



Qualitative Testing for Amino Acids and Proteins

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Purpose of the Experiment

Study the properties and reactions of amino acids and proteins with reagents used to classify and identify these compounds.

Background Information

We divide the food we consume into three main classes: **carbohydrates**, the body's most readily available energy source; **lipids**, the body's principal energy reserve; and **proteins**, the body's source of energy for growth and cellular maintenance. Proteins also make up the second largest portion of cells, after water. They are large polymeric compounds that cells synthesize from various building blocks called **amino acids**. The general structure for an amino acid is shown in Figure 1. Note that all amino acids contain carboxylic acid groups ($-\text{COOH}$), amino groups ($-\text{NH}_2$), and substituent, or replaceable, side chains ($-\text{R}$).

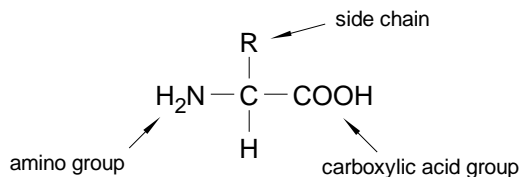


Figure 1 General structure for an amino acid

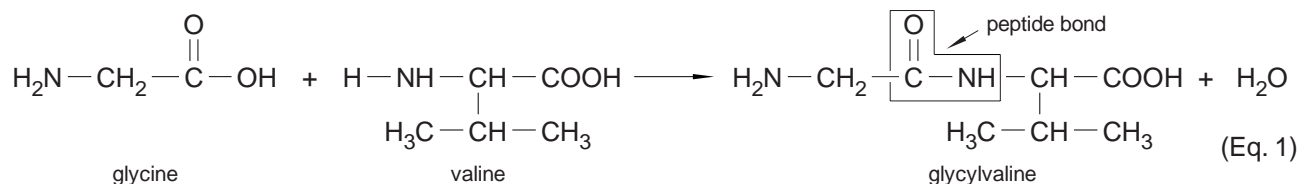
Twenty different amino acids, which differ only in the structures of their side chains, are used by human

cells to build proteins. The side chain structure determines the class of the amino acid: nonpolar, neutral, basic, or acidic. Human cells can synthesize most of the amino acids they need to build proteins. However, about 8 amino acids called **essential amino acids** cannot be synthesized by human cells and must be obtained from food.

Amino acids incorporated into proteins are covalently linked by **peptide bonds**. Peptide bonds are amide bonds formed between the carboxylic acid group of one amino acid and the amino group of a second amino acid. Equation 1 on the next page shows a peptide linkage formed between glycine and valine. The peptide bond is indicated.

Note that, because every amino acid contains at least one amino group and one carboxylic acid group, it is possible for a peptide bond to form in two different ways. For example, with glycine and valine, it is also possible for the peptide bond to form between the carboxylic acid group of valine and the amino group of glycine, producing valylglycine.

Proteins are composed of hundreds of amino acids linked by peptide bonds, forming a **peptide chain**. We define the direction in which the amino acids link by



referring to the two ends of the chain as the N-terminus and the C-terminus. The **N-terminus** is the terminal amino acid in the chain that contains the only amino group not part of a peptide bond. The **C-terminus** is the other terminal amino acid in the chain, containing the only carboxylic acid group not part of a peptide bond. Note that the N-terminus and the C-terminus are not determined by the side chains. The number of constituent amino acids and the order in which they are linked starting from the N-terminus, are referred to as the protein's **primary structure**.

I. Amino Acid and Protein Solubility

The physical properties of amino acids and proteins are mainly a result of their structure, both in the solid state and in various solutions. In this part of the experiment you will investigate the solubility of selected amino acids and proteins in various solutions. Using your data you will compare amino acid and protein structural characteristics.

Solubility as a Function of Solution pH

The presence of amino and carboxylic acid groups enables amino acids to accept protons from and donate protons to aqueous solution, and, therefore, to act as acids and bases. Because proteins contain both acidic and basic side chains, they too can donate or accept protons. A molecule that functions simultaneously as an acid and a base is known as an **amphoteric molecule**. In neutral aqueous solutions, amino acids act as amphoteric molecules. For example, an amino acid with a neutral side chain contains two charges: one positive, due to the protonation of the amino group, and one negative, due to the dissociation of the carboxylic acid proton. This double ionic form of an amino acid is the **zwitterionic form**. Figure 2 shows an amino acid in the zwitterionic form.

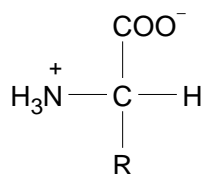


Figure 2 The zwitterionic form of an amino acid

Amino acids in zwitterionic form have many physical properties that are also associated with ionic salts. For example, zwitterionic amino acids are colorless, nonvolatile, crystalline solids with melting points above 200 °C, usually melting with decomposition. They are soluble in water but not in nonpolar organic solvents such as cyclohexane. Compared to organic amines and carboxylic acids of similar molecular weight, amino acids have much lower melting points and are highly soluble in polar organic solvents, but only slightly soluble in water. The amino and carboxylic acid groups of constituent amino acids, as well as the nature of various side chains, allow proteins to possess some of these same properties. However, there are many other factors that must be considered when discussing protein solubility.

The solubility of amino acids and proteins is largely dependent on the solution pH. The structural changes in an amino acid or protein that take place at different pH values alter the relative solubility of the molecule. In acidic solutions, both amino and carboxylic groups are protonated. In basic solutions, both groups are unprotonated. Figure 3 shows an amino acid in acidic, neutral, and basic solutions.

The pH value at which the concentrations of anionic and cationic groups are equal is the isoelectric point for that amino acid or protein. Amino acids and proteins are least soluble at their isoelectric points. Most of the proteins found in human tissues and fluids have isoelectric points below pH 7.0 (below human body pH) and therefore exist mostly in their anionic forms.

II. Chemical Reactions of Amino Acid and Protein Functional Groups

Certain functional groups in amino acids and proteins can react to produce characteristically colored products. The color intensity of the product formed by a particular group varies among proteins in proportion to the number of reacting functional, or free, groups present and their accessibility to the reagent. In this part of the experiment, you will use various color-producing reagents (dyes) to qualitatively detect the presence of certain functional groups in amino acids and proteins.

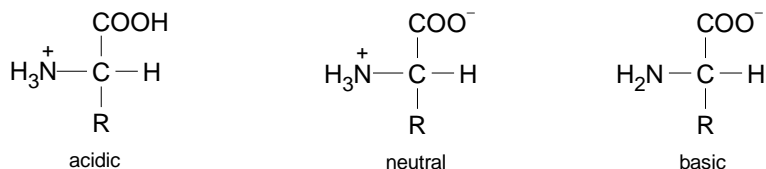


Figure 3 An amino acid in acidic, neutral, and basic solutions

Ninhydrin Test

Amino acids contain a free amino group and a free carboxylic acid group that react together with **ninhydrin** to produce a colored product. When an amino group is attached to the first, or **alpha**, carbon on the amino acid's carbon chain, the amino group's nitrogen atom is part of a blue-purple product, as shown in Equation 2. Proteins also contain free amino groups on the alpha-carbon and can react with ninhydrin to produce a blue-purple product.

Amino acids that have secondary amino group attachments also react with ninhydrin. However, when the amino group is secondary, the condensation product is yellow. For example, the amino acid proline, which contains a secondary amino group, reacts with ninhydrin, as shown in Equation 3. Blue-purple and yellow reaction products positively identify free amino groups on amino acids and proteins.

Biuret Test

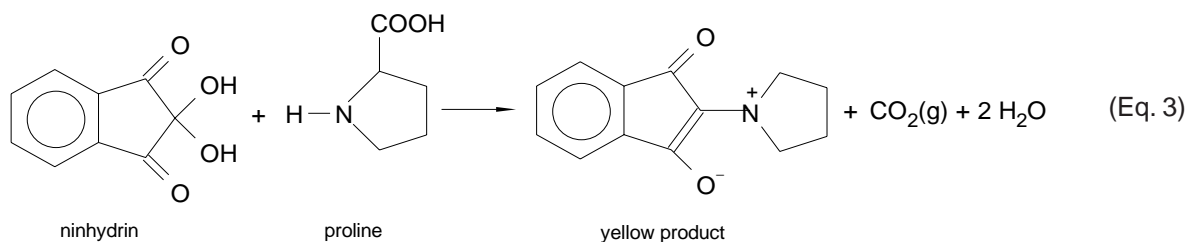
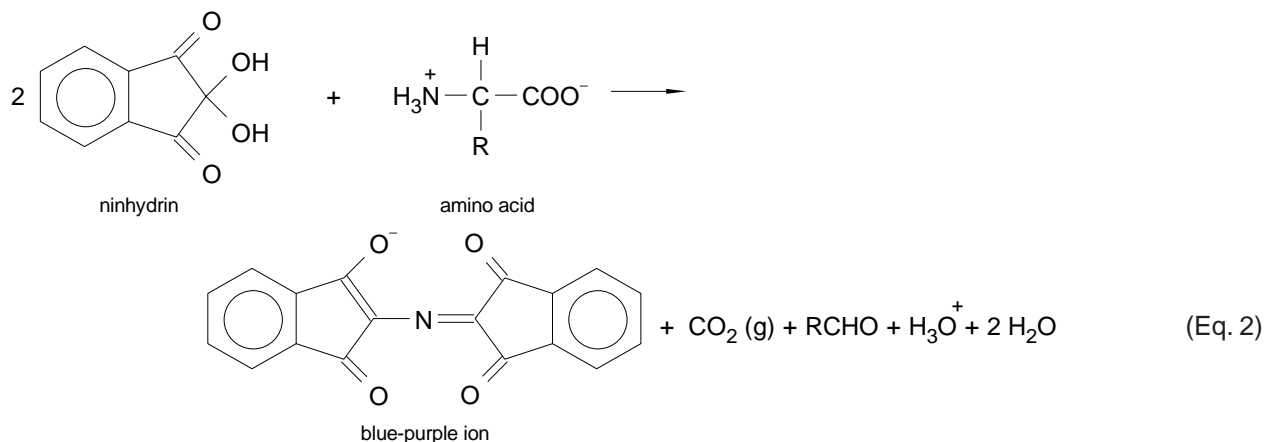
The **biuret test** for proteins positively identifies the presence of proteins in solution with a deep violet color. Biuret, $\text{H}_2\text{NCONHCONH}_2$, reacts with copper(II) ions in

a basic solution to form a deep violet complex. The peptide linkages in proteins resemble those in biuret and also form deep violet complexes with basic copper(II) ions in solution. The general or biuret complex formed between the protein linkages and the copper(II) ion of the biuret test is shown in Figure 4 on the next page.

Xanthoproteic Test

Some amino acids contain aromatic groups that are derivatives of benzene. These aromatic groups can undergo reactions that are characteristic of benzene and benzene derivatives. One such reaction is the nitration of a benzene ring with nitric acid. The amino acids tyrosine and tryptophan contain activated benzene rings and readily undergo nitration. The amino acid phenylalanine also contains a benzene ring, but the ring is not activated and therefore does not readily undergo nitration.

This nitration reaction, when used to identify the presence of an activated benzene ring, is commonly known as the **xanthoproteic test**, because the product is yellow. *Xanthoproteic* comes from the Greek word *xanthos*, which means "yellow." The intensity of



In this experiment you will observe some physical and chemical properties of amino acids and proteins. You will use various chemical reactions to classify and identify different functional groups present in amino acids and proteins.

Procedure

Chemical Alert

concentrated nitric acid—toxic, corrosive, and strong oxidant
 concentrated sulfuric acid—toxic and corrosive
 0.1M copper(II) sulfate—toxic, skin and respiratory irritant
 3M hydrochloric acid—toxic and corrosive
 Hopkins–Cole reagent contains glyoxylic acid—toxic
 Millon's reagent contains mercury(II) nitrate in concentrated nitric acid—highly toxic, corrosive, and strong oxidant
 0.5% ninhydrin–ethanol reagent—toxic and irritant
 3M sodium hydroxide—toxic and corrosive
 2% sodium nitroprusside—highly toxic

Note: Wear departmentally approved eye protection while doing this experiment.

I. Amino Acid and Protein Solubility

Solubility in Water

1. Label 6 clean, dry 18 × 150-mm test tubes with the names of the following substances: glycine, glutamic acid, lysine, tyrosine, gelatin, and casein. Place 0.05 g of each substance in the corresponding test tube. Add 2 mL of distilled or deionized water to each of the test tubes. Stopper the test tubes and shake vigorously for 2 min. Record your observations on Data Sheet 1. Save these mixtures for the next step.

Solubility as a Function of Solution pH

Caution: 3M sodium hydroxide solution is toxic, corrosive, and can cause burns. Prevent eye, skin, and clothing contact. If you spill any base, immediately notify your laboratory instructor.

2. Add 1 mL of 3M sodium hydroxide solution to the glycine test tube. Stopper the test tube and shake vigorously for 1 min. Record your observations on Data Sheet 1.

Caution: 3M hydrochloric acid is a corrosive, toxic solution that can cause skin irritation. Prevent eye, skin, and clothing contact. Avoid inhaling the vapors or ingesting the substance. If any acid is spilled, clean it up following the directions of your laboratory instructor.

3. Add a small piece of litmus paper or pH paper to the test tube. Add 3M hydrochloric acid dropwise to the glycine test tube just until the litmus paper indicates an acidic solution pH. Stopper the test tube and shake vigorously for 1 min. Record your observations on Data Sheet 1.

4. Add 15 more drops of 3M hydrochloric acid to the glycine test tube. Stopper the test tube and shake vigorously for 1 min. Record your observations on Data Sheet 1.

5. Repeat Steps 2–4 using the remaining mixtures prepared in Step 1.

6. Remove the litmus paper from the test tubes with a forceps and place the paper in a waste container indicated by your laboratory instructor. Dilute the mixtures in the test tubes with water and discard down the drain. Wash the test tubes with soap or detergent solution, and rinse three times with tap water and once with distilled water. Allow the test tubes to drain to remove as much water as possible.

II. Chemical Reactions of Amino Acid and Protein Functional Groups

Caution: Several of the following tests require you to place reaction solutions in a boiling-water bath. Be careful not to come in contact with the steam or the hot apparatus, and be sure not to knock the boiling-water bath over.

Note: Add a couple of boiling chips to the beaker of water in the boiling-water bath to prevent bumping while the water is boiling.

Note: Shake the amino acid and protein solutions well before dispensing them into the test tubes.

7. To set up a boiling-water bath, place an 800-mL beaker through an 80-mm iron ring, letting the lip of the beaker sit on the edge of the ring. Clamp the ring and beaker to a support stand. Place ceramic-centered wire gauze on a 60-mm iron ring or a tripod, and position the ring or tripod so it supports the beaker. Add 350 mL water to the beaker, along with two boiling chips. Place a Bunsen burner under the beaker, and heat the water to boiling.

Ninhydrin Test

Caution: Ninhydrin–ethanol reagent is flammable, toxic, and an irritant. Keep away from Bunsen burner flames. Prevent eye, skin, and clothing contact. Avoid inhaling the vapors or ingesting the reagent.

8. Label 6 clean, drained 18 × 150-mm test tubes with the names of the following solutions: 2% glycine, 1% tyrosine, 2% proline, 2% casein, 2% gelatin, and 2% albumin. Obtain the solutions from your laboratory instructor and place 15 drops of each in the corresponding test tube. To each of the test tubes, add 5 drops of 0.5% ninhydrin–ethanol reagent solution.

9. Using a test tube holder, place the test tubes into the boiling-water bath prepared in Step 7. Allow the test tubes to stand in the bath for 5 min. Using the test tube holder, remove the test tubes from the bath and place them in a test tube rack. Record your observations on Data Sheet 2.

10. Discard the test solutions containing ninhydrin into the container provided by your laboratory instructor and labeled “Discarded Ninhydrin Solutions.” Wash the test tubes with soap or detergent solution, and rinse three times with tap water and once with distilled or deionized water. Allow the test tubes to drain to remove as much water as possible.

Biuret Test

Caution: 0.1M Copper(II) sulfate solution is toxic and an irritant. Prevent eye, skin, and clothing contact. Avoid inhaling the vapor and ingesting the solution. If you spill any of this solution, immediately notify your laboratory instructor.

11. Label 5 clean, drained 18 × 150-mm test tubes with the names of the following solutions: 2% glycine, 2% arginine, 2% gelatin, 2% casein, and 2% albumin. Obtain the solutions from your laboratory instructor and place 15 drops of each in the corresponding test tube. Add 5 drops of 3M sodium hydroxide and 2 drops of 0.1M copper(II) sulfate solution to each test tube. Using a separate glass stirring rod for each test tube, mix the contents. Record your observations on Data Sheet 2.

12. Discard the test solutions containing the biuret mixtures into the container provided by your laboratory instructor and labeled “Discarded Biuret Test Solutions.” Wash the test tubes with soap or detergent solution, and rinse three times with tap water and once with distilled or deionized water. Allow the test tubes to drain to remove as much water as possible.

Xanthoproteic Test

Caution: Concentrated HNO₃ is a toxic, corrosive substance that can cause severe burns and discolor your skin. Prevent eye, skin, and clothing contact. Avoid inhaling vapors and ingesting the compound. If you spill any acid, immediately notify your laboratory instructor.

Note: The following test must be performed under a **fume hood**. Avoid inhaling the vapors produced when heating the reaction mixture.

13. Label 5 clean, drained 18 × 150-mm test tubes with the names of the following solutions: 1% tyrosine, 2% tryptophan, 2% glycine, 2% albumin, and 2% gelatin. Obtain the solutions from your laboratory instructor and place 15 drops of each in the corresponding test tube.

Caution: In the next step, you will be adding concentrated nitric acid to an aqueous solution. Take extreme care during this step.

14. Working under a **fume hood**, cautiously add 10 drops of concentrated nitric acid to the 1% tyrosine test tube. Using a test tube holder, place the test tube into the boiling-water bath. Warm the contents gently for 1–2 min, using the holder to swirl the test tube. Remove the test tube from the boiling-water bath. Place it into a test tube rack and allow it to cool. Record your observations on Data Sheet 2.

15. Repeat Step 14 using the other solutions.
16. To each of the cooled solutions from Steps 14 and 15, cautiously add 3M sodium hydroxide until the solution tests slightly basic using litmus or pH paper. Record your observations on Data Sheet 2.
17. Dilute the contents of each test tube with water, and discard down the drain. Wash the test tubes with soap or detergent solution, and rinse three times with tap water and once with distilled or deionized water. Allow the test tubes to drain to remove as much water as possible.

Millon's Test

Caution: Millon's reagent contains mercury and HNO₃ and is very toxic, corrosive, a strong oxidant, an irritant, and can cause burns. Possibility of cumulative effects if ingested. Contact with combustible material can cause fires. Prevent eye, skin, and clothing contact. Avoid inhaling the vapors and ingesting the reagent. If you spill any solution, immediately notify your laboratory instructor.

18. Label 6 clean, drained 18 × 150-mm test tubes with the names of the following solutions: 1% tyrosine, 2% tryptophan, 2% glycine, 2% albumin, 2% gelatin and 2% casein. Obtain from your laboratory instructor 2 mL of each solution. Add each solution to the corresponding test tube. Add 3 drops of Millon's reagent to each test tube. Using a test tube holder, place the test tubes into the boiling-water bath. Heat the solutions just to their boiling points, then use a test tube holder to remove the test tubes from the bath and place them in a test tube rack. Record your results on Data Sheet 2.
19. Discard the test solutions containing Millon's reagent into the container provided by your laboratory instructor and labeled "Discarded Millon's Solutions." Wash the test tubes with soap or detergent solution and rinse three times with tap water and once with distilled or deionized water. Allow the test tubes to drain to remove as much water as possible.

Hopkins–Cole Test

Caution: Concentrated sulfuric acid is toxic, corrosive, and causes severe burns. Prevent eye, skin, clothing, and combustible material contact. Avoid ingesting the substance. If you spill any acid, immediately notify your laboratory instructor.

Caution: In the following test, you will be adding concentrated sulfuric acid to an aqueous solution. Take extreme care during Step 21.

Caution: Hopkins–Cole reagent is toxic, corrosive, and hygroscopic. Can cause burns. Prevent eye, skin, and clothing contact. Avoid inhaling vapors and ingesting the compound. If you spill any solution, immediately notify your laboratory instructor.

20. Label 4 clean, drained 18 × 150-mm test tubes with the names of the following solutions: 1% tyrosine, 2% tryptophan, 2% albumin and 2% gelatin. Obtain from your laboratory instructor 2 mL of each solution, and add each to the corresponding test tube. Add 2 mL of Hopkins–Cole reagent to each test tube.

Note: If a purple ring does not form at the interface of the two layers in each test tube, gently tap the side of the test tube once to slightly mix the two layers. If a purple ring still doesn't form, consider it a negative result.

21. Holding the 1% tyrosine test tube at a 45° angle, cautiously and slowly add 30 drops of concentrated sulfuric acid down the inside wall of the test tube. Do not mix the two layers that form. Record your observations on Data Sheet 2.
22. Repeat Step 21 using the remaining reaction mixtures.
23. Discard the test solutions containing Hopkins–Cole reagent into the container provided by your laboratory instructor and labeled "Discarded Hopkins–Cole Solutions." Wash the test tubes with soap or detergent solution, and rinse three times with tap water and once with distilled or deionized water. Allow the test tubes to drain to remove as much water as possible.

Nitroprusside Test

24. Label 4 clean, drained 18 × 150-mm test tubes with the names of the following solutions: 2% glycine, 2% cysteine, 2% albumin, and 2% casein. Put a blank label on a fifth clean test tube. Obtain from your laboratory instructor 2 mL each of 2% glycine and 2% cysteine, and 4 mL each of 2% albumin and 2% casein. Place each solution in the corresponding test

tube. Add 2 mL of 3M sodium hydroxide solution to each of the 5 test tubes.

Caution: 2% Sodium nitroprusside is highly toxic. Prevent eye, skin, and clothing contact. Avoid inhaling and ingesting the compound. If you spill any nitroprusside, immediately notify your laboratory instructor.

25. Add 1 mL of 2% nitroprusside solution to each test tube, including the blank. Record your observations on Data Sheet 2.

26. Discard the test solutions containing the nitroprusside reagent into the container provided by your laboratory instructor and labeled "Discarded Nitroprusside Solutions." Wash the test tubes with soap or detergent solution, and rinse three times with tap water and once with distilled or deionized water. Allow the test tubes to drain to remove as much water as possible.

Caution: Wash your hands thoroughly with soap or detergent before leaving the laboratory.

Post-Laboratory Questions

(Use the spaces provided for the answers and additional paper if necessary.)

1. Explain how solution pH affected the solubility results recorded on the second part of Data Sheet 1 for tyrosine. Use structures to help explain the results.

3. Vasopressin, a substance produced by the human body that regulates blood volume and pressure, is a nonapeptide with the following amino acid sequence. Describe the results you would expect when vasopressin is tested with the following reagents.

Note: The amino acid asparagine, ASN, is structurally similar to glutamine. The only difference is that asparagine has one fewer CH_2 group in its side chain than glutamine.

GLY-ARG-PRO-CYS-ASN-GLN-PHE-TYR-CYS
human vasopressin

2. An unknown protein yielded the following test results using the procedure in this experiment. Based on these results, what amino acids must be present in the protein?

<i>test</i>	<i>observation</i>
ninhydrin	yellow solution
xanthoproteic	(+) yellow solution
Millon's	(+) red precipitate
Hopkins-Cole	(-) colorless
nitroprusside	(+) purple solution

<i>test</i>	<i>observation</i>
(1) xanthoproteic	
(2) Millon's	
(3) Hopkins-Cole	
(4) nitroprusside	

Data Sheet 1

I. Amino Acid and Protein Solubility

Solubility in Water

observations

glycine

glutamic acid

lysine

tyrosine

gelatin

casein

Solubility as a Function of Solution pH

3M NaOH solution

*3M HCl solution
(weakly acidic)*

*3M HCl solution
(strongly acidic)*

glycine

glutamic acid

lysine

tyrosine

gelatin

casein

Data Sheet 2

II. Chemical Reactions of Amino Acid and Protein Functional Groups

Ninhydrin Test

observations

2% glycine

1% tyrosine

2% proline

2% casein

2% gelatin

2% albumin

Biuret Test*observations*

2% glycine

2% arginine

2% gelatin

2% casein

2% albumin

Xanthoproteic Test*observations**after heating**after adding 3M NaOH*

1% tyrosine

2% tryptophan

2% glycine

2% albumin

2% gelatin

Millon's Test*observations*

1% tyrosine

2% tryptophan

2% glycine

2% albumin

2% gelatin

2% casein

Hopkins–Cole Test*observations*

1% tyrosine

2% tryptophan

2% albumin

2% gelatin

Nitroprusside Test*observations*

2% glycine

2% cysteine

2% albumin

2% casein

blank solution

Pre-Laboratory Assignment

1. Describe the hazards associated with the following procedures.

(1) xanthoproteic test

(2) Millon's test

(3) Hopkins–Cole test

(4) nitroprusside test

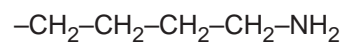
2. Briefly explain the meanings of the following terms as they relate to this experiment.

(1) essential amino acids

(2) peptide bond

(3) isoelectric point

3. The R-group of the amino acid lysine is



Draw the complete structure of lysine, including the sites of ionization, in:

(1) strongly acidic solution

(2) neutral solution

(3) strongly basic solution

4. Predict the results of the following tests on lysine.

(1) solubility in acidic solution

(2) ninhydrin

(3) biuret

(4) nitroprusside

