruvic acid, .60 for dimethylpyruvic acid, .75 for cinnamoylformic acid, .21 for α-keto-glutaric acid, and .16 for oxalacetic acid with

PWB solutions. For WDB solutions, the values are .46, .78, .95, .07, and .04 respectively.

James and Martin (95) describe gas-liquid partition columns for the separation of volatile fatty acids from formic to dodecanoic acid. Complete resolution is obtained of all normal acids (and iso acids up to at least C_6) on a 4 ft column and of all the isomers of valeric acid on an 11 ft column. They also describe a method for the micro-estimation of volatile fatty acids isolated as their sodium salts and a recording burette which may be coupled with a photoelectric control circuit to render the analysis automatic.

The above method presents modifications to the method of Martin and Synge (96).

The most recent paper on the subject of chromatography of acids was published by Kinnory, Takeda, and Greenberg (97). They describe a column chromatographic method in which .05 N H_2SO_4 on silica gel is used as the stationary phase and a benzene-ethyl ether mixture as the solvent system. This procedure successfully separates the tri- and dicarboxylic acids, citric, α-ketoglutaric, succinic, fumaric, and malic; the short chain monocarboxylic acids, acetic, propionic, and butyric; the α-keto acids, pyruvic, α-ketopyruvic, and α-ketoisovaleric; and also, lactic acid. The recoveries of the acids range from 80 to 100%, most of them being over 90%. The positions of the individual acid peaks on the chromatogram are reproducible.

APPENDIX C

MISCELLANEOUS METHODS FOR DETERMINATION OF CERTAIN ACIDS

ACETIC ACID

Hutchens and Kass (98) describe a quantitative, colorimetric, microanalytical method for determination of acetic acid (80 to 250γ) or monofluoroacetic acid (100 to 400γ). They outline a procedure for removal of known inorganic ions interfering with color development or measurement and discuss limitations of the method.

The method involves mixing (a) suspected acetate sample, (b) .01 N iodine, (c) 5% $La(NO_3)_3$ and (d) 1 N NH_4OH. This results in the formation of basic lanthanum acetate which adsorbs iodine, giving a characteristic blue color.

FORMIC ACID

Pickett, Ley and Zygmuntowicz (99) describe a procedure for the manometric determination of formic acid by oxidation with ceric sulfate in the presence of catalytic amounts of palladium. The error, with .5 mg of formic acid, is less than $\pm$ 3%. Compounds which might interfere in this determination were investigated and methods are given for determining formic acid in samples containing these compounds. The procedure gives satisfactory recoveries of formic acid in bacterial cultures and suspensions.

FUMARIC ACID

Marshall, Orten and Smith (100) describe a satisfactory method for the determination of small amounts of fumaric acid in biological materials by partition chromatography. The procedure can be used to determine as little as .05 mg, and is specific. Satisfactory recoveries of small amounts of fumaric acid added to the urine, plasma, casein hydrolysate, and muscle were obtained. The procedure also seems suitable for succinic, malic, and citric acids.

MALIC ACID

Hummel (101) states that the condensation of malic acid with orcinol in the presence of concentrated sulfuric acid, to form the highly fluorescent homoumbelliferone (7-hydroxy-5-methyl-coumarin) is relatively specific for malic acid, and the quantitative precipitation of calcium malate by alcohol as a means of fractionation from biological materials gives it additional specificity. If, in conjunction with this treatment, a small amount of 2,4-dinitro-phenylhydrazine is added, most of the simple carbohydrate impurities, which give an interfering amber color with orcinol, become soluble in alcohol and thus may be removed. Further information on the use of orcinol may be obtained from Vasseur (102).

The method of Leininger and Kats (103) for the determination of malic acid depends upon heating with 2-naphthol in 92% sulfuric acid. The blue fluorescence is believed to be due to the formation of 5,6-benzo-coumarin. This has been corroborated by Goodwin and Kavanagh (104) who obtained identical pH vs fluorescence curves for 5,6-benzo-coumarin and a solution of malic acid treated with 2-naphthol and sulfuric acid.

The fluorometric determination of malic acid by the method of Barr (105) also depends upon the formation of coumarin derivatives.

OXALACETIC ACID

Edson (106) describes micro-methods for the determination of β-keto acids (acetoacetic and oxalacetic) and of β-hydroxy-butyric acid. These are suitable for tissue slice work. This method uses the aniline citrate method of Ostern (107).

PROPIONIC ACID

McNair (108) describes a method for the determination of propionic

acid in which it is oxidized to oxalic acid. Such a method is especially valuabe in the determination of propionic acid in the presence of formic and acetic acids.

FATTY ACIDS IN MIXTURES

The following publications, Osburn and Werkman (109), (110) and Werkman (111), (112), (113), (114) review the literature and discuss methods for quantitative determination of volatile fatty acids in fermenting liquors. In these papers, the application of the partition method to two-acid mixtures is shown, together with theoretical discussions of the possibilities of the partition method in general.

The method presented by Osburn, Wood and Werkman (115) deals specifically with the problems presented under the special conditions involved in the fermentative production of propionic acid. In the procedure described, the partition method is combined with a modified mercuric oxide oxidation for the separate determination of formic acid.

FUMARIC, MALIC, AND SUCCINIC ACIDS IN FERMENTATION BROTHS

Lemjakov (116) has found it possible to obtain reliable analytical results for fumaric acid by applying the mercurous fumarate method directly to the fermented substrate without extraction. The differences in the results of the analysis of samples of a substrate and of other samples of the same substrate, to which were added known quantities of fumaric acid correspond to the amounts of added fumaric acid. Large scale practical application of this technique is satisfactory. The results have been further checked by polarographic determination of the fumaric acid and concordant results have been obtained by both methods. The polarographic method appears to be the more accurate as well as the more rapid, and requires less sample. The mercurous nitrate method requires 10 ml, whereas 2 ml are sufficient for the polarographic method.

Preliminary experiments showed fumaric acid to be destroyed quickly by permanganate in cold acid solution, but malic and succinic acids were not. Malic acid is often present in a fumaric acid fermentation.

Recalling the titration of oxalic acid with permanganate and the building of catalytically active intermediary products, Lemjakov conducted an experiment with a mixture of fumaric and malic acids; complete oxidation of both acids occurred. On the basis of these observations, a new method for the determination of succinic acid was devised, which eliminated the extraction necessay in the usual methods.

BIBLIOGRAPHY

1. Fletcher and Hopkins; J PHYSIOL; 35, 247 (1907)
2. Wolf; J PHYSIOL; 48, 341 (1914)
3. Boas; DEUT MED WOCH; 19, 940 (1893)
4. Jerusalem; BIOCHEM Z; 12, 361 (1908)
5. von Fürth and Charnass; BIOCHEM Z; 26, 199 (1910)
6. Ripper; MONATSHFT CHEM; 21, 1079 (1900)
7. Baumberger and Field; PROC SOC EXP BIOL and MED; 25, 87 (1927)
8. Denigès; ANN CHIM PHYS; (series viii) 18, 149 (1910)
9. Ryffel; J PHYSIOL (SOC PROC); 39, v (1909 - 10)
10. Harrop; PROC SOC EXP BIOL MED; 17, 162 (1919 - 20)
11. Mendel and Goldscheider; BIOCHEM Z; 164, 163 (1925)
12. Friedemann, Cotonio and Shaffer; J BIOL CHEM; 73, 335 (1927)
13. Clausen; J BIOL CHEM; 52, 263 (1922)
14. McCready, Mitchell and Kirk; MIKROCHEMIE VER MIKROCHIM ACTA; 28, 23 (1939)
15. Friedemann and Graeser; J BIOL CHEM; 100, 291 (1933)
16. Eegriwe; Z ANAL CHEM; 95, 323 (1933)
17. Miller and Muntz; J BIOL CHEM; 126, 413 (1938)
18. Koenemann; J BIOL CHEM; 135, 105 (1940)
19. Peters and Van Slyke; QUANTITATIVE CLINICAL CHEMISTRY - METHODS: Williams and Wilkins Co.; Baltimore (1932)
20. Gordon and Quastel; BIOCHEM J; 33, 1332 (1939)
21. Winnick; J BIOL CHEM; 142, 451 (1942)
22. Conway; MICRODIFFUSION ANALYSIS and VOLUMETRIC ERROR, 3rd ed; p 241; London; Crosby Lockwood and Son Ltd (1950)
23. Long; BIOCHEM J; 40, 27 (1946)
24. Elsden and Gibson; BIOCHEM J; 58, 154 (1954)

25. Neuberg, Strauss and Lipkin; ARCH BIOCHEM; 4, 101 (1944)

26. Friedemann and Kendall; J BIOL CHEM; 82, 23 (1929)

27. Barker and Summerson; J BIOL CHEM; 138, 535 (1941)

28. Van Slyke; J BIOL CHEM; 32, 455 (1917)

29. Russell; J BIOL CHEM; 156, 463 (1944)

30. Hullin and Noble; BIOCHEM J; 55, 289 (1953)

31. Berzelius; ANN PHYSIK U CHEM; 36, 1 (1835)

32. Simon; COMPT REND ACAD; 125, 534 (1897)

33. Quastel; BIOCHEM J; 18, 365 (1924)

34. Posternak; COMP REND SOC PHYSIQ HIST NAT GENEVA; 44, 519 (1927)

35. Alvarez; CHEM NEWS; 91, 209 (1905)

36. Garzarolli-Thurnlackh; MONATSH CHEM; 20, 467 (478) (1899)

37. Anderson, Peterson and Fred; SOIL SC; 25, 123 (1928)

38. Fischer; BER CHEM GES; 17, 572 (1884)

39. Fischer and Jourdan; BER CHEM GES; 16, 2241 (1883)

40. de Jong; REC TRAV CHIM PAYS-BAS; 19, 280 (1900)

41. MacLean; BIOCHEM J; 7, 611 (1913)

42. Simon and Piaux; BULL SOC CHIM BIOL; 6, 477 (1924)

43. Hahn, Fischbach and Haarmann; Z BIOL; 88, 89 (1929)

44. Hyde; BER CHEM GES; 32, 1810 (1899)

45. Neuberg and Kobel; BIOCHEM Z; 216, 493 (1929)

46. Simon and Neuberg; BIOCHEM Z; 232, 479 (1931)

47. Kostytschew and Soldatenkov; PHYSIOL CHEM; 168, 124 (1927); 176, 287 (1928)

48. Beilstein and Wiegand; BER CHEM GES; 17, 840 (1884)

49. Fernbach and Schoen; COMPT REND ACAD; 158, 1719 (1914)

50. Holleman; CHEM ZENTR; 2, 194 (1904)

51. Levene and Meyer; J BIOL CHEM; 17, 443 (1914)

52. Bleyer and Braun; BIOCHEM Z; 183, 310 (1927)

53. Mazé and Ruot; COMPT REND SOC BIOL; 79, 706 (1916)

54. Denis; AM CHEM J; 38, 561 (577) (1907)

55. Evans and Witzemann; J A C S; 34, 1086 (1096) (1912)

56. Lieben; BIOCHEM Z; 135, 240 (1923)

57. Hatcher and Hill; TR ROY SOC CANAD; 22, sect 3, 211 (1928)

58. Wieland; ANN CHEM; 436, 229 (1924)

59. Neuberg; BIOCHEM Z; 43, 500 (1912)

60. Warburg, Kubowitz and Christian; BIOCHEM Z; 227, 245 (1930)

61. Cook; BIOCHEM J; 24, 1526 (1930)

62. Wislicenus; ANN CHEM; 126, 225 (1863)

63. Debus; ANN CHEM; 127,332 (1863)

64. Laufberger; BIOCHEM Z; 181, 220 (1927)

65. Krishna and Sreenivasaya; BIOCHEM J; 22, 1169 (1928)

66. Kendall and Friedemann; J INFECT DIS; 47, 176 (1930)

67. Dakin and Dudley; J BIOL CHEM; 15, 127 (1913)

68. Neuberg and Schwenk; BIOCHEM Z; 71, 104

69. Neuberg and Gorr; BIOCHEM Z; 166, 442 (1925)

70. Barrenscheen and Dregus; BIOCHEM Z; 233, 305 (1931)

71. Case; BIOCHEM J; 26, 753 (1932)

72. Peters and Thompson; BIOCHEM J; 28, 916 (1934)

73. Pi-Suñer and Farrán; BIOCHEM Z; 287, 113 (1936)

74. Lu; BIOCHEM J; 33, 249 (1939)

75. Larsson and Liljedahl; SVENSK KEM TIDSKR; 52, 67 (1940)

76. Bueding and Wortis; J BIOL CHEM; 133, 585 (1940)

77. Fornaroli and Pardi; BULL SOC ITAL BIOL SPER; 15, 511 (1940)

78. Kato and Li; AM J DIS CHILD; 61, 1222 (1941)

79. Dische, Weil and Landsberg; J BIOL CHEM; 208, 23 (1954)

80. Elsden; BIOCHEM J; 40, 252 (1946)

81. Moyle, Baldwin and Scarisbrick; BIOCHEM J; 43, 308 (1948)

82. Peterson and Johnson; J BIOL CHEM; 174, 775 (1948)

83. Lugg and Overell; NATURE; London; 160, 87 (1947)

84. Lugg and Overell; AUST J SCI RES A; 1, 98 (1948)

85. Lederer; AUST J SCI; 11, 208 (1949)

86. Fink and Fink; PROC SOC EXP BIOL; New York; 70, 654 (1949)

87. Reid and Lederer; BIOCHEM J; 50, 60 (1951-52)

88. Brown and Hall; NATURE; London; 166, 66 (1950)

89. Brown; BIOCHEM J; 47, 598 (1950)

90. Friedemann; J BIOL CHEM; 123, 161 (1938)

91. Williams and Kirby; SCIENCE; 107, 481 (1948)

92. Fisher, Parsons and Morrison; NATURE; London; 161, 764 (1948)

93. Bryant and Overell; NATURE; London; (1951)

94. Wieland and Fischer; NATURWISSENSCHAFTEN; 36, 219 (1949)

95. James and Martin; BIOCHEM J; 50, 679 (1952)

96. Martin and Synge; BIOCHEM J; 35, 1358 (1941)

97. Kinnory, Takeda and Greenberg; J BIOL CHEM; 212, 379 (1955)

98. Hutchens and Kass; J BIOL CHEM; 177, 571 (1949)

99. Pickett, Ley and Zygmuntowicz; J BIOL CHEM; 156, 303 (1944)

100. Marshall, Orten and Smith; J BIOL CHEM; 179, 1127 (1949)

101. Hummel; J BIOL CHEM; 180, 1225 (1949)

102. Vasseur; ACTA CHEM SCAND; 2, 693 (1948)

103. Leininger and Kats; ANAL CHEM; 21, 1375 (1949)

104. Goodwin and Kavanagh; ARCH BIOCHEM; 27, 152 (1950)

105. Barr; PLANT PHYSIO; 23, 443 (1948)

106. Edson; BIOCHEM J; 29, 2082 (1935)

107. Ostern; Z PHYSIOL CHEM; 218, 160 (1933)

108. McNair; J A C S; 54, 3249 (1932)

109. Osburn and Werkman; IND ENG CHEM, ANAL ED; 3, 264 (1931)

110. Ibid; 4, 421 (1932)

111. Werkman; IND ENG CHEM, ANAL ED; 2, 302 (1930)

112. Werkman; IOWA STATE COLL J SCI; 4, 459 (1930)

113. Ibid; 5, 1 (1931)

114. Ibid; 5, 122 (1931)

115. Osburn, Wood and Werkman; IND ENG CHEM, ANAL ED; 5, 247 (1933)

116. Lemjakov; ANAL CHEM; 26, 1227 (1954)

Thesis
H162 Halligan 23369
Micro-estimation and determina-
tion of lactic acid in biological
fluids.