PRACTICAL METHODS

IN

BIOCHEMISTRY

BY

FREDERICK C. KOCH

Late Frank P. Hixon Distinguished Service Professor Emeritus of Biochemistry, University of Chicago. Late Director of Biochemical Research, Armour & Co., Chicago

AND

MARTIN E. HANKE

Associate Professor of Biochemistry, University of Chicago

SIXTH EDITION

LONDON

BAILLIÈRE, TINDALL & COX, LTD.

1953

PREFACE TO THE SIXTH EDITION

This edition contains several new experiments which have been found to work well in our medical courses in the last few years: separation and identification of amino acids by paper chromatography and ion exchange resins; studies in enzyme kinetics; manometric study of succinic dehydrogenase in a modified Warburg apparatus; saponification number with diethylene glycol as solvent; the estimation of pepsin by the hemoglobin method; and the microbiological estimation of methionine. There has been some reorganization of chapter headings and corresponding relocation of certain experiments. Under a new chapter heading, Some Biochemical Preparations, have been gathered several preparations which previously appeared under other headings, and several new preparations have been added: crystalline egg albumin, cytochrome c, hippuric acid, and creatinine. Under the heading, Manometric Methods with the Van Slyke-Neill Apparatus, is included a general discussion of this versatile instrument; the old experiments on estimation of CO_2, O_2, and urea in blood and plasma; and finally several new experiments on the estimation of amino nitrogen, amino acids with decarboxylases, and the analysis of gas mixtures. Under the heading, Enzymes, are the preparation of arginase and crystalline urease; several new experiments on the kinetics of these enzymes and of ptyalin, including the estimation of Michaelis constants and enzyme-inhibitor constants; and finally the previously mentioned experiment on succinic dehydrogenase. In the chapter on Colorimetric Methods for Vitamins, new and improved procedures for ascorbic acid and vitamin A have been introduced. The chapter on Microbiological Methods has been reorganized with new experiments on the estimation of methionine and cobalamine. Other minor additions are a colorimetric method for blood glucose on 0.1 ml. samples of blood; an experiment on the effect of pH on the distribution of chloride between blood cells and plasma; and the estimation of serum proteins by the biuret method.

Acknowledgment is due to other members of the staff of the Department for initiating new experiments, and to students whose experience has been very helpful in determining the practicability of certain procedures. The proof reading by Maude Martin Hanke has been invaluable.

MARTIN E. HANKE

PREFACE TO THE FIRST EDITION

This laboratory manual is intended to present for medical students the more important qualitative and quantitative chemical aspects of cell constituents, of cell activities and of the composition of blood, secretions, and excretions. An attempt has been made to improve the application of certain qualitative reactions by conducting the tests in a comparative manner on known concentrations of the substances under investigation. The data obtained should be more significant and the observing student may thereby develop a better quantitative sense and a more critical attitude toward the interpretation of laboratory methods and observations. As a result of this mode of presentation many solutions of known strengths must be provided. It is assumed that the majority of these are prepared by the laboratory staff. Nevertheless, detailed instructions for the preparation of these solutions and reagents are given in the appendix so that the student should be able to prepare the reagents himself and use them intelligently. In the strictly quantitative procedures for blood and urine, I have attempted to give only the more accurate of the rapid and practical methods rather than confuse the student with numerous methods, some of which are of doubtful or historical value or which may be of use only in very special studies. Although this manual is intended primarily as the practical companion to Professor A. P. Mathews' textbook, nevertheless it contains considerable explanatory matter in order to help correlate the theoretical and laboratory aspects of the subject matter. However, no attempt has been made to interpret the significance of the results in blood and urine analysis.

The author should acknowledge that much use is made of the manual previously published by Professor A. P. Mathews as a part of his textbook on physiological chemistry, of numerous standard textbooks, larger works, current journals, and monographs. He also wishes to acknowledge his indebtedness to associates and assistants who have helped from time to time to make these laboratory instructions more reliable.

F. C. Koch.

1934

INTRODUCTION

The beginner in biochemistry should have good fundamental training in chemistry, physics, and general biology. Chemistry is so important that the preliminary work should include a good course in the theoretical and practical aspects of quantitative analysis, a working knowledge of the application of the fundamental laws of physical chemistry to solutions and gases, and the mastery of the more important fundamental facts and concepts of organic chemistry. Biochemistry is the application of these laws, concepts, facts, and methods to the study of any field in biology. Every biological activity involves some chemical change in one or more constituents in the system under consideration. These biological systems are more or less specific in character so that frequently some information can be obtained from rather crude qualitative studies. More often, however, it is only when such methods become truly quantitative in character that we make a true advance in our knowledge which ultimately is applied quantitatively in medicine, agriculture, or industry. It is, therefore, particularly important that the student develop the habits of thinking quantitatively and of manipulating neatly and accurately. The student may recall that in quantitative analysis and in physical chemistry the detailed laboratory instructions must be followed most carefully, and that, similarly, in organic chemistry the best yield (often less than 50 per cent of the theoretical) and purity of product are obtained only after the process has been worked out carefully after numerous trials. In biochemistry, the situation is many times more complex; due, first, to the labile character of the numerous organic compounds present in biological systems; second, to the presence of catalytic agents which hasten these processes, and third, to the peculiar colloidal condition of many of the constituents therein. It should be obvious then that we find it necessary to work out detailed methods and to adhere to these details still more carefully in biochemistry. In many cases these instructions are based upon empirically devised experiments partly because the systems are so complex in nature and partly because of the present limited state of our knowledge. In other words, in biochemistry we aim to combine the quantitative methods of the chemist with the comparative modes of study of the biologist.

Although the instructions in this manual are written on the assumption that the student understands the terminology used for standard solutions of various kinds and their values in calculations, and that he is familiar with general preparative and quantitative laboratory procedures, it was thought desirable, nevertheless, to include such information and instructions in the appendix.

TABLE OF CONTENTS

PART I. THE CHEMISTRY OF CELL CONSTITUENTS

PART II. THE CHEMISTRY OF THE DIGESTIVE TRACT

PART III. BLOOD AND URINE

PART IV. ENZYMES, VITAMINS AND HORMONES

PART I
THE CHEMISTRY OF CELL CONSTITUENTS

CHAPTER 1

CARBOHYDRATES

The action of bases on carbohydrates. The carbohydrates are very weak acids and as such form salts with bases. These salts vary remarkably in stability depending on the character of the carbohydrate. Those carbohydrates which contain a free aldehyde or ketone group yield very unstable salts with bases whereas all the other carbohydrates which do not contain such free groups form very stable salts with bases. The unstable form of carbohydrate salts obtained from aldoses and ketoses presents a very complex series of reactions, the speed, character, and extent of which depend (a) on the concentration and character of the aldose or ketose, (b) on the concentration and character of the base, (c) on the time and temperature, and (d) on the presence or absence of various oxidizing agents and of salts. Thus, we may have (a) a simple tautomeric change such as the conversion of d-glucose into levulose or vice versa, (b) the oxidation of an aldose, as glucose, into the corresponding acid, gluconic acid, (c) the intermolecular oxidation-reduction reactions with the formation of acids and alcohols, (d) the intramolecular oxidation-reduction reactions with the formation of saccharinic acids, (e) the breaking of the monosaccharide chain into smaller reducing particles, and (f) the oxidation or condensation of these smaller particles.[1,2,3,4]

Experiment 1. Bases form caramel and humus from reducing sugars (Moore's Test). To test tubes transfer 1 cc. of M/30 glucose, lactose, saccharose, and 0.7 per cent starch solution. To each next add 1 cc. of 1 per cent NaOH solution. Mix each well and immerse all at the same time in boiling water. Heat for five minutes and observe the odor and color from time to time.

Experiment 2. Only those carbohydrates which form humus with bases easily reduce cupric ions. Prepare a Fehling's mixture by taking 1 cc. of each Fehling's solution and dilute the mixture to 20 cc. To each tube from experiment 1, now add 5 cc. of the diluted Fehling's solution. Mix each well and continue the heating for another five minutes. The formation of a yellowish or reddish cuprous oxide precipitate is due to the reducing action of the carbohydrate in alkaline solution.

Experiment 3. The tautomeric change of aldoses to ketoses and vice versa in weakly alkaline solutions. Prepare 6 test tubes as in-

[1] J. Nef, *Ann. der Chem.*, 1907, 357, 214 and 1913, 403, 204.
[2] A. P. Mathews, *Am. J. Physiol.*, 1907, 19, 199 and *J. Biol. Chem.*, 1909, 6, 3.
[3] H. A. Spoehr, *The Carbohydrate Economy of Cacti*, 1919, pp. 10–23.
[4] W. L. Evans, *Chem. Rev.*, 1929, 6, 281.

dicated in table 1. Note that you are using one-tenth the concentration of levulose as of glucose. Shake each tube well, then add a layer of toluene about ½ inch deep, stopper and set aside over night. The toluene is added to retard oxidation from the air and to retard bacterial decomposition in the (b) tubes.

After standing for the proper length of time, prepare fresh Seliwanoff's reagent as follows: To 3.6 cc. of the 0.5 per cent solution of resorcinol in alcohol add 12 cc. concentrated HCl, dilute to 36 cc. with water and mix well. Now add 5 cc. of this reagent to each tube above, mix the contents of each tube well and immerse in boiling water at the same time. Compare the speed of development, intensity, and shade of color in the different tubes. The most intense color, yellow to red, is an indication of the highest ketose concentration because ketoses react more readily than aldoses in strongly acid solutions to yield hydroxy-

TABLE 1

PREPARATION OF TEST TUBES IN EXPERIMENT 3

TUBE NUMBER	0.5 CC. OF SATURATED $Ba(OH)_2$ AND 0.5 CC. OF	TUBE NUMBER	0.5 CC. OF DISTILLED WATER AND 0.5 CC. OF
1a	M/100 levulose	1b	M/100 levulose
2a	M/10 glucose	2b	M/10 glucose
3a	M/10 lactose	3b	M/10 lactose

methyl furfural, which then condenses to a colored compound with resorcinol. Note that 1b is more intense than 1a, but that 2a is more intense than 2b, and 3a more so than 3b. In other words levulose, a ketose, has been converted in part into a non-ketose; glucose, an aldose, has yielded more ketose; and lactose, a disaccharide aldose has also yielded some ketose.

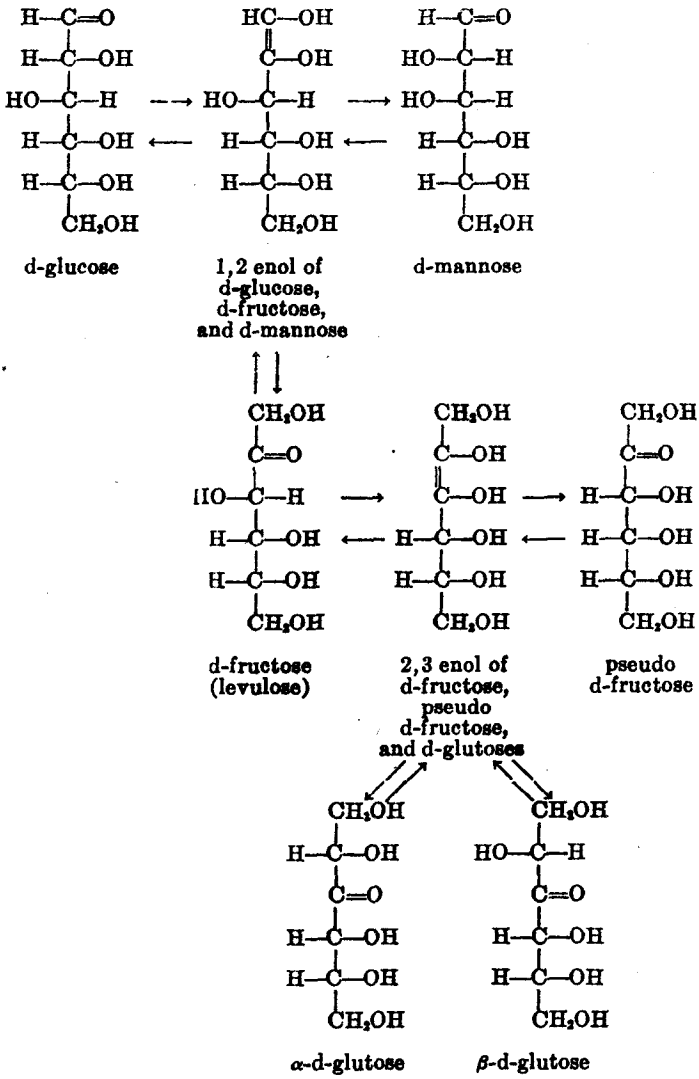
This is referred to as the de Bruyn and van Ekenstein[5] rearrangement. These rearrangements are, however, limited to the first three carbon atoms beginning with the aldehyde group. In the case of d-glucose and levulose the limited rearrangements, and the reversibility thereof are illustrated by the structural formulae on the next page. A similar rearrangement and reversibility exists between galactose, d-talose, d-tagatose, d-sorbose, and α- and β-galtose.

Experiment 4. Aldoses and ketoses in alkaline solutions take up atmospheric oxygen to form acids (however, acids are also formed without oxygen gas.[6] (This is intended as a demonstration.) Two

[5] *Rec. des. trav. chim. des Pays-Bas, 1895,* **14,** *203.*

[6] A. P. Mathews, *J. Biol. Chem.*, 1909, **6**, 3.

500-cc. Florence flasks are provided with two-hole rubber stoppers. Through one hole passes a bent tube provided with a glass stopcock



and through the other an open glass manometer tube about 10 inches long. The latter tube contains a 6-inch column of mercury.

Into each flask are introduced 50 cc. of an approximately normal NaOH solution and 5 grams glucose. After complete solution, 10-cc.

amounts from each flask are titrated with normal acid by phenolphthalein. Flask A is stoppered at once and after adjusting the inside pressure to atmospheric pressure the stopcock is closed. In flask B, displace the air by a rapid current of hydrogen from a tank and stopper and adjust to atmospheric pressure in the same way. Both flasks are next shaken rather vigorously for three hours at room temperature. During the course of the shaking, note the differences in the color and the pressures as shown by the manometers. Also finally titrate 10-cc. volumes of each solution.

The results in table 2 are typical values.

TABLE 2

RESULTS IN EXPERIMENT 4

	FLASK A SHAKEN IN AIR	FLASK B SHAKEN IN HYDROGEN
Negative pressure (mm. mercury)	40	0
Original titration for 50 cc	63 cc. N	63 cc. N
Titration for 50 cc. after 3 hours' shaking	55.85 cc. N	57.5 cc. N
Acids formed (differences)	7.15 cc. N	5.5 cc. N
Difference between A and B	1.65 cc. N	

Experiment 5. Further evidence that bases dissociate reducing sugars into reducing particles and that these particles may then be oxidized with ease even in an acid solution. Carefully prepare tubes 1 and 2 as indicated below making all measurements by means of pipettes or burettes:

	Tube 1 cc.	Tube 2 cc.
M/10 glucose	1	1
N/2 NaOH	0.8	0
N/2 H_2SO_4	0	1.1

Stopper loosely and immerse in boiling water for 2 minutes. Then add:

	Tube 1 cc.	Tube 2 cc.
N/2 H_2SO_4	1.1	0
N/2 NaOH	0	0.8

Mix well the contents of each tube. Each now contains an excess of 0.3 cc. N/2 H_2SO_4 in a volume of about 2.9 cc. or, in other words, each solution is now approximately of N/19 strength in acidity. To each tube now add 1 cc. of a 2 per cent solution of ammonium molybdate,

shake and immerse in the boiling water. The reaction is very striking and almost immediate. The blue color is due to the reduction of colorless molybdic acid into blue molybdous acid.

Experiment 6. Reduction tests as applied to carbohydrates.

a. The majority of the oxidizing solutions used in the detection or estimation of reducing carbohydrates are alkaline in reaction and most of them contain copper as the oxidizing agent. From the evidence obtained in the previous experiments it is safe to infer that the function of the base is to break up the carbohydrate into more vigorous and a greater number of reducing particles. It is clear that the base is not necessary for the oxidation of the dissociated sugar, but it may be necessary to detect the reduced state of the oxidizing agent. The latter is the case where we have cuprous oxide precipitated in the reduction test. The experiments below are given in order to demonstrate the function of sodium potassium tartrate, sodium citrate, glycerin, and ammonia in Fehling's, Benedict's, Haines', and Purdy's (also called Pavy's or Long's) tests respectively.

TABLE 3

PREPARATION OF TEST TUBES IN EXPERIMENT 6A

TUBE	2 PER CENT $CuSO_4$	BASE	OTHER ADDITIONS
	cc.		
1	1	1 cc. 1 per cent NaOH	1 cc. 10 per cent sodium potassium tartrate solution
2	1	1 cc. 10 per cent Na_2CO_3	1 cc. 20 per cent sodium citrate solution
3	1	1 cc. 10 per cent Na_2CO_3	1 cc. 20 per cent sodium citrate and 1 cc. 10 per cent NaCNS
4	1	1 cc. 1 per cent NaOH	2 cc. glycerol
5	1	1 cc. 1 per cent NaOH	3 cc. NH_4OH (sp. gr. 0.90)
6	1	1 cc. 1 per cent NaOH	0
7	1	1 cc. 1 per cent NaOH	0

Prepare seven test tubes as indicated in table 3. Shake each tube well and note the solution of the precipitate and the change in color in the first five tubes. Observe that we have two types of substances which dissolve $Cu(OH)_2$ in *alkaline* solutions. Those of type 1 contain alcohol groups and those of type 2 are ammonia compounds.

To each of the first four tubes and to tube 6 now add 1 cc. of a M/100 glucose solution and shake. To tube 5, add 1 cc. M/10 glucose solution and mix. Next immerse the seven tubes in boiling water. Note that tubes 1, 2, and 4 soon contain a precipitate of yellow or red cuprous oxide and that tube 3 contains a white precipitate of cuprous sulpho-

cyanate (CuCNS), that the contents of tube 5 become colorless without any precipitate and that tubes 6 and 7 contain a brown to black precipitate. In tubes 1, 2, and 4, the OH ions cause the precipitation of the cuprous ions (as Cu_2O) as rapidly as they are formed by the action of the dissociated glucose. The same is true in tube 6, but here we do not have the solvent necessary to keep the cupric ions from being precipitated also and as a result we have a mixture of yellow to red cuprous oxide and brown to black cupric oxide. The result is that we have mainly the black CuO as found in tube 7 where no glucose was added and that even when fair amounts of cuprous oxide are formed the detection thereof is obscured by the preponderating black CuO. In tube 3 the CNS ions are the precipitating ions instead of OH as in tubes 1, 2, and 4. In tube 5, the blue $(Cu[NH_3]_4)^{++}$ has been changed to the

TABLE 4

PREPARATION OF TEST TUBES IN EXPERIMENT 6c

TUBE	FEHLING'S MIXTURE	GLUCOSE SOLUTION
1	5 cc. of 1:10 dilution	1 cc. M/100
2	5 cc. of 1:10 dilution	1 cc. M/250
3	5 cc. of 1:10 dilution	1 cc. M/500
4	5 cc. undiluted	1 cc. M/100
5	5 cc. undiluted	1 cc. M/250
6	5 cc. undiluted	1 cc. M/500

colorless $(Cu[NH_3]_2)^+$ or, in other words, NH_4OH dissolves cuprous as well as cupric hydrates or oxides, but the former solution is colorless while the latter is dark blue. The change of the dark blue solution to the colorless is proof that all cupric copper has been reduced to cuprous.

b. Application of Fehling's solution on aqueous carbohydrate solutions. Note that this reagent as made without dilution is approximately 1.25 N in NaOH and that the cupric hydroxide is kept in solution by sodium potassium tartrate (see appendix). Prepare 40 cc. of diluted Fehling's solution by mixing 2 cc. of each of the two solutions 1 and 2 and diluting to 40 cc. Measure off five 2-cc. portions of the well mixed diluted solution into different test tubes and then add respectively 2 cc. of M/500 solutions of levulose, glucose, lactose, saccharose, and 0.7 per cent starch paste. After shaking, immerse all in boiling water and heat for 15 minutes. Note the order in which reduction appears in the different tubes as shown by the formation of yellow or red hydrated cuprous oxide. Also observe the variation in intensity in reduction.

c. Best conditions for the detection of low concentrations of glucose by Fehling's test. Prepare six tubes as indicated in table 4. Immerse in boiling water. Note how much more readily one can detect the reduction when the diluted Fehling's mixture is used. This is an important factor in the detection of reducing sugars by Fehling's solution.

d. The detection of glucose in urine by Fehling's solution. Although this test is by no means specific for glucose, nevertheless it becomes of more practical value when applied under the specific conditions given below. With more urine or longer boiling a positive test might be obtained with normal urine; this is not due to the glucose but to other reducing agents found in normal urine. Note that the dilution of the reagent, the amount of urine and time of boiling are all important.

To 2 cc. of the Fehling's 1:1 mixture add 5 cc. distilled water. Heat to boiling, then add 0.5 cc. of the albumin-free diabetic urine and boil for 1 to 2 minutes. If the greenish yellow to red precipitate does not form, add another 0.5 cc. of the urine and again boil for one or two minutes. With urine containing 0.1 per cent of glucose a greenish precipitate should form very soon. On cooling and standing a reddish precipitate should separate in the course of 15 to 30 minutes.

e. Application of Benedict's qualitative reagent on an aqueous glucose solution. Note that this reagent is approximately 2 N in sodium carbonate content and that the cupric hydroxide is kept in solution by sodium citrate (see appendix). Into each of three tubes measure off 5 cc of the Benedict qualitative reagent and then add respectively 1 cc. M/100, 1 cc. M/250, and 1 cc. M/500 glucose. Place in boiling water and heat for 10 minutes. How does the delicacy compare with Fehling's test?

f. The detection of glucose in urine by Benedict's qualitative reagent. This test is not specific for glucose but it becomes much more reliable when applied as given here. See (d) above for reasons. Heat 5 cc. of the reagent to boiling. To the boiling hot mixture add 8 drops of a diabetic urine which is albumin free. Boil vigorously for 1 to 2 minutes. Set aside to cool. If glucose is present in concentrations of 0.05 per cent or higher, reduction will be indicated by a greenish tinge to a reddish precipitate depending on the concentration of glucose. More than 8 drops of urine should not be used unless the urine is very dilute in nitrogenous constituents such as uric acid and creatinine. These substances are also reducing agents and if present in abnormal concentrations would lead to the reduction of Benedict's solution.

g. Application of Haines' solution on aqueous glucose solutions.

Note that this reagent is approximately 0.35 N in NaOH content and that the cupric hydrate is kept in solution by glycerol (see appendix). This reagent is not perfectly stable so that a blank test should be made very frequently. Prepare four test tubes, each containing 5 cc. of Haines' solution. To these add respectively 1 cc. water, 1 cc. M/100, 1 cc. M/250, and 1 cc. M/500 glucose solutions. Place in boiling water and note the rate and intensity of reduction. Compare this with similar tests by Benedict's and Fehling's reagents.

h. The detection of glucose in urine by Haines' solution. This test is no more specific than Fehling's or Benedict's. As applied here it is claimed to detect truly pathological urines of the diabetic type. See (d) above. To 5 cc. of urine add 5 to 6 drops of 10 per cent NaOH solution. Mix and centrifugate or allow to sediment by standing to remove calcium and magnesium phosphates. Gently boil 5 cc. of the Haines' solution. Next remove from the flame and while holding the test tube at an angle of about 30° with the horizontal add by means of a dropper or pipette 10 to 20 drops of the clear phosphate-free urine. Set aside in an upright position. If the urine contains more than 0.1 per cent glucose, a brick red or yellowish ring will form *at once* at the junction between the reagent and urine.

Experiment 7. The oxidation of aldoses and ketoses in slightly acid solution by cupric ions (Barfoed's test).[7, 8, 9]

This reagent is a solution of cupric acetate in approximately 0.15 N acetic acid. The reagent was originally devised to detect glucose in dextrin but later it was claimed to be specific for monosaccharides. The facts are that all aldose and ketose sugars reduce this reagent but that hexoses act more rapidly and more vigorously than reducing disaccharides for equivalent molar concentrations. There is even some danger of reduction of the reagent with saccharose because the saccharose is very easily hydrolyzed by the dilute acid reagent and the liberated levulose is exceedingly reactive in this test. If used in analytical studies it must be applied very cautiously under very specific conditions.

Prepare six test tubes as indicated in table 5, place in boiling water and heat for 30 minutes. Note when reduction first occurs in each tube:

Experiment 8. The iodometric estimation of aldoses in presence of ketoses.[10]

Principle. In neutral, very slightly acid or slightly alkaline solutions free

[7] H. McGuigan, *Amer. J. Physiol.*, 1907, **19**, 175; A. P. Mathews and H. McGuigan, *ibid.*, 1907, **19**, 199.

[8] H. Tauber and I. S. Kleiner, *J. Biol. Chem.*, 1932, **99**, 249.

[9] W. H. Welker, *J. Amer. Chem. Soc.*, 1915, **37**, 2227.

[10] F. A. Cajori, *J. Biol. Chem.*, 1922, **54**, 617.