A COURSE IN

PRACTICAL BIOCHEMISTRY

FOR STUDENTS OF MEDICINE

BY

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PRACTICAL BIOCHEMISTRY

实用生物化学

PREFACE TO THE FIFTH EDITION

As in previous editions, we have made few alterations, on the principle that a practical text designed to be used in training students should, if anything, tend to err on the conservative side, employing old tested methods rather than newer, less tested ones.

In this edition we have revised the tests concerned with the clotting of blood, in order to illustrate the chief features of Quick's theory.

We have also included brief accounts of the estimation of hippuric acid excretion as a measure of liver function, and of the estimation of urobilinogen in urine, and have revised the description of a basal metabolism determination to conform more closely to present clinical apparatus.

No attempt has been made to incorporate an account of any of the photo-electric colorimeters, since it is felt that while these expensive types of apparatus can be demonstrated to students, yet there can be but few laboratories in which individual medical students are permitted to carry out experiments with them.

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WINNIPEG.

PREFACE TO THE FIRST EDITION

THIS book consists of a series of exercises designed to train medical students in the practical tests and procedures used in biochemical work. It is in no sense a reference book, though it should afford a useful introduction to the larger texts such as Hawk and Bergeim's "Practical Physiological Chemistry." To this we have been especially indebted as a reference book both for practical teaching and for clinical procedures for many years.

The purposes of a course in Practical Biochemistry such as that given in this volume should be in the first place to demonstrate the accuracy of a reasonably large number of statements taught in lecture courses, in the second place to afford some training in the technique of the quantitative procedures used in the science, and in the third place to continue to inculcate those methods of scientific accuracy of observation, deduction, and measurement in which presumably previous training has been given in the more basic sciences of chemistry and physics.

Such a course designed for medical students is circumscribed (and justifiably circumscribed) by the more limited time which their curriculum affords for the study of biochemistry than can be allowed in a pure science course, and by the desirability of utilising such portions of the subject and such procedures as have a bearing upon their subsequent medical course and the clinical applications of the subject. Hence certain more complex procedures, such as the electrometric determination of hydrogen-ion concentration, have been deliberately excluded as unnecessary and relatively undesirable in a course for which only a limited amount of time should be provided.

This book is designed to be used with any of the smaller theoretical text-books of biochemistry, although it more particularly follows the order of the "Text-book of Biochemistry" by the senior author. The work covered is that

which has been given for the past several years to the students of medicine in the University of Manitoba, with a few additions to facilitate that selection of work which every teacher prefers. All methods given have been tested repeatedly.

In the quantitative section only one approved method has been usually detailed for each estimation. References to others have been given, with comments on them in so far as our experience seemed to warrant such comments. The order of the quantitative exercises has been selected mainly from the standpoint of gradually increasing difficulty; thus exercises on urine constituents precede those on blood. The exercises illustrate as many different types of procedure as possible, with the exception of gravimetric procedures.

Matter extraneous to the purposes outlined has been excluded as far as possible, since large and costly handbooks seem both undesirable and unnecessary for the student at this stage; he merely becomes confused with a multiplicity of detail. He is presumed to be well acquainted with the ordinary types of chemical apparatus, and the illustrations have therefore been limited to a minimum.

Demonstrations have been introduced throughout the course. These we have found to serve two useful purposes; many new procedures are most easily taught to students in groups before these students themselves attempt them; in tests involving use of costly chemicals the wastage is very materially decreased.

A note at the head of each exercise indicates the time that we have found by experience to be adequate.

In the quantitative exercises calculation formulae have been rigidly excluded, since we have long found that the majority of students use them without comprehension, and often through lack of comprehension use them wrongly.

Colorimeters, and certain other pieces of apparatus, are too costly to be purchased in large numbers for students' courses by most universities. A simple system of alternating groups of students renders such large expenditures unnecessary.

No attempt has been made to obtain unusually fine crystal preparations for the micro-photographs, but rather we have sought to reproduce such pictures as students themselves, following the directions in the text carefully, may reasonably expect to obtain.

Our thanks are due to Professor William Boyd, of the Department of Pathology of this University, and his technician, Miss L. Nason, who have made these micro-photographs from our preparations, and to Messrs. J. & A. Churchill, for permitting us to reproduce the first two plates from Cameron's " Biochemistry."

We desire to express our appreciation of the co-operation of Messrs. J. & A. Churchill, whereby the rapid publication of this volume has been greatly facilitated.

A. T. C.
F. D. W.

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PART I

QUALITATIVE PROCEDURES

GENERAL INSTRUCTIONS

STUDENTS should not be satisfied with any result which is not in accordance with that stated in the text-book. If repetition does not bring agreement, assistance should be sought to explain the discrepancy. One of the most frequent sources of error is the use of **dirty test-tubes.**

Students should learn as soon as possible to use only small quantities of materials and of reagents. A test, especially a test requiring the application of heat, can always be carried out more satisfactorily in a test-tube one-third filled with liquid than in one half filled. Students should familiarise themselves with the appearance of 1, 2 and 5 c.c. of liquid in a test-tube.

Dilute reagents should always be used unless concentrated reagents are specifically called for in the text.

Reagents must never be poured back into reagent bottles. Since many reagents are expensive, only the small amounts actually required should be used.

All procedures in which noxious or unpleasant odours may be produced should be carried out in a fume-chamber.

All procedures in which ether is being evaporated must be carried out away from the vicinity of flames.

When concentrated acids or alkalies have to be pipetted, connect a 6 or 8-in. length of rubber tubing to the pipette and apply suction to the tubing (and not directly to the pipette).

Since some students only learn to appreciate inhibitory instructions by the unpleasant results following failure to observe them a few simple first aid procedures are given in Appendix III.

EXERCISE I

DETECTION OF THE ELEMENTS IN ANIMAL AND PLANT MATERIAL

(One or two Three-hour Periods)

THE delicacy of the tests employed determines to a great extent what elements can be detected in animal and plant material; the more delicate the tests, the greater the list of elements which can be shown to be present.

In this Exercise a few of the easier tests are detailed; these apply to the elements present in largest amount, with the exception of oxygen; the presence or absence of this element can only be determined by quantitative procedures.

Experiment 1. Place a little stearic acid (an organic compound) in a porcelain crucible and heat it over a Bunsen flame *in the fume-chamber*. It melts, chars, burns with a smoky flame (both charring and the smoky flame indicating the presence of **Carbon**), and on continued heating completely oxidises, leaving no **Ash.** Repeat with a little cane sugar. Similar results are obtained.

Experiment 2. Repeat (1), using a little powdered sodium chloride (an inorganic salt). There is no charring. The sodium chloride may partly fuse, but remains otherwise unaltered.

Now take a little of a salt of an organic acid, such as sodium citrate, and treat it in the same way. Charring takes place. The carbon gradually disappears, and a colourless or slightly grey **Ash** is left.

Plant and animal materials are usually mixtures of organic and inorganic constituents. If subjected to similar treatment part will burn away, and ash will remain.

Experiment 3. Repeat (1), using a tiny piece of lean meat. The charring, as before, signifies the presence of **Carbon.** An unpleasant odour—the odour of " burning flesh "—becomes noticeable, and this is characteristic of *proteins* containing **Nitrogen.** After ten or fifteen minutes a slightly grey ash remains. (The greyness is due to traces of unburnt carbon.)

Repeat, using instead of the meat a fragment of cabbage leaf. Similar results will be obtained.

Comparison of the results of Experiments 1 and 3 illustrates the fact that cane sugar contains no nitrogen and no

mineral constituents. The following are more specific tests for certain of the elements :—

Carbon and Hydrogen

Experiment 4. Warm some cupric oxide in a dry test-tube to drive off any water that may have condensed with it. Then mix with it in a dry mortar about one-fifth the amount of powdered lactose (milk sugar). Transfer sufficient of the mixture to fill one-third of a dry test-tube. Close this with a perforated cork through which passes a bent glass tube. Allow the other end of the tube to dip into either lime water or baryta water in a second test-tube. Gently heat the test-tube containing the mixture over a Bunsen flame ; gas will bubble through the lime (or baryta) water, and gradually a cloudiness will appear. This is due to the formation of insoluble carbonate, and that indicates the presence of carbon dioxide in the gas evolved, and therefore of carbon in the original material.

Note that drops of *water* condense on the upper (cool) end of the heated tube. These obviously indicate the presence of **Hydrogen** in the original material (provided that material was properly dried).

Nitrogen

Experiment 5. Whenever a preliminary heating of plant or animal material gives the characteristic odour of burning flesh (horn, hair, feathers, etc.), this in itself is reasonable evidence that the element nitrogen is present. Its presence can be confirmed in either of the two following ways :—

(i.) Take a little dried (commercial) egg or blood albumin, and mix it with ten to fifteen times its volume of soda lime in a mortar. Transfer enough to a test-tube to one-third fill it, and heat over a Bunsen flame in the fume-chamber. The smell of ammonia should be noticeable. Hold at the mouth of the tube a piece of moistened red litmus paper. It will be turned blue. (The ammonia gas dissolves to give an alkaline solution.) Then hold at the mouth of the tube a glass rod that has been dipped in a **test-tube** containing $\frac{1}{2}$ c.c. of concentrated hydrochloric acid. (*Do not dip the rod in the bench reagent bottle.*) Dense fumes result. The ammonia and hydrochloric acid vapours have combined to give **solid** ammonium chloride.

The formation of ammonia in this test indicates the presence of nitrogen in the original substance. Most plant and animal materials when heated with strong alkali or acid liberate their nitrogen as ammonia.

(ii.) Mix thoroughly some dried albumin with about ten times its weight of a mixture of equal parts of magnesium powder and anhydrous sodium carbonate. One-tenth fill a dry test-tube with this mixture and heat in the fume-chamber. Gradually increase the heat until finally the mixture is kept at red heat for half a minute. Then immediately dip the red-hot tube into a beaker containing about 3 to 4 c.c. of distilled water. The bottom of the tube breaks up to small fragments, and its contents partly dissolve in the water. Filter. Add to the filtrate 2 drops of a cold saturated solution of ferrous sulphate (freshly prepared) and a drop of ferric chloride solution. Warm the mixture for two or three minutes, then cool it, and then just acidify with concentrated hydrochloric acid. The solution becomes bluish-green, and gradually a precipitate of " Prussian blue " (ferric ferro-cyanide) separates out.

In this test during the fusion sodium cyanide is formed from part of the nitrogen of the albumin, and this, treated with ferrous sulphate and ferric chloride and warmed, is converted to " Prussian blue." The production of this coloured compound therefore indicates the presence of *nitrogen* in the substance that is being tested.

Sulphur

Experiment 6. Note that in the following tests a negative result for " loosely combined sulphur " does not prove that sulphur is absent from the substance under test. A fusion test is essential to determine absolutely the presence or absence of sulphur.

(i.) Add 2 drops of a solution of neutral lead acetate to a few cubic centimetres of sodium hydroxide solution. A precipitate of lead hydroxide is first formed and then redissolves. Add a very little powdered albumin to this solution and warm. The mixture turns black. Part of the sulphur in the albumin molecule has been split off to form sulphide, and this reacting with lead ions has given insoluble black lead sulphide. The sulphur capable of reacting in this way is spoken of as " *loosely combined sulphur.*"

(ii.) Fuse some of the albumin-magnesium powder-sodium carbonate mixture as in Experiment 5 (ii.), and after breaking the tube in water and filtering add to the filtrate a *freshly prepared* solution of sodium nitroprusside. A reddish-violet colour appears. The sulphur of the albumin has been converted into sodium sulphide, and sulphides give this colour reaction with the nitroprusside.

(iii.) Take enough dried albumin to cover $\frac{1}{2}$ in. of a penknife blade, place it in a porcelain crucible, then add $\frac{1}{2}$ in. of stick potassium hydroxide and about as much potassium nitrate as albumin. Heat strongly. Cool. Treat with water and filter.

Acidify the filtrate with hydrochloric acid. Add barium chloride (4 or 5 drops). A white precipitate of barium sulphate is produced. The sulphur present in the albumin has been oxidised to sulphate.

Phosphorus

Experiment 7. Fuse some casein, or some nucleo-protein, or some lecithin (all of which contain phosphorus in organic combination) just as in Experiment 6 (iii.). Cool, treat with water and filter. Mix 3 c.c. of the filtrate in a test-tube with 2 c.c. of concentrated nitric acid and 4 or 5 c.c. of ammonium molybdate solution, and warm gently for some time. A yellow crystalline precipitate of ammonium phospho-molybdate gradually separates along the side of the tube. The formation of this precipitate indicates the presence of **Phosphorus** in the original substance.

Iodine

Experiment 8. Mix somewhat less desiccated thyroid tissue than will be held on a (copper) farthing or one-cent piece with five or six times its weight of anhydrous sodium carbonate in a porcelain crucible, add 2 drops of concentrated sodium hydroxide and heat over a Bunsen flame in the fume-chamber, gradually increasing the heat to redness. After ten minutes cool and place in a beaker containing 15 to 20 c.c. of distilled water. Warm. Filter. Transfer part of the filtrate to a test-tube, slightly acidify with concentrated hydrochloric acid, add 1 c.c. of chloroform and then 2 or 3 drops of chlorine water. Shake up and allow the layers to separate. The chloroform layer will become pinkish-violet in colour.

The fusion liberates iodine from organic combination to form sodium iodide. Chlorine displaces iodine from this salt, and iodine in chloroform is coloured as described. Excess of chlorine must be avoided, since it forms with iodine colourless iodine chloride.

Iron

A simple test for the detection of iron is given in Exercise XII., Experiment 9.

EXERCISE II

THE CARBOHYDRATES

(Four or five Three-hour Periods)

THE important groups of carbohydrates that will be studied are the simple sugars (monosaccharides), typified by *glucose*, the complex sugars (disaccharides), typified by *cane sugar* and *lactose*, and the polysaccharides, typified by *starch* and *cellulose*.

In each of these divisions it is necessary to study also other compounds closely related to the types—fructose, galactose, and arabinose, related to glucose, maltose, related to lactose, and glycogen, the dextrins, and inulin, related to starch.[1]

Glucose

The tests for glucose depend upon both chemical properties and physical properties. On account of its easy oxidisability, it brings about certain "reductions" and is hence a reducing sugar. It reacts with phenylhydrazine to form a characteristic yellow crystalline "osazone," which can be identified under the microscope. It is decomposed by certain ferments in yeast with production of ethyl alcohol and carbon dioxide gas, so that the evolution of gas serves as a test for it. It rotates the plane of polarisation of polarised light, and can therefore be tested for in the polarimeter. It is readily soluble in water.

The following tests should be carried out with a 1 per cent. solution of glucose :—

General Test for Carbohydrates (Molisch's Test)

Experiment 1. Add to 5 c.c. of glucose solution in a test-tube 2 drops of the special *Molisch's reagent* (made by dissolving 5 gm. of α-naphthol in 100 c.c. of ethyl alcohol). Shake. Pour care-

[1] Before and while carrying out the tests in this Exercise the chapters on carbohydrates in a theoretical text-book should be consulted, *e.g.* Cameron's "Biochemistry," 6th edit., Chapter IV.

fully down the side of the inclined test-tube 5 c.c. of concentrated sulphuric acid. With care scarcely any mixing takes place.

A reddish-violet colour is produced at the surface between the two solutions. (A greenish colour is to be neglected.)

This test is given by all soluble carbohydrates, and a similar colour is developed on the surface of insoluble carbohydrates under appropriate conditions.

Reduction Tests

Copper solutions are reduced with precipitation of cuprous oxide (red or yellow according to variations in the experimental conditions), certain silver solutions are reduced with precipitation of metallic silver, and certain bismuth solutions with precipitation of metallic bismuth. Such results are due to the ease with which glucose and related sugars can be oxidised. This is usually attributed to the presence (or potential presence) of an aldehyde or ketone radical in the carbohydrate molecule.

Trommer's Test

Experiment 2. To 3 c.c. of glucose solution in a test-tube add an equal volume of 40 per cent. sodium hydroxide. Then add drop by drop a *very dilute* solution of copper sulphate. A deep blue solution is produced. Continue adding copper sulphate until there is the faintest turbidity. Warm nearly to boiling. A yellowish-red precipitate of cuprous oxide separates.

Reduction of Fehling's Solution

FEHLING'S SOLUTION. This solution decomposes fairly rapidly, so that its two parts must be kept separately. Part I consists of 34·65 gm. crystallised copper sulphate, $CuSO_4$, $5H_2O$, dissolved in water, and water added to 500 c.c. Part II consists of 125 gm. of potassium hydroxide and 173 gm. Rochelle salt (sodium potassium tartrate) dissolved in water, and water added to 500 c.c.

Experiment 3. Mix 1 c.c. of each of the two parts of Fehling's solution in a test-tube. Note the appearance of a deep blue colour, due to the formation of a complex copper tartrate. Add an equal quantity of water. Boil. No change of colour and no reduction should take place. If any does occur the Fehling's solution has decomposed and must be replaced by fresh solution. If the solution is proved to be satisfactory, then—

As before, mix equal quantities of the two solutions (1 c.c. of