Laboratory Manual for BIOCHEMISTRY

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by

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DEDICATION

To those who know the challenge of biochemical research and have met the challenge with their labors and to those who aspire to do biochemical research, this book is dedicated.

PREFACE

The importance of laboratory work in the biochemical curriculum cannot be overemphasized. It is unfortunate that in many instances the laboratory work associated with biochemistry courses has not kept pace with the rapid development in the subject material. It is significant that, although a number of excellent textbooks of biochemistry have appeared in the last decade, relatively few new laboratory manuals have been published during this period. In biochemistry as in all areas of science simple mastery of the subject material is of limited value unless there is a concurrent development of the laboratory skills and manipulative techniques necessary to put the acquired knowledge to proper use. It was in the laboratory that the modern concepts of biochemistry were first given birth.

The primary purpose of this manual is to provide a suitable introduction to biochemical laboratory work for the beginning student. The manual is designed to fulfill the needs of a one-semester course in biochemistry which deals primarily with the chemistry and metabolism of the proteins, carbohydrates and lipids and is planned to provide a broad general training with a proper balance between isolation procedures, quantitative determinations, chemical tests, methods and metabolic experiments. In the preparation of this manual no attempt was made to slant the experiments toward any area of applied biochemistry or to make it a comprehensive work. Consequently, it is not intended to be used as a reference work for laboratory procedures and a number of excellent and important experiments have been omitted. The primary consideration in selecting the material to be included in the manual was the student who will perform the experiments and the development of his laboratory skills and techniques. Several criteria were used: 1) The experiment should illustrate a fundamental principle of biochemistry; 2) The experiment should be simple so that the beginning student can complete it with a reasonable chance of success; 3) The experiment should be reliable; and 4) The experiment should require a minimum of complex equipment.

It is hoped that the student in carrying out the experiments in this manual will not only be able to understand thoroughly the technique of the individual experiment and to master it but will also extend his vision beyond the horizon of the single experiment and will develop a greater appreciation for biochemical experimentation and the substances encountered in biochemistry. It is further hoped that these simple experiments will serve as a proper introduction to the biochemistry laboratory and will provide sufficient stimuli for the student so that he will desire to learn more about the techniques and methods used in biochemical research.

The author is indebted to Dr. Walter E. Militzer, who generously made available the material in his book, "Manual for Biochemistry"; to his wife, Rogene, who aided in the preparation of the manuscript; to Mr. Gordon Luikart, who assisted in making some of the drawings; and to Dr. James Mattoon, who contributed many helpful suggestions and advice.

Lincoln, Nebraska   R. B. J.
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1 - SOME OF THE CHEMICAL PROPERTIES OF PROTEINS

Although proteins are all composed of amino acids linked together by peptide bonds, these substances exhibit remarkable variations in their chemical, physical and biological properties. The purpose of this experiment is to familiarize the student with some of the common chemical properties of proteins, especially those properties by which proteins can be distinguished one from another. The protein molecule is so complex that it is often difficult to interpret the observed chemical behavior of these compounds. The protein will in general, however, reflect the chemical properties of the amino acids which it contains. Many of the common color reactions of the proteins depend upon the occurrence of a specific amino acid in the protein.

The proteins or their derived products which will be employed in this experiment are egg albumin, one of the chief proteins of egg; casein, the principle protein of milk; gelatin, a well-known protein which can be obtained from connective tissue and tendon; peptone, a group of complex intermediate molecular weight substances of unknown structure which can be obtained by hydrolysis of protein; and glycine, a simple amino acid which can be derived from protein.

The Elementary Composition of Proteins

Proteins, unlike most organic solids, do not melt on heating but instead char and burn. The formation of free carbon during the charring process serves to establish the presence of carbon in the protein. Often during the charring of the protein water droplets may condense in the cold upper portion of the tube. This may be due in part to the expelling of water of hydration of the protein, but is also the result of the combustion of the hydrogen in the protein by oxygen in the air to water. This indicates that the original protein contained hydrogen.

The presence of nitrogen in the form of an amino group in the protein can be established by fusing the protein with alkali. This liberates ammonia from the protein, which can be detected by litmus paper.

EXPERIMENTAL

Charring of Protein at High Temperatures

Add a small quantity of casein to a dry test tube and heat gradually over a free flame until the protein begins to char and the odor of burning hair is detected. Hold the tube at an angle so that the lip of the test tube is not heated by the flame. Avoid excess heating of the protein. The odor of charring or burning of proteins is a familiar one and very characteristic for proteins. The black appearance of the protein in the bottom of the tube confirms the presence of carbon in the protein and the condensation of water on the wall of the cool portion of the tube indicates the presence of hydrogen in the protein.

Liberation of Nitrogen in the Form of Ammonia
on Fusion of Protein with Alkali

Grind with a mortar and pestal a small amount of sodium hydroxide to a fine powder, carefully avoiding contact of the strong alkali to the skin. Place a small quantity of casein in a pyrex test tube and add to this twice this amount of the powdered sodium hydroxide. Heat slowly and carefully over a free flame. The odor of ammonia can be detected which indicates the presence of nitrogen in the protein. Test the effect of the fumes on moistened red litmus paper to confirm the presence of ammonia.

QUESTIONS

1. What is the percentage of carbon in proteins?

2. What is the percentage of hydrogen in proteins?

3. Do all proteins give the above tests?

Color Reactions of Proteins

BIURET REACTION (Piotrowski's Reaction)

Copper ion will form a complex with protein in a manner similar to that which it forms with ammonia. The complex in the case of the protein is red or violet rather than deep blue as in the case of ammonia. Simple amino acids and dipeptides do not react with copper ion to give the red or violet color. In order to form the red or violet color at least two peptide bonds are required in the molecule. The simplest substance which possesses the required structure is biuret ($NH_2CONHCONH_2$) and it is for this reason that this reaction has been referred to as the biuret reaction. This is one of the few protein tests which requires a protein-like structure for a positive result.

EXPERIMENTAL

Carry out the biuret reaction on 2% solutions of glycine, peptone, casein, gelatin, and egg albumin. To 2 cc. portions of the protein solutions and 2 cc. of 6 N sodium hydroxide add one drop of a 0.5% copper sulfate solution. Mix well and add more copper sulfate, drop by drop, until a distinct pink or violet color develops or until a precipitate of copper hydroxide is formed. If the red or violet color which constitutes the biuret reaction does not come at once, allow to stand 15-20 minutes. Note any differences in the color given by the various protein solutions.

QUESTIONS

1. Describe the chemical basis for the biuret test.

2. Why must the solution be alkaline to observe the biuret test?

3. What is the minimum number of amino acid residues which a peptide must contain in order to give a positive biuret test?

4. Do all proteins give a positive biuret test?

5. Explain why ammonia interferes with the biuret reaction.

XANTHOPROTEIC TEST

This reaction is specific for proteins containing aromatic amino acids, especially tyrosine and tryptophan. If sulfuric acid is added to the concentrated nitric acid, the phenylalanine in the proteins will also react. For further information on this reaction see the experiment on the identification of amino acids in an amino acid unknown.

EXPERIMENTAL

Carry out the xanthoproteic reaction on 2% solutions of glycine, peptone, casein, gelatin, and egg albumin. To 2-3 cc. of protein solution in a test tube add 2-3 cc. of concentrated nitric acid. Heat cautiously to boiling or until any precipitate which formed on addition of the acid is dissolved. Cool the solution and add 6 N sodium hydroxide in excess. The yellow color deepens into orange.

QUESTIONS

1. Why does nitric acid stain the skin yellow?

2. Do all proteins show the xanthoproteic test?

MILLON'S TEST

Millon's test is specific for the phenol group (C_6H_5OH) and the amino acid tyrosine contains this group as an integral part of the molecule. Consequently, proteins containing tyrosine will give a positive Millon's test. Millon's reagent is a complex mixture of mercury salts which form when mercury is dissolved in nitric acid. The color is believed to be due to the formation of mercury salts of the weakly acidic phenol group. For further information regarding the Millon's test see the experiment on the identification of amino acids in an amino acid unknown.

EXPERIMENTAL

Carry out the Millon's test on 2% solutions of glycine, peptone, casein, gelatin, and egg albumin. To 2-3 cc. of the solution add a few drops of Millon's reagent. Warm the solution gradually over the free flame but do not place the tube directly in the flame. Bring the solution gradually to the boiling point. The presence of tyrosine is indicated by the development of a faint pink color which may gradually develop into a pure red and remain red after boiling. If the color does not develop it may be necessary to add more of the Millon's reagent, but low concentrations of the reagent are more desirable. The color may disappear on continued heating so it is important to heat the solution gradually so that one does not miss the development of the color.

QUESTIONS

1. Explain the differences observed in the reaction of egg albumin and gelatin with Millon's reagent.

2. What is the tyrosine content of egg albumin and gelatin?

HOPKINS-COLE TEST

Proteins which contain tryptophan will give a positive Hopkins-Cole test. The color is due to the condensation of the indole ring of the tryptophan with an aldehyde present in the Hopkins-Cole reagent. Further information regarding this test can be found in the experiment on the identification of amino acids in an amino acid unknown.

EXPERIMENTAL

Perform the Hopkins-Cole test on 2% solutions of glycine, peptone, casein, gelatin, and egg albumin. Place 2-3 cc. of the protein solution and 3 cc. of the Hopkins-Cole reagent (magnesium salt of glyoxylic acid) in a test tube and mix thoroughly. In a second test tube place 5 cc. of concentrated sulfuric acid. Incline the tube containing the solution of protein and reagent and into it carefully pour the sulfuric acid. If this is done carefully there is a layer of sulfuric acid and a layer of the protein. Heat in a boiling water bath for 2 minutes and allow to stand. A reddish violet color develops at the junction of the two solutions if tryptophan is present.

QUESTIONS

1. Name two proteins which will not give a positive Hopkins-Cole test.

2. Write the structure of glyoxylic acid.

EHRLICH DIAZO REACTION

Diazotized sulphanilic acid will condense with the phenolic and imidazole rings of tyrosine and hystidine in proteins to give colored products. With histidine a deep cherry red color forms in concentrations even as low as 1 part histidine in 20,000 parts of water. Tyrosine reacts to give a less intense orange red color. Tyrosine when converted into the benzoyl derivative no longer gives the test, but benzoyl histidine does.

EXPERIMENTAL

Carry out the Ehrlich diazo reaction on 2% solutions of glycine, peptone, casein, gelatin, and egg albumin. To 1 cc. of 0.5% sulfanilic acid solution in 2% hydrochloric acid add an equal volume of 0.5% sodium nitrite solution, mix well and after about half a minute add 1 cc. of the protein solution. Again mix and then add enough ammonium hydroxide or sodium carbonate to make the mixture distinctly alkaline. Histidine gives a red to orange color and tyrosine gives an orange color.

QUESTIONS

1. Write the reaction for the diazotization of sulfanilic acid.

2. Name a protein which will not give a positive Ehrlich diazo reaction.

REDUCED SULFUR TEST

Proteins containing cysteine or cystine, when treated with strong alkali, will form hydrogen sulfide which can be detected by forming black insoluble lead sulfide or by its characteristic "rotten egg" odor.

EXPERIMENTAL

Run the reduced sulfur test on 2% solutions of glycine, peptone, gelatin, casein, and egg albumin. To 2 cc. of the protein solution add about twice the volume of 6 N sodium hydroxide. Place about 1/2 ml. (about 10 drops) of 2% lead acetate in the solution and heat to boiling. Continue boiling for 3 to 5 minutes. It may be necessary to heat the protein longer and to allow it to stand for some time after heating for the black deposit of lead sulfide to appear.

QUESTIONS

1. Write the chemical reactions for the reduced sulfur test as carried out with the amino acid cysteine.

2. Name two proteins which will not give a positive reduced sulfur test.

MOLISCH TEST

The Molisch test is a general test for carbohydrates. Consequently, glycoproteins, which are conjugated proteins which contain carbohydrates as the prosthetic groups, will give a positive Molisch reaction. In this test the carbohydrate is dehydrated by strong nonoxidizing acid to furfural or a furfural derivative which condenses with the α-naphthol to form a red to violet colored product.

EXPERIMENTAL

Perform the Molisch reaction on 2% solutions of glycine, peptone, casein, gelatin, and egg albumin. To 2 ml. of the protein solution add 2 drops of 5% α-naphthol solution, and carefully pour concentrated sulfuric acid into the solution so that it forms a layer at the bottom of the tube. At the junction of the two layers a purple ring will form if carbohydrate is present.

QUESTIONS

1. What is the formula of α-naphthol?

2. Name three glycoproteins.

ORGANIC PHOSPHOROUS TEST

Conjugated proteins which contain phosphorous as a prosthetic group will on digestion with sulfuric acid liberate inorganic phosphate. The inorganic phosphate can be detected by formation of the characteristic yellow ammonium phosphomolybdate.

$$H_3PO_4 + 12(NH_4)_2MoO_4 + 21\ HNO_3 \longrightarrow (NH_4)_3PO_4\cdot 12\ MoO_3 + 12\ H_2O + 21\ NH_4NO_3$$

EXPERIMENTAL

Carry out the determination on 2% solutions of glycine, peptone, casein, egg albumin and gelatin. Pipette 5 ml. of the solution to a large pyrex 8-inch test tube. Add 1 ml. of 6 N sulfuric acid and concentrate the solution by boiling over an open flame. Shake the tube continuously to prevent bumping. If excessive foaming occurs during the concentration, this can be minimized by the addition of a drop of octyl alcohol to the tube. Continue heating until white fumes of sulfur trioxide appear. The protein may char at this point. Add carefully a drop or two of concentrated nitric acid making sure that the mouth of the tube is directed away from you during the addition. Again heat to the charring point and repeat the addition of nitric acid. Continue heating until the solution remains colorless when the dense sulfur trioxide fumes are visible in the flask. Cool and add about 5 cc. of distilled water. Add strong ammonium hydroxide until the solution is just alkaline to litmus. Adjust the solution so that it is acid to litmus by the addition of 6 N nitric acid. Heat the solution to 70° C. and add 2 ml. of the acid ammonium molybdate reagent. On standing the formation of the yellow ammonium phosphomolybdate indicates the presence of phosphorous.

QUESTIONS

1. What class of proteins contain phosphorous?

2. Name three proteins which contain phosphorous.

3. Describe the chemical linkage as to how the phosphorous is linked to the protein.

Precipitation Reactions of Proteins

COAGULATION BY HEAT

Most proteins are instable at temperatures above 50° C. At these temperatures the molecule undergoes subtle changes which render it less soluble in water. Certain proteins will on heating precipitate and form large insoluble aggregates. This property of the protein is called coagulation.

EXPERIMENTAL

Carry out the reaction on a 2% solution of gelatin, casein, egg albumin, glycine, and peptone. Add 1 ml. of 5% sodium chloride to 2-3 ml. of the protein solution. Heat to boiling and observe the amount of coagulation in each tube.

QUESTIONS

1. Explain the difference between denaturation and coagulation.

2. Do all proteins coagulate on heating?

PRECIPITATION OF PROTEINS BY THE ALKALOIDAL REAGENTS

The alkaloidal reagents are so called because of their property to precipitate alkaloids. Among these reagents are ferrocyanic acid, tannic acid, picric acid, phosphotungstic acid, phosphomolybic acid, sulfosalicylic acid, dinitrosalicylic acid, metaphosphoric acid and trichloroacetic acid. The anions of these acids will combine with proteins which have a net positive charge, i.e., on the acid side of the isoelectric point of the protein, to form insoluble complexes in which the protein and anions are held together by electrostatic linkages. These reactions are very useful in removing proteins from biological fluids for analysis of nonprotein substances.

EXPERIMENTAL

Carry out the following reactions on 2% solutions of glycine, peptone, gelatin, casein, and egg albumin. Acidify 5 cc. of the protein solution with acetic acid and add 2 ml. of 5% potassium ferrocyanide solution. Note the precipitate. Repeat the procedure employing 2 ml. of 10% trichloroacetic acid in place of the 2 ml. of potassium ferrocyanide. Finally carry out the procedure with 2 ml. of saturated picric acid in place of the potassium ferrocyanide.

QUESTIONS

1. Describe a method whereby one can prepare a protein-free solution of a biological fluid such as blood.
2. Indicate whether alkaline or acid conditions favor the precipitation of protein by alkaloidal reagents. Explain your answer.

PRECIPITATION OF PROTEINS BY FORMING SALTS OF HEAVY METALS

Certain proteins on the alkaline side of their isoelectric point will combine with heavy metals to form insoluble metal proteinates.

EXPERIMENTAL

Carry out the reaction on 2% solutions of glycine, peptone, gelatin, egg albumin, and casein. To 5 cc. of the protein solution add very slowly, a drop at a time, a 1% solution of ferric chloride. Note the effect and then add an excess of ferric chloride. Repeat, using 2% copper sulfate, 2% lead acetate, 2% silver nitrate and 1% mercuric chloride. In the case of the copper sulfate add a few drops of 6 N sodium hydroxide to each tube.

QUESTIONS

1. Indicate as to whether alkaline or acid conditions favor the precipitation of protein by heavy metals. Explain your answer.
2. Would egg white be a good antidote for poisoning by the heavy metals? For which metals would it work the best?

PRECIPITATION OF PROTEINS BY STRONG MINERAL ACIDS

Strong mineral acids will denature certain proteins and cause them to become insoluble in aqueous solution. This property of proteins is often used in the clinics to determine if albumin is present in the urine. The procedure is known as the Heller's ring test.

EXPERIMENTAL

To 3 cc. of the egg-white solution in a test tube, add strong nitric acid, pouring it down the side of the tube to form a layer under the protein solution. Observe the precipitate which forms at the junction of the two liquids. Repeat the reaction with concentrated hydrochloric acid. Carry out similar tests with a solution of peptone and one of gelatin.

QUESTIONS

1. Is albumin a normal constituent of urine?

2. Do all proteins show the Heller's ring test?

SALTING OUT OF PROTEINS

Proteins are less soluble in solutions of high ionic strength so that some of them can be precipitated by the addition of a very soluble ionized salt. Ammonium sulfate is often employed for salting out reactions, but sodium sulfate is also used. The globulins are precipitated by 1/2 saturated ammonium sulfate while the albumins precipitate from a saturated ammonium sulfate solution. Crude egg albumin contains globulins which can be separated from the albumin by this method.

EXPERIMENTAL

To 25 ml. of distilled water in a beaker add slowly with stirring 1 g. of crude egg albumin. Stir for several minutes and allow to stand for 5 minutes. Filter the solution through a fine texture filter paper. Pipette 5 ml. of the slightly cloudly supernatant into a 12 ml. centrifuge tube and add 5 ml. of saturated ammonium sulfate. The solution should now be half saturated with ammonium sulfate. Shake the tube and notice the cloudiness appearing in the solution due to the precipitation of the globulin-like proteins in the crude egg albumin. Allow the tube to stand for 5 minutes and then centrifuge for 10 minutes. Pour off the supernatant liquid into a second 12 ml. centrifuge tube and add small amounts of solid ammonium sulfate with vigorous shaking until the solution is saturated with ammonium sulfate. This requires about 4 g. of ammonium sulfate. When the solution is saturated some of the large ammonium sulfate crystals can be observed at the bottom of the tube after vigorous shaking. Centrifuge the solution for 10 minutes to collect the albumin.

QUESTIONS

1. What is ionic strength?

2. What is the difference between a pseudoglobulin and an euglobulin?

REAGENTS

1. Ammonium sulfate (saturated)
2. Ammonium sulfate
3. Trichloroacetic acid (10%)
4. Sulphanilic acid (0.5%)
5. Sodium nitrite (0.5%)
6. Lead acetate (2%)
7. Copper sulfate (0.5%)
8. Ammonium hydroxide (conc.)
9. α-Naphthol (5%)
10. Gelatin (2%)
11. Casein (2%)
12. Peptone (2%)
13. Glycine (2%)
14. Egg albumin (2%)
15. Sodium hydroxide
16. Casein
17. Millon's reagent
18. Hopkins-Cole-Benedict reagent
19. Octyl alcohol
20. Ammonium molybdate (2.5% in 3 N sulfuric acid)
21. Picric acid (saturated)
22. Potassium Ferrocyanide (5%)
23. Ferric Chloride (1%)
24. Copper Sulfate (2%)
25. Silver Nitrate (2%)
26. Mercuric Chloride (1%)

DATA SHEET

REACTIONS OF THE PROTEINS

Date____________ Desk No.________ Name____________________

Protein	Biuret	Xantho-proteic	Millon's	Hopkins-Cole	Ehrlich Diazo	Reduced Sulfur
Egg Albumin						
Gelatin						
Casein						
Peptone						
Glycine						

Protein	Heat Coagula-bility	Ppt. by Alkaloidal Reagents	Ppt. by Heavy Metal Ions	Molisch	Phosphorous
Egg Albumin					
Gelatin					
Casein					
Peptone					
Glycine					