Laboratory Manual of

Biochemistry

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Gilbert C. H. Stone
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FIFTH EDITION

W. B. SAUNDERS COMPANY

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Preface to the Fifth Edition

Changes in the present edition are due to our extensive experiences with procedures introduced in the last edition as well as to a desire to introduce, wherever possible, new techniques which have become a part of modern biochemistry and which require apparatus available in almost all college chemistry departments. As in the past, wherever possible, the quantitative approach has been emphasized.

Among the new experiments are the use of circular filter paper chromatography of amino acids (p. 26), preparation of a crystalline protein, ferritin (p. 31), estimation of succinic acid dehydrogenase activity (p. 51), effect of malonic acid as a competitive inhibitor of succinic acid dehydrogenase (p. 52), incorporation of acetate-C^{14} into cholesterol of respiring rat liver slices (p. 55), use of the Beckman model DU spectrophotometer (p. 89), determination of blood urea by nesslerization (p. 105), determination of hemoglobin by the cyanomethemoglobin method (p. 127), and the preparation of rabbit antiserum to crystalline ferritin to illustrate quantitative immunochemical procedures (p. 144).

In accord with many requests, we have also included short discussions of the chemistry of various tests, insofar as these are known, together with appropriate formulae. Discussion of the Van Slyke manometric apparatus and directions for its use have been deleted, since the authors' experience lead them to believe that a student will learn to use this apparatus properly only if given personal instruction.

THE AUTHORS

New York City

Contents

1

Carbohydrates

(Details concerning the preparation of all reagents will be found in the Appendix, page 152.)

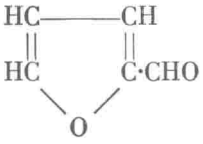
The carbohydrates include polyhydroxyaldehydes and ketones, and compounds which can be converted into such aldehydes or ketones by hydrolysis. Glyceraldehyde, $CHO{\cdot}CH_2OH{\cdot}CH_2OH$, may be regarded as the "mother" compound of the polyhydroxyaldehydes; and dihydroxyacetone, $CH_2OH{\cdot}CO{\cdot}CH_2OH$, may be considered as the lowest member of the sugars having a keto group.

The more important of these carbohydrates may be classified into: monosaccharides, oligosaccharides and polysaccharides.

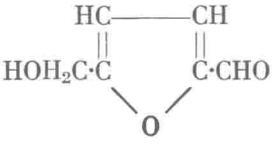
For Experiments 1 to 9 use 1 per cent solutions of glucose, fructose, galactose, maltose, lactose, sucrose and starch.

Experiment 1. Molisch Test

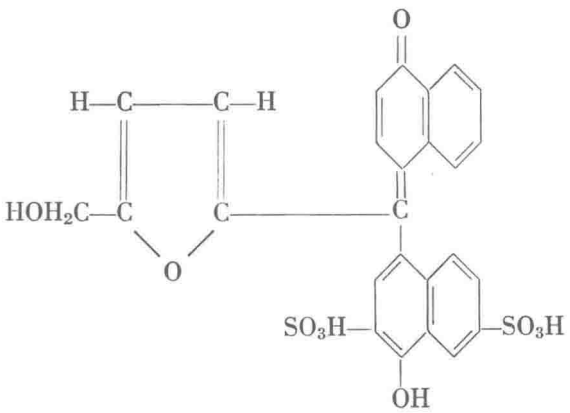
The Molisch test is a general test for carbohydrates. It is effective for any compound which can be dehydrated to furfural or a



substituted furfural, such as hydroxymethylfurfural, by concentrated sulfuric acid.



The color is due to condensation products of furfural or its derivatives with α-naphthol. The proposed formula for one such product is:



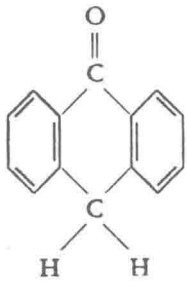
Thymol may also be used as a reagent instead of α-naphthol. Thymol is more stable than α-naphthol and does not become colored on long standing.

Add 2 drops of the Molisch reagent to 2 ml. of the sugar solution and mix thoroughly. Incline the tube, and pour down its side, slowly and carefully, 5 ml. of concentrated sulfuric acid.

(*a*) What is the color of the ring formed at the interface of the two liquids?
(*b*) What group of substances give the Molisch test?
(*c*) Why do many proteins give the Molisch test?

Experiment 2. Anthrone Test*

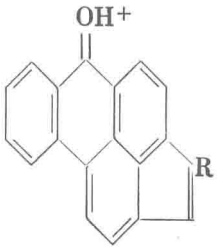
The anthrone test is another general test for carbohydrates. Anthrone, which is the keto form of 9-hydroxyanthracene, reacts with carbohydrates to



produce a green to blue-green color. A suggested formula† concerning the nature of this colored compound is:

* R. Dreywood: Ind. and Eng. Chem., Analytical Ed., **18**:499, 1946.

† From Davidson and Perlman: A Guide to Qualitative Organic Analysis. Brooklyn College Press, 1954.



Add carefully 2 ml. of a 0.2 per cent solution of anthrone in concentrated sulfuric acid to 0.2 ml. of each of the carbohydrate solutions. Also add 2 ml. of a 0.2 per cent anthrone solution to a test tube containing some filter paper pulp. Shake each tube carefully and allow to stand. Notice the color immediately and after a half hour. If a milkiness is produced, dilute carefully with glacial acetic acid or 50 per cent sulfuric acid. (Note: The final sulfuric acid concentration in the test solution should always be greater than 50 per cent.)

(*a*) Is there a change in intensity of the color upon standing?
(*b*) If the test solution contains other organic compounds besides carbohydrates, brown colors may be produced. Why?

Experiment 3. Picric Acid Test

Reducing sugars convert picric acid to red picramic acid.

Mix 2 ml. of each of the carbohydrate solutions with 1 ml. of saturated picric acid solution and 0.5 ml. of 1 *M* sodium carbonate. Place the samples, at the same time, into a boiling water bath, and continue boiling until a color change develops.

(*a*) Which of the sugars you have tried gives the test?
(*b*) Write the equation for the conversion of picric acid to picramic acid.
(*c*) Why can this test be made the basis for a colorimetric determination of sugar in the blood?

Experiment 4. Benedict Test

The Benedict and Barfoed tests are based on the reduction of Cu^{++} to Cu^{+}. In copper reduction tests in alkaline solution, complexing agents such as citrate (Benedict's solution) or tartrate (Fehling's solution) are added to form deep blue soluble complex ions with Cu^{++}. This is done to prevent the precipitation of $CuCO_3$ in sodium carbonate solution (Benedict's reagent), and $Cu(OH)_2$ or CuO in sodium hydroxide solution (Fehling's solution). The oxidation products of carbohydrates in alkaline solution are quite complex and numerous. Not all of them have been identified. Unlike maltose and lactose, sucrose does not reduce Benedict's solution, because it has no free aldehyde or keto group.

Add 8 drops of each of the carbohydrate solutions to 5 ml. of Benedict's qualitative reagent, and shake each tube. Place all the tubes at the same time into a boiling water bath and continue boiling for three minutes. Allow to cool and compare.

(*a*) What is the color of the precipitate?
(*b*) Discuss the chemistry of the test.
(*c*) What compounds other than those of copper may be used?
(*d*) What is the function of the sodium citrate?
(*e*) How does Benedict's reagent differ from Fehling's reagent?
(*f*) What substance in the urine interferes with the Fehling test?

Experiment 5. Barfoed Test

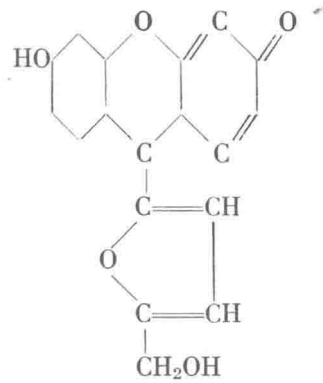
By use of the Barfoed reagent (which contains copper acetate in acetic acid), it is possible to distinguish monosaccharides from disaccharides by controlling such conditions as pH and time of heating.

Add 1 ml. of each of the carbohydrate solutions to 3 ml. of freshly prepared Barfoed's reagent. Place the test tubes at the same time into a boiling water bath and boil for one minute or longer, if necessary, until reduction is noticed.

(*a*) Which of the sugars are oxidized?
(*b*) What is the objection to boiling the solution for too long a time?
(*c*) How does Barfoed's reagent differ from Benedict's reagent?
(*d*) Can the Barfoed test be used in place of the Benedict test for the detection of sugar in the urine?

Experiment 6. Seliwanoff Test

There are several more or less specific reactions for certain carbohydrates; e.g., the Seliwanoff test and the mucic acid test. The Selinwanoff reaction is due to the conversion of fructose by hot hydrochloric acid to levulinic acid and hydroxymethylfurfural, and to the condensation of the latter compound with resorcinol to give a compound of the following type:



Sucrose, which is easily hydrolyzed to glucose and fructose, gives a positive Seliwanoff reaction. Upon continued boiling, aldoses will give a red color with the Seliwanoff reagent, because of their gradual conversion to ketoses by the HCl.

Add 3 drops of each of the carbohydrate solutions to 3 ml. of the Seliwanoff reagent. Place the tubes at the same time into a boiling water bath, and heat until a color develops in one or more of the solutions.

(*a*) Which solution gives the test in the shortest time?
(*b*) Could this test be used to distinguish sucrose from fructose?
(*c*) When glucose or maltose solutions containing the Seliwanoff reagent are heated for a long time, a color will also be produced. What is the reason for this?

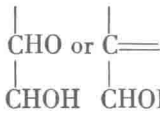
Experiment 7. Galactose. Mucic Acid Test

Galactose may be distinguished from the other monosaccharides by its reaction with HNO_3. Oxidation of other monosaccharides yields soluble dicarboxylic acids, whereas galactose produces an insoluble mucic acid.

Place about 50 mg. of galactose in a test tube and 50 mg. of glucose in another tube. Add 1 ml. of distilled water and 1 ml. of concentrated nitric acid to each. Heat in a boiling water bath in the hood for one and one-half hours. Add 5 ml. of water to each of the tubes, and allow them to stand overnight.

(*a*) Write the equations for the two reactions.
(*b*) What other sugar gives the mucic acid test?

Experiment 8. Phenylhydrazine Test

Carbohydrates (except mannose) which have the  groupings form osazones with phenylhydrazine. Glucose and fructose give the same osazones because these monosaccharides have the same spatial arrangements of the H and OH groups on carbons 3, 4, 5 and 6. Mannose does not form an osazone in water solution, but instead it forms an insoluble phenylhydrazone.*

It is not always possible to identify the sugars according to the melting points of their osazones, because some of these melting points vary only by several degrees. Furthermore, many of the osazones melt with decomposition. The crystal forms of several of the osazones are sufficiently different to help in their identification.

Using a small graduated cylinder, add 5 ml. of a freshly prepared phenylhydrazine solution to a test tube containing 2 ml. of glucose solution and to another tube containing 2 ml. of fructose solution. Place the tubes in a boiling water bath for thirty minutes. Allow the tubes to cool slowly. Remove a small quantity of crystals with a pipette, transfer them to a slide, cover with a slip, and examine under both powers of a microscope. Make drawings of the crystals.

(*a*) How do the crystalline forms of the two sugars compare?
(*b*) Write the equations for the formation of the osazones of glucose and fructose.
(*c*) Which sugars do not give osazones?
(*d*) What is the object of adding sodium acetate to the phenylhydrazine hydrochloride?

* Cason, James: Essential Principles of Organic Chemistry. Englewood, N. J., Prentice-Hall, Inc., 1956, p. 210.

Experiment 9. Inversion of Sucrose

Heat 10 ml. of 1 per cent sucrose solution and 2 drops of concentrated sulfuric acid in a test tube for three minutes. Cool. Neutralize the hydrolysate with saturated sodium carbonate solution, using litmus as the indicator, and perform the Benedict test.

(*a*) Does sucrose give a positive Benedict's reaction?
(*b*) How would the hydrolysate behave towards phenylhydrazine mixture, Barfoed's and Seliwanoff's reagents?
(*c*) Explain the reactions under (*b*).
(*d*) Write the equation for the inversion of sucrose.
(*e*) What methods other than the one above can be used for hydrolyzing sucrose?

Experiment 10. Starch-Iodine Test

The iodine test may be used to distinguish starch from glycogen. The nature of the starch-iodine bonding is not known. One theory suggests the formation of an adsorption complex of starch and iodine, and another suggests that a compound may be formed.

Add 3 ml. of a 1 per cent starch solution to each of three test tubes. To the first tube add 2 drops of water, to the second add 2 drops of 6 *N* hydrochloric acid, and to the third add 2 drops of 6 *N* sodium hydroxide. Mix. To each tube add one drop of a 0.01 *M* iodine solution. Note the color. Heat a tube in which color develops. Cool. Note any change.

(*a*) What substances other than starch give a color with iodine?
(*b*) $3I_2 + 6NaOH \rightarrow 5NaI + NaIO_3 + 3H_2O$
$5NaI + NaIO_3 + 6HCl \rightarrow 3I_2 + 6NaCl + 3H_2O$
What do these equations suggest with regard to the best conditions for performing the test?
(*c*) How would you compare the sensitivity of this test with that of the anthrone test?

Experiment 11. Hydrolysis of Starch

Mix 10 ml. of a 1 per cent starch solution with 3 ml. of 3 *M* hydrochloric acid, and immerse the tube in a boiling water bath. At three minute intervals remove a drop and test (using a test tablet) with 0.01 *M* iodine, and compare with a control. Continue the testing (observing all the color changes) until the point where the iodine color remains unchanged upon the addition of a drop of the hydrolysate. Neutralize a portion of the solution after hydrolysis, with sodium carbonate, and perform the Benedict test.

(*a*) What intermediate substances are formed during the starch hydrolysis?
(*b*) How do these intermediate substances behave towards iodine and Benedict's reagent?

Experiment 12. Preparation of Potato Starch

Wash and peel potatoes. Dice 250 gm. of potato and homogenize for 30 seconds in a motor blender with 200 ml. water. Strain the mixture through

several layers of cheese cloth into a 500 ml. beaker. What is the residue? Discard.

Add 200 ml. water; stir; allow to stand undisturbed until the starch has settled. Decant the supernatant and suspend the starch in 200 ml. water. Decant after it has settled. Suspend the starch in 100 ml. 95 per cent ethanol. Decant. Add fresh alcohol and filter on a Buchner funnel. Spread on a watch glass to dry.

Experiment 13. Time-Course of Starch Hydrolysis

See p. 99 for a discussion of the determination of glucose (Folin-Wu), and p. 82 for photoelectric colorimetry.

1. Prepare 4 test tubes labeled 1 through 4, each with a stopper. Into each, pipette 1.0 ml. of 0.25 *M* Na_2CO_3 and 8.0 ml. water.
2. Prepare a boiling water bath in a 1000-ml. beaker.
3. Suspend 1.0 gm. of potato starch in 100 ml. of 0.5 *N* HCl in an Erlenmeyer flask fitted with a loose stopper. Shake vigorously and immediately place the flask into the water bath. Note the exact time.
4. At exactly 5 minutes pipette 1 ml. of the hydrolysate and add to test tube 1. Mix.
5. Repeat at 15, 30 and 45 minutes.
6. Prepare 9 Folin-Wu tubes labeled Bl, St_1, St_2, St_3, St_4, 1, 2, 3 and 4.
7. Into Folin tube Bl place 2.0 ml. of distilled water; into tube St_1 place 2.0 ml. of the glucose standard containing 0.05 mg. glucose per ml.; into tube St_2 place 2 ml. of the glucose standard solution containing 0.1 mg. glucose per ml.; into St_3 place 2.0 ml. of the glucose standard solution containing 0.2 mg. glucose per ml., and into St_4 place 2.0 ml. of the glucose standard solution containing 0.3 mg. glucose per ml. (Note: Bl = blank, St = standard.)
8. Into each of the Folin tubes labeled 1 through 4, pipette 2.0 ml. of each of the neutralized hydrolysates.
9. To each Folin tube add 2 ml. of the alkaline copper tartrate reagent. Mix by lateral shaking. Place the tubes in a boiling water bath for 6 minutes. Cool in a beaker of cold water.
10. To each Folin tube add 2 ml. phosphomolybdic acid reagent. Mix. When the evolution of CO_2 has ceased, carefully add water to the 25-ml. mark, then stopper and invert several times.
11. Insert the proper filter in the photometer (photoelectric colorimeter) and allow the instrument to warm for 3–5 minutes. Zero the colorimeter with the solution in Folin tube Bl.
12. Read each standard in the photometer. With the data obtained from the readings of the various Folin tubes prepare a calibration curve.
13. Read each unknown in the photometer, and obtain the glucose value of each from the graph.
14. Plot mg. glucose per 100 ml. hydrolysate for each time interval.
15. To each of the neutralized hydrolysates add 1 drop of 1 *N* HCl and 1 drop of iodine solution. Observe the color as compared with that obtained with an unhydrolyzed starch solution.

Experiment 14. Isolation of Glucosazone from a Starch Hydrolysate

Transfer 1 gm. of starch with the aid of 100 ml. of 1 *M* hydrochloric acid to a 250 ml. flask, attach a reflux condenser to the flask, and boil the contents gently to hydrolyze the starch.

At five minute intervals remove a drop of the liquid and test with iodine (Experiment 10). When the blue (starch-iodine) color no longer appears, perform the Benedict test (Experiment 4) on a neutralized sample of the liquid.

Continue boiling gently for thirty minutes after a positive Benedict's reaction has been obtained.

Transfer the hydrolysate to a beaker and, while stirring carefully, add solid sodium carbonate until the hydrolysate is neutral to litmus.

Do several carbohydrate tests on small portions of the neutralized hydrolysate (see Experiments 3, 5, and 6).

To prepare the glucosazone, add 90 ml. of phenylhydrazine mixture to the neutralized hydrolysate and boil gently over a free flame for thirty minutes. (**Caution!** phenylhydrazine is poisonous and should be handled with care. Avoid spilling the reagent or getting it on the skin. In case of such an accident, wash off with dilute acetic acid, followed by water.)

Recrystallize the osazone by dissolving it in a minimum quantity of hot 50 per cent alcohol. Add decolorizing carbon or norite, filter while hot, and cool. Examine the crystals under the microscope. Air-dry and submit the sample to the instructor.

Experiment 15. Glycogen

Grind several fresh oysters with sand in a mortar. Transfer to an evaporating dish, add 10 ml. of water, slightly acidify with acetic acid, and boil gently for fifteen minutes. (**Do not char or evaporate to dryness!**) If necessary, add 1 or 2 ml. of water. Filter. Test 2 ml. of the filtrate with 0.01 *M* iodine. In order to test the glycogen with the dilute iodine solution, use a control solution containing 2 ml. of water. Add iodine solution, drop by drop, and in the same amounts, to each. Note the development of color.

Hydrolyze 5 ml. of glycogen solution. For the hydrolysis and subsequent testing, repeat as under Experiment 14.

(*a*) Compare the iodine-glycogen test with the iodine-starch test.

(*b*) Compare glycogen and starch as to their reaction with (1) phenylhydrazine; (2) Benedict's solution, and (3) anthrone.

(*c*) In addition to the use of acid, what other methods can be used for hydrolyzing starch or glycogen?

Experiment 16. The Identification of Sucrose in the Presence of Glucose

To a mixture of 2 ml. of 1 per cent sucrose and 2 ml. of 1 per cent glucose, add 6 ml. of Benedict's qualitative reagent and immerse in a boiling water bath for ten minutes. Cool. Filter. Add 6 ml. of Benedict's solution to the filtrate and immerse in a boiling water bath again. Cool. If no reduction takes place, what does this show?

If reduction does occur, filter and treat the filtrate with Benedict's solution

as before. Continue the process until no more reduction occurs. Add concentrated hydrochloric acid, a little at a time, to the filtrate until it is neutral. Now add 2 drops of concentrated hydrochloric acid and boil for three minutes. Cool. Neutralize with 0.1 *M* sodium hydroxide. Add 6 ml. of Benedict's solution and immerse in a boiling water bath.

(*a*) What is the result?
(*b*) Explain what has happened.
(*c*) What sugars are obtained when sucrose is hydrolyzed?
(*d*) How could you prove your answer under (*b*)?

Experiment 17. Identification of an Unknown Sugar

Devise your own procedure to identify the carbohydrate your instructor gave you as an "unknown."

POLARIMETRY

Ordinary light is a wave motion in which the vibrations take place in all planes perpendicular to the direction of propagation. Light caused to pass through certain substances emerges changed in such a manner that it vibrates in but a single plane, and is said to be *plane polarized.* Calcite (crystalline calcium carbonate) is frequently used to polarize light. However, the crystal causes the light to be polarized in two planes perpendicular to each other. Two wedge-shaped pieces of calcite cemented together with Canada balsam constitute a Nicol prism. Such a prism will remove one of the polarized rays and not the other (Fig. 1). The light on entering the prism is divided into

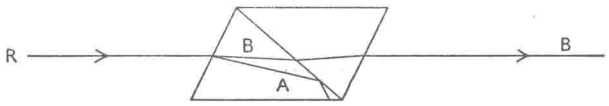


Fig. 1.

two rays, *A* and *B*. Ray *A* is bent more than *B* and is reflected from the cemented surface and absorbed in the black varnish coating the prism. Ray *B* emerges from the prism plane, polarized.

Many substances in solution have the power to rotate the plane of polarized light. Such substances are said to be *optically active.* If the rotation is measured under standardized conditions, the specific rotation is given by

$$\left[\alpha\right]_{D}^{t} = \frac{100\,\lambda}{lc}$$

where

α = specific rotation.
t = the temperature at which the measurement is made
D = the line for sodium vapor (5890 Å)
λ = observed angle of rotation
l = length of the tube in decimeters
c = the number of grams of material dissolved in 100 ml. of solution

If the specific rotation of a substance is known, the concentration of the sugar solution of unknown concentration is determined by the following relationship:

$$c = \frac{100\,\lambda}{\left[\alpha\right]_D^t l}$$

The percentage of substance in solution (parts by weight in 100 parts by weight of solution) may be obtained if the density (d) of the solution is known, by substituting $p \times d = c$ and solving for p.

$$\text{Therefore, } p = \frac{100\,\lambda}{\left[\alpha\right]_D^t \times l \times d}$$

The instrument used to determine the extent of rotation is the *polarimeter*. A special form designed for the examination of sugar solutions is called a *saccharimeter*. These instruments vary in design, but in principle are much the same. Monochromatic light (usually sodium D line) from the source, *L* (Fig. 2), is passed through the lens, *B*, which renders the rays parallel. It then passes through a Nicol prism, *P*, called a polarizer. The thin quartz

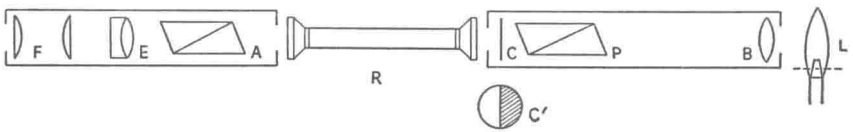


Fig. 2.

plate, *C*, half covers the field and is of such thickness as to alter the phase of the light by half a wavelength. The light still remains plane polarized, but half the field is shadowed. The light then traverses the column of liquid to be examined in the tube, *R*, and thence through the analyzer, *A*. The field of view is observed through the telescope, *E F*. Near the eyepiece is a recording disc which registers in fractions of a degree the rotation of the analyzer when the two light fields are of equal intensity. (See also Fig. 3.)

Substances which rotate the beam of polarized light to the right are called *dextrorotatory*, and those rotating it to the left, *levorotatory* (not to be confused with the D- and L- forms, which indicate chemical structural relationships and not rotations).

Some freshly prepared sugar solutions change their rotation with time. This is called *mutarotation*. When measuring the rotation of such substances, it is important to make certain that equilibrium among the various forms has taken place.

Before using the instrument, consult the specific directions which come with the polarimeter or saccharimeter, or consult your instructor.

Calculation of Specific Rotation: Twenty grams of sucrose are weighed and

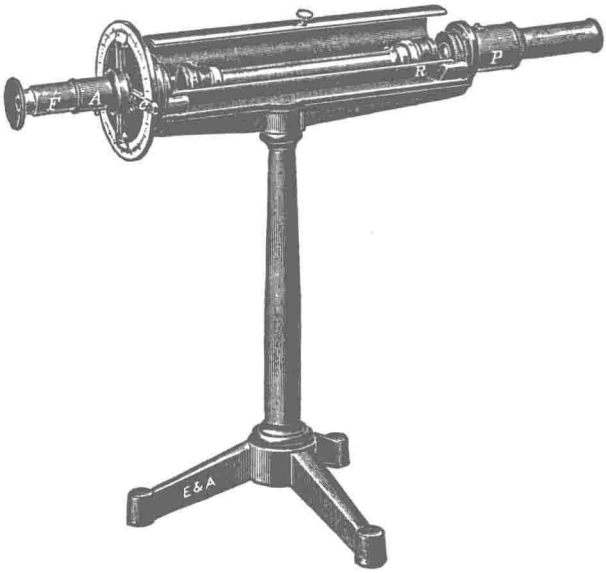


Fig. 3.

dissolved in water to 100 ml. This solution gives an angular rotation for sodium light of +53.2° in a 400 mm. tube at 20°C.

Calculate the specific rotation of sucrose for the given concentration.

Experiment 18. Determination of Specific Rotation

Rinse a polarimeter tube twice with 5 to 10 ml. portions of distilled water, and then fill to the top with water, and stopper. Place in the polarimeter and rotate the analyzer of the polarimeter until both halves of the circular field seen through the eyepiece are equally illuminated. Change the analyzer setting and again bring the analyzer to the point where the field is uniform and obtain another reading. In this way take three readings and calculate the average. This is the zero reading of the polarimeter.

Dissolve 2 gm. of D-glucose in about 95 ml. of distilled water in a 100 ml. volumetric flask. Add several drops of concentrated ammonium hydroxide, or some solid sodium carbonate, until 1 drop of the sugar solution gives a red color to 2 drops of phenolphthalein solution (which is used as an outside indicator). Now make up to 100 ml. with distilled water. Mix well.

Rinse the polarimeter tube at least twice with 5 to 10 ml. of the clear, colorless sugar solution, fill with that solution, and take at least three readings in the polarimeter as before. Calculate the average of these readings. Calculate the difference between the zero reading and the reading of the optically active solution. Record the temperature.

Repeat this procedure with sucrose and maltose. Calculate the specific rotation of each sugar and compare with those given in the literature.

(*a*) Why is it necessary to add ammonium hydroxide or sodium carbonate to each reducing sugar before reading in the polarimeter?