Laboratory Experiments in BIOCHEMISTRY

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BY

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PREFACE

Many students specializing in agriculture, home economics, biology, and chemistry are required to take at least one general course in biochemistry. Most of these students do not expect to become specialists in biochemistry, but they desire to learn some of the more important facts of biochemistry which either can be applied to their particular field, or used to increase their general knowledge of science.

This laboratory manual has been designed for students who want a broad, general background in elementary biochemistry. It is used in a one-semester course in general biochemistry taught to sophomore students in the Purdue School of Agriculture. The laboratory work in this sophomore course consists of one three-hour laboratory period per week. The students who enroll in the course have had general and organic chemistry, zoology, botany and bacteriology, but have had no quantitative analysis.

In choosing the experiments, an attempt has been made to show the diversity of the field of biochemistry. Not all of the experiments can be preformed by the average student in the allotted time of fifteen three-hour periods. If, however, the instructor dispenses most of the reagents from burettes, the number of experiments that can be assigned will be increased.

The reader will observe that the laboratory directions have been printed only on the left-hand pages of the manual. The authors feel that this arrangement of printed matter will encourage the student to enter his observations on the right-hand pages of the manual at the time that he is actually carrying out the experiments in the laboratory.

The authors are grateful to Drs. D. A. Burns, D. Gambal, A. R. Pappenhagen, H. E. Parker, N. E. Lloyd, H. Renis, E. D. Schall and R. A. Shaw for their suggestions during the preparation of this revision.

<div style="text-align:center">

E. T. Mertz

J. W. Porter

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GENERAL INSTRUCTIONS

The following instructions are to acquaint and guide you in the procedure to be followed in performing and reporting laboratory experiments. You are to perform each experiment individually.

1. PROMPTNESS: At the beginning of each laboratory period some time will be devoted to a discussion of the work for the day. If tardy, you will, of course, miss some of the discussion. In addition, the assignment for the following week will be given during this discussion period.

2. PREPARATION: Inasmuch as your assignments will be given one week in advance, it is expected that you will acquaint yourself with the objective and procedure for each experiment to be performed in any given week. Careful planning of your work will enable you to understand each experiment and to complete it with a minimum of wasted effort.

3. CLEANLINESS AND ORDERLINESS: The condition of your laboratory table and equipment is some indication as to whether you are an exacting student. Therefore, your instructor will consider these factors in his appraisal of your work.

4. CHEMICALS: Use only the quantity of each chemical that is necessary for each experiment. Do not pour chemicals back into reagent bottles.

5. QUESTIONS: Your instructor is provided for the purpose of supervision. Therefore, do not hesitate to ask questions. You are expected, however, to make certain you cannot answer your questions with some additional thought before you monopolize his time.

6. METHOD OF RECORDING DATA: The directions for each experiment have been printed on the left-hand pages of your manual. The right-hand pages (blank) are to be used for recording the following data:

    a. Title of experiment
    b. Date
    c. Objective of experiment
    d. Observations

    Your experimental observations should be recorded in the form of brief, but complete, statements or properly labeled tables, depending upon the nature of the experiment. All observations should be recorded in the same sequence as they are made.

    The observations which you record in your notebook will be checked by your instructor before you leave the laboratory. If the data in your notebook are not complete, you will be given an incomplete for the experiment in question.

7. REPORTS: A written report will be due one week after the completion of each experiment. This report is to be written on ruled paper and handed to the instructor in a manila folder bearing your name and section.

The report should be organized as follows:

TITLE

DATE_____   NAME _____

1. Objective - a brief statement of the purpose of carrying out the experiment.

2. Procedure - refer to the appropriate pages in the laboratory manual unless you
    change the experimental procedure. If you do change it, state the changes
    and your reasons why.

3. Results - briefly state what happened in the experiment. Tables or graphs may be
    helpful in correlating the data in some cases.

4. Conclusions - include equations for known chemical reactions. Briefly tell what
    facts can be derived from your experimental results.

5. Discussion - a general discussion of the applicability of the experiment, its limita-
    tions, and the significance of your results. Answer any questions that are
    asked in the laboratory manual. End your report with any comments that you
    might have about the experiment - both pro and con - so that we might make
    the laboratory more useful and interesting.

HELPFUL HINTS

1. Be careful of your grammar and spelling as these faults will lower the instructor's
    opinion of your report.

2. Be brief and concise - say what you want to say in as few words as possible. Be
    sure to read your report carefully after it is written to be sure you know what is
    said.

3. Keep a good record of your experimental results while in the laboratory and this will
    aid you in writing a report.

4. Don't copy from the book. Say what you want in your own words and your report
    will be shorter and more valuable.

LOCKER ITEMS

2 Graduated Cylinders, 100 ml.	6 Slides, Microscope
1 Beaker, 150 ml.	1 Spatula
2 Beakers, 250 ml.	1 Sponge
1 Beaker, 400 ml.	2 Stirring Rods
1 Beaker, 600 ml.	1 Support, Test Tube
1 Beaker, 800 ml.	1 Tongs
1 Beaker, 1000 ml.	1 Towel
1 Bottle, Wash (Plastic)	2 Triangles
1 Clamp, Test Tube	20 Tubes, Test, Small
3 Flasks, Erlenmeyer, 250 ml.	4 Tubes, Test, Large
1 Flask, Florence, 1000 ml.	2 Watch Glasses, 3"
2 Funnels, Pyrex, 60°	2 Wire Gauze
1 Pipette, Mohr, 10 ml.	2 Bottles, Pyrex, 1000 ml.
1 Pipette, Volumetric, 5 ml.	2 Burettes, 50 ml.
1 Policeman	1 Burner, Bunsen

Inspect your apparatus before you begin work. Report any deficiencies to your instructor. When you have checked and signed this slip you are responsible for the above equipment.

Desk No. _____   Date _____

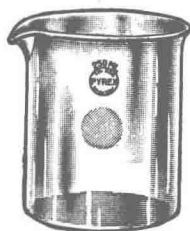I, _____ , have received the above items in good condition.

Additional items needed during the semester may be obtained, upon request, from the stockroom.
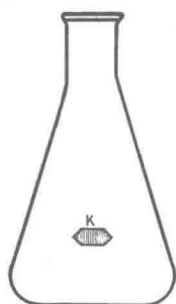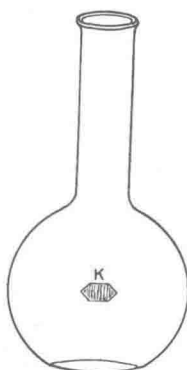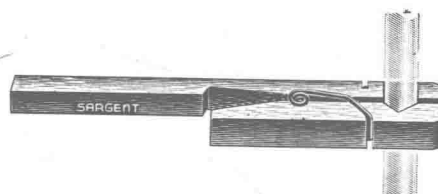
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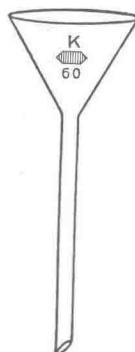
GRADUATE

BEAKER

BOTTLE, WASH

FLASK, ERLENMEYER

FLASK, FLORENCE
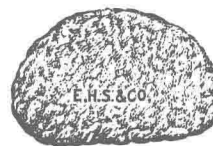
CLAMP, TEST TUBE

FLASK, VOLUMETRIC
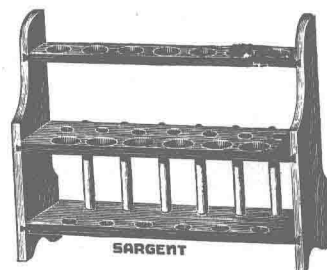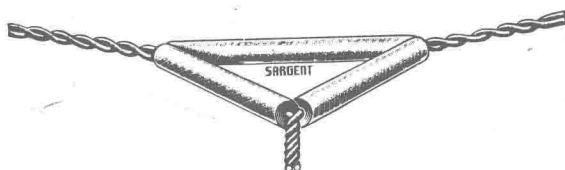
FUNNEL

PIPETTE, MOHR

PIPETTE, VOLUMETRIC

POLICEMAN AND
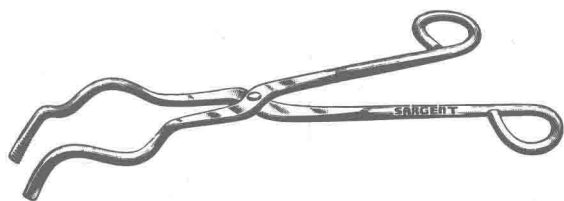GLASS ROD

SPATULA

SPONGE

SUPPORT, TEST TUBE

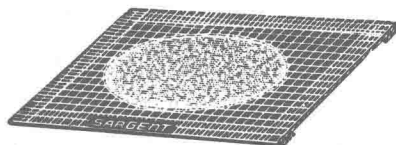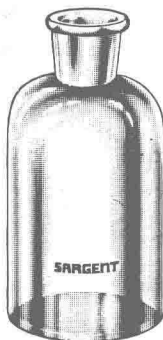WATCH GLASS

TRIANGLE

TONGS

WIRE GAUZE

BOTTLE
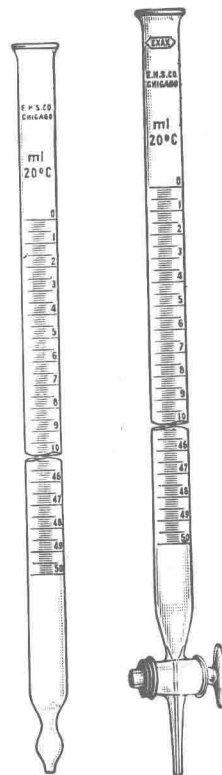
BURETTES

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Chapter I
HYDROGEN IONS, pH, AND BUFFERS

I. INTRODUCTION

A. Hydrogen and hydroxyl ions:

Many compounds are dissociated into electrically charged particles called ions when they are placed in water. The dissociation of the compound results in an equal number of positive and negative charges. Those ions with positive charges (K^+, Mg^{++}, H^+, Fe^{+++}, etc.) are attracted to the negative pole of an electrical circuit (cathode) and are called cations. Those with negative charges, (Cl^-, OH^-, SO_4^{--}, PO_4^{---}, etc.) are attracted to the positive pole of an electrical circuit (anode) and are called anions. Compounds which dissociate into ions are called electrolytes and fall into three main classes: acids, bases and salts. All acids have the same cation (hydrogen ion), all bases have the same anion (hydroxide ion), whereas salts are formed by the reaction of an acid with a base and are made up of the anion of an acid and the cation of a base.

The hydrogen and hydroxide ion concentrations of water extracts of biological materials have a marked influence on the reactivity of many compounds in the extract. It is, therefore, important that we measure hydrogen and hydroxide ion concentrations with accuracy.

Acids contribute hydrogen ions, and bases contribute hydroxyl ions to water solutions; however, both hydrogen and hydroxyl ions are always present due to a slight dissociation of water itself. This is shown in the following reversible reaction:

$$HOH \rightleftharpoons H^+ + OH^-$$

The degree of dissociation for water has been determined, and it has been found that pure water contains 1×10^{-7} grams of hydrogen ions and 1×10^{-7} gram equivalents, or $17 \times 1 \times 10^{-7}$ grams, of hydroxyl ions per liter. This is such a small amount that the amount of undissociated water per liter may be considered to be constant (unity) in the equilibrium equation for water:

$$\frac{(\text{conc. } H^+) \text{ X } (\text{conc. } OH^-)}{(\text{conc. undissoc. } H_2O)} = K_1 \text{ ; let conc. undissoc. } H_2O = K_2$$

then: $(\text{conc. } H^+) \text{ X } (\text{conc. } OH^-) = K_1 K_2 = K$

or in pure water: $1 \times 10^{-7} \times 1 \times 10^{-7} = K = 1 \times 10^{-14}$

When an acid is added to pure water, the hydrogen ions that it contributes must satisfy the above equilibrium equation for water. Thus, if 9×10^{-7} gm. of hydrogen ions are contributed by the acid per liter, the hydroxyl ion concentration must decrease proportionately to maintain the constant, K, at 1×10^{-14}:

$$(9 \times 10^{-7} + 1 \times 10^{-7}) \times \frac{1}{10} \times 10^{-7} = 1 \times 10^{-14}$$

$$1 \times 10^{-6} \times 1 \times 10^{-8} = 1 \times 10^{-14}$$

Addition of an alkali would cause a downward shift in the hydrogen ion concentration in a similar fashion.

B. pH

Because of the difficulty in expressing hydrogen ion concentration in the form of negative exponents, Sorensen proposed the term, pH, which is related to hydrogen ion concentration as follows:

$$pH = \log \left(\frac{1}{H} + \right)$$

The relation of pH to hydrogen ion and hydroxyl ion concentration is seen from the following scale:

pH	0	4	7	10	14
H^+ in gm/L.	1	1×10^{-4}	1×10^{-7}	1×10^{-10}	1×10^{-14}
OH^- in gm/L. (X17)	1×10^{-14}	1×10^{-10}	1×10^{-7}	1×10^{-4}	1

Since pH 7 represents the hydrogen ion concentration of pure water, it is called the neutral point on the pH scale. pH 0 to 7 is the acid side of the scale (more H^+ than OH^- present in aqueous solution), and pH 7-14 is the alkaline side of the scale (more OH^- than H^+ present in aqueous solution).

The pH scale described above adequately covers hydrogen ion and hydroxyl ion concentrations encountered in biological systems. Thus, gastric juice from the stomach of animals has a pH of about 1-2, tissue juice about 7.5, blood about 7.3 and intestinal juice about 8.0; in plants, lemon juice has a pH of 2.2, tomato juice about 4.2, flower petals about 6, and many other plant tissues about 6-6.5.

1. Determination of pH.

Since the measurement of hydrogen ion concentration in aqueous solutions in the pH range 0-14 covers not only strongly acid solutions, but also strongly alkaline solutions as well, it is not necessary to determine hydroxide ions directly. We need only to measure the hydrogen ion concentration, and we can obtain the hydroxide ion concentration by substituting in the equation

$$(H^+) \times (OH^-) = 1 \times 10^{-14}$$

$$(OH^-) = \frac{1 \times 10^{-14}}{(H^+)}$$

The methods used most commonly for the determination of hydrogen ions are (a) colorimetric and (b) electrometric.

a. Colorimetric

Certain dyes change color or change from a colored to a colorless form in water solution when they are subjected to a change in pH of 1 to 2 units.

The names of some of these dyes, which are called <u>indicators</u>, the pH range in which they change color, and the color change involved, are given in Table 1.

TABLE 1

pH RANGE OF SOME COMMON INDICATORS*

Reagent No.	Indicator	Range	Color	Composition of Solution (%)
1	Thymol blue	1.2 - 2.8	red-yellow	0.04
2	Brom phenol blue	3.0 - 4.6	yellow-blue	0.04
3	Chlor phenol red	4.8 - 6.4	yellow-red	0.04
4	Brom thymol blue	6.0 - 7.6	yellow-blue	0.04
5	Phenol red	6.8 - 8.4	yellow-red	0.02
6	Cresol red	7.2 - 8.8	yellow-red	0.02
7	Phenolphthalein	8.3 -10.0	colorless-red	0.02

*Clark, Lubs and Cohen indicators as modified by Danielson and Van Kloester.

<u>Colorimetric</u> estimations of pH are based on the use of indicators such as those listed in Table 1. Indicators do not change color abruptly at given values of pH, but undergo rather gradual changes in color and depth of color, between definite limits of hydrogen ion concentration. These limits vary with different indicators. pH values lying between the useful limits for a given indicator are said to lie within the range of the indicator. Thus, phenolphthalein undergoes definite changes in shade between pH values of 8.3 and 10.0. It is evident that if one were to construct a color scale, by placing phenolphthalein in solutions of graded pH values lying between 8.3 and 10.0 (buffered solutions), he would be able to determine the pH of an unknown solution (if it is within the phenolphthalein range) by adding the same amount of indicator to it, and matching the color so obtained against the standard phenolphthalein color scale. The limit of accuracy for this method is about 0.2 of a pH unit. The number of indicators available is very large, and their individual pH ranges run from as low as 0.1 to as high as 13.0. By careful selection, one may eliminate the majority of them, and still be able to cover the useful range for hydrogen ion work.

b. <u>Electrometric</u>

The pH of a solution may be determined <u>electrometrically</u> by measuring the potential (E.M.F.) set up between that solution and gaseous hydrogen. From this value the pH may be calculated. The hydrogen electrode method is rather delicate and difficult to operate, and other methods have been devised which are more desirable. The most popular method is that which employs a glass electrode. The concentration of the active hydrogen ions in the un-known solution causes a change in the potential of the half cell enclosed within the small glass diaphragm. The change in the potential of the half cell is pro-portional to the effective hydrogen ion concentration and thus can be used as a measure of the pH. Most glass electrodes measure pH with an accuracy of $\pm$ 0.02 of a pH unit.
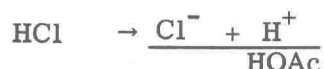
C.   Buffers

Buffer solutions are mixtures containing weak acids and their salts, or weak bases and their salts. These buffers have the power to maintain a nearly constant pH upon the addition of acid or base. An example of a buffer is a mixture of sodium acetate and acetic acid. If HCl is added to this buffer, we get:
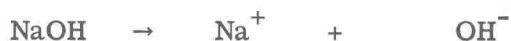
$$NaOAc + HCl \rightarrow NaCl + HOAc$$

The addition of HCl causes the formation of acetic acid, which is only slightly dissociated, with the result that there is little or no change in the hydrogen ion concentration.

This may be formulated in a slightly different way:

$$NaOAc \rightarrow Na^+ + AcO^-$$

$$HCl \rightarrow \underline{Cl^- + H^+}$$
$$HOAc$$

With the addition of alkali, we have:

$$NaOH + HOAc \rightarrow NaOAc + H_2O \text{ or}$$

$$NaOH \rightarrow Na^+ + OH^-$$

$$HOAc \rightarrow \underline{AcO^- + H^+}$$
$$H_2O$$

Several sets of buffer solutions can be prepared which have various pH ranges, and obviously each set has its advantages for specific purposes. A very convenient buffer solution series that may be used for colorimetric pH determinations can be prepared by mixing solutions of secondary sodium phosphate and citric acid. Table 2 gives the quantities of these solutions that must be mixed to obtain a buffer solution of the desired pH.

TABLE 2

BUFFER SOLUTIONS
(MC ILVAIN'S STANDARDS)

pH	ml 0.2 Molar $Na_2H\,PO_4$	ml. 0.1 Molar Citric Acid	pH	ml. 0.2 Molar $Na_2H\,PO_4$	ml. 0.1 Molar Citric Acid
2.2	0.20	9.80	5.2	5.36	4.64
2.4	0.62	9.38	5.4	5.58	4.42
2.6	1.09	8.91	5.6	5.80	4.20
2.8	1.58	8.42	5.8	6.05	3.95
3.0	2.05	7.95	6.0	6.31	3.69
3.2	2.47	7.53	6.2	6.61	3.39
3.4	2.85	7.15	6.4	6.92	3.08
3.6	3.22	6.78	6.6	7.27	2.73
3.8	3.55	6.45	6.8	7.72	2.28
4.0	3.85	6.15	7.0	8.24	1.76
4.2	4.14	5.86	7.2	8.69	1.31
4.4	4.41	5.59	7.4	9.08	0.92
4.6	4.67	5.33	7.6	9.37	0.63
4.8	4.93	5.07	7.8	9.57	0.43
5.0	5.15	4.85	8.0	9.72	0.28

For example, if one wishes to prepare a buffer with a pH of 4.2, this would be achieved by mixing with accuracy 4.14 ml. of 0.2 M $Na_2H PO_4$ and 5.86 ml. of 0.1 M citric acid (microburette or graduated pipette). If a larger quantity of the buffer solution is desired, simply mix multiple quantities of these values.

II. EXPERIMENTAL DETERMINATION OF pH

Determine the pH of an unknown buffer solution colorimetrically as follows:

Place about 1 ml. of the solution in a test tube, add one drop of indicator No. 4* and note whether the indicator assumes its "acid" or its "alkaline" color. If "alkaline" proceed to test, with additional 1 ml. quantities of solution, indicators No. 5, 6, and 7, until the proper one is found. If "acid" proceed to test, in turn, indicators No. 3, 2, and 1. When you have determined which indicator is the correct one to use, prepare buffer solutions corresponding to its range.

Place 5 ml. of each buffer solution required (based on tests with indicators) in small labeled test tubes, and add 5 drops of the proper indicator to each tube. Each tube must contain the same quantity of indicator.

Place 5 ml. of the unknown solution in a similar test tube, and add 5 drops of the proper indicator to it. Determine the pH value of the solution by matching the color so obtained against the indicator colors of the buffered solutions. Record the pH. Check the correctness of the pH value obtained by the indicator method by determining the pH value electrometrically.

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* See Table 1, page 3.

Chapter II
ACID-BASE TITRATIONS

I. INTRODUCTION

Most acids and bases do not ionize completely when placed in water. For example, only a small part (about 1%) of the molecules of acetic acid split into ions when placed in solution. Potentially, however, a gram molecular weight of acetic acid can yield as many hydrogen ions as a gram molecular weight of hydrochloric acid. This actually happens when acetic acid is neutralized with sodium hydroxide. In this reaction, the formation of undissociated water allows the reaction to go to completion and therefore acetic acid yields all of its potential hydrogen ions as available or titratable ions:

$$HC_2H_3O_2 \ + \ NaOH \ \rightarrow \ NaC_2H_3O_2 \ + \ HOH$$

$$HCl \qquad + \ NaOH \ \rightarrow \ NaCl \qquad + \ HOH$$

It can be seen that gram molecular weights of acetic acid and hydrochloric acid are neutralized by the same quantity of a strong base, i.e., they yield equivalent amounts of titratable hydrogen ions. Gram molecular weights of acetic and hydrochloric acids are then equivalent, and when dissolved in the same quantity of solution, they have the same normality.

The equivalent weight of any compound can be determined by dividing the gram molecular weight of the compound by the number of hydrogen ions (H^+) or hydroxyl ions (OH^-) furnished actually or potentially by each molecule of the compound. If the equivalent weight of an acid or base is dissolved in sufficient water to make exactly 1 liter of solution, this solution will be a one normal solution (See Chapter XI), and contains one equivalent weight of acid or base. A milliequivalent weight is the weight in grams of the acid or base in 1 milliliter of 1 normal solution. On the basis of these definitions, one liter of any 1 $\underline{N}$ acid will exactly neutralize 1 liter of any 1 $\underline{N}$ base to form a salt and water.

Mixing 1 liter of 1 $\underline{N}$ solution of acid A with 1 liter of 1 $\underline{N}$ solution of base B does not always give a salt solution having a pH of 7. This happens only when the acid A and the base B ionize to the same extent in water. Strong acids (HCl, HNO_3, etc.) and strong bases (NaOH, KOH, etc.) ionize to about the same extent and hence give salt solutions of pH 7. Weak acids (HOAc, etc.) and weak bases (NH_4OH, etc.) ionize to about the same extent and thus also give salt solutions of pH 7. However, salts produced by the interaction of equal volumes of 1 $\underline{N}$ solutions of a strong acid with a weak base have a pH of less than 7 (usually about 5) and salts produced by the interaction of equal volumes of 1 N solutions of a weak acid with a strong base have a pH of more than 7 (usually about 9). These pH values obtained at the end-point or stoichiometric point (equivalent amounts of acid and base used in the titration) are apparently caused by a slight hydrolysis of the salt into equivalent amounts of the original acid and base.

When an acid of unknown normality is to be analyzed by titrating it against a base of known normality, the end-point of the titration is the pH at which equivalent amounts of the acid and base are present. This end-point pH value has been determined either (1) colorimetrically with indicators or (2) electrometrically, with a hydrogen, or glass electrode assembly, for most acid-base titrations.

Characteristic neutralization curves are obtained with four combinations of acid and base: (I) strong acid titrated with strong base (II) strong acid titrated with weak base (III) weak acid titrated with strong base and (IV) weak acid titrated with weak base. These characteristic curves are reproduced in Figure 1. The end-point of any acid-base titration is that pH at which equivalent amounts (equal volumes of the same normality) of acid and base are present. Inspection of the titration curves in Figure 1 show that the end-point pH for strong acid - strong base titrations is 4-10, for strong acid - weak base titrations 4-6, for weak acid - strong base titrations 8-10, and for
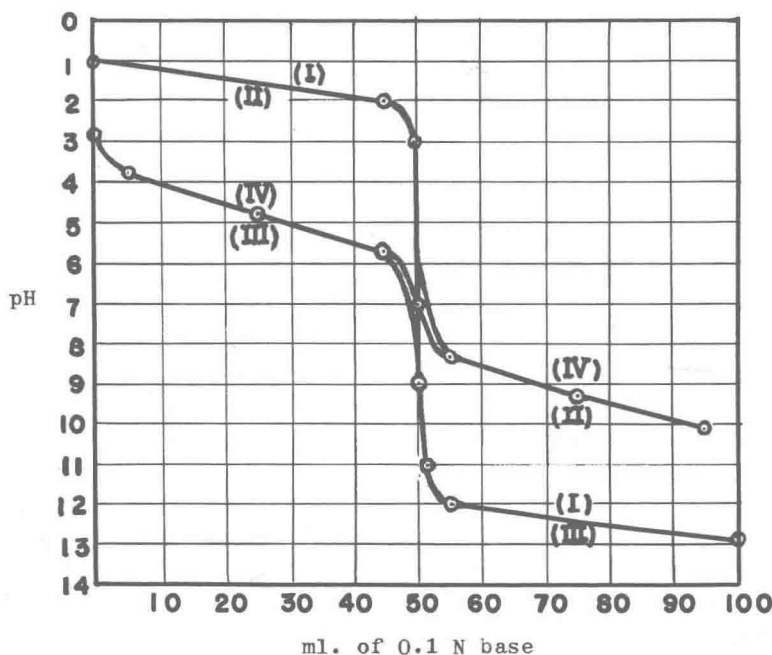

Figure 1

NEUTRALIZATION CURVES

(I) HCl-NaOH;   (II) HCl-NH₄OH;   (III) HOAc-NaOH;   (IV) HOAc-NH₄OH;

To 50 ml. portions of 0.1 $\underline{N}$ acid, the amounts of 0.1 $\underline{N}$ base indicated by the abscissa were added.

weak acid - weak base titrations, 7. By referring to the pH range of color change for the indicators listed in Table 1, p. 3, select the indicator (or indicators) that would give the greatest accuracy for visual end-point titrations with the four combinations of acid and base discussed above.

II. EXPERIMENTS ON THE TITRATION OF ACIDS AND BASES

A.   Titration of a strong acid with a strong base.

Accurately measure 10 ml. of 0.1 $\underline{N}$ HCl (burette) into a small Erlenmeyer flask. Add 2 drops of phenophthalein. Titrate with 0.1 $\underline{N}$ NaOH to the first appearance of a pink color which persists for 30 seconds. To another 10 ml. portion of 0.1 $\underline{N}$ HCl solution, add 2 drops of brom phenol blue, and titrate with NaOH to the appearance of a blue color. Assume that the normality of the NaOH is not known, and calculate the apparent normality of the NaOH as found with the

2 indicators. Explain your results on the basis of the titration curves in Figure 1, p. 7, and the data on indicators in Table 1, p. 3.

B. Titration of a strong acid with a weak base.

Accurately measure 10 ml. of 0.1 N HCl into a small Erlenmeyer flask. Add 2 drops of phenolphthalein. Titrate with 0.1 N ammonium hydroxide to the pink end-point. To another 10 ml. portion of 0.1 N HCl, add 2 drops of brom phenol blue, and titrate with 0.1 N ammonium hydroxide solution to the blue end-point. Assume that the normality of the NH_4OH is not known, and calculate the apparent normality of the NH_4OH as found with the 2 indicators. Explain your results on the basis of the titration curves in Figure 1 and the data in Table 1.

C. Titration of a weak acid with a strong base.

Using 0.1 N acetic acid in place of the 0.1 N HCl, repeat Exp. A. Assume that the normality of the sodium hydroxide is not known, and calculate the apparent normality of the NaOH as found with each indicator. Explain your results on the basis of the titration curves in Figure 1 and the data in Table 1.

D. Titration of a weak acid with a weak base.

Using 0.1 N acetic acid in place of the 0.1 N HCl, repeat Exp. B. Assume that the normality of the ammonium hydroxide is not known, and calculate the apparent normality of the NH_4OH as found with each indicator. Explain your results on the basis of the titration curves in Figure 1 and the data in Table 1.

E. Titration of a buffer (NaOAc - HOAc).

1. Add 2 drops of brom phenol blue to 10 ml. of water having a pH of 7. How many drops of 0.1 N hydrochloric acid are required to bring the solution to a yellow color? Add 2 drops of phenolphthalein to 10 ml. of distilled water. (pH = 7). How many drops of 0.1 N sodium hydroxide are required to bring the solution to a pink color?

2. Into each of two beakers A and B, place 1.0 ml. of 0.01 N acetic acid, 1 ml. of 0.1 N sodium acetate, and 8 ml. of distilled water (pH of buffer solution= 5.6). To beaker A add brom phenol blue, to beaker B add phenolphthalein. Determine how many drops of 0.1 N hydrochloric acid are required to bring the solution in beaker A to the yellow color end-point of brom phenol blue; determine how many drops of 0.1 N NaOH are required to bring the solution in beaker B to the pink color end-point of phenolphthalein. Compare these values with those obtained in (1), and explain the differences.