Laboratory manual of biological chemistry : with supplement / by Otto Folin.

LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

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WITH SUPPLEMENT

BY

OTTO FOLIN

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IN HARVARD MEDICAL SCHOOL

FIFTH EDITION

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PREFACE TO FIFTH EDITION

Several years have elapsed since the fourth edition of this manual was published. During the intervening period many of the analytical methods used in the department of biological chemistry in Harvard Medical School have been modified, and new methods have been developed. In order to bring the manual up to date it has therefore been necessary to introduce more extensive revisions and re-writing than in any previous edition. The newer methods applicable to blood analysis are particularly important, because in some cases they yield values which are materially different from those obtained by the older technique. Three micro methods based on the use of 0.1 or 0.2 cc. of blood are described. These methods for the determination of sugar, nonprotein nitrogen, and uric acid have already come into extensive use both for clinical purposes and for research. Some previously unpublished material is described, among which may be mentioned a revised method for the determination of creatinine in blood.

I am indebted to Drs. M. A. Logan and I. S. Danielson and especially to Professor Fiske, for many improved details and also for some of the major revisions. Professor Bloor, in this as in every previous edition, has contributed the methods for the determination of fats and cholesterol in blood.

OTTO FOLIN

PREFACE TO FIRST EDITION

This manual of biological chemistry for medical students in Harvard Medical School has been revised annually for the past seven years, and it is believed now to meet our needs sufficiently well to warrant publication.

For many years I have been interested in the development of analytical methods applicable to metabolism investigations. The most serviceable of my older methods and some of the newer methods have been taught to our medical students; these are described in the main body of the manual. Others not heretofore included have been incorporated in the supplement, so that nearly all the newer methods devised in the department are now described in this manual.

In connection with the revisions referred to above I am indebted for valuable help to W. R. Bloor, W. Denis, C. J. Farmer, L. J. Morris, F. B. Kingsbury, F. S. Hammett, R. D. Bell, and C. H. Fiske, as well as to my older friend, P. A. Shaffer.

OTTO FOLIN

BOSTON

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INTERNATIONAL ATOMIC WEIGHTS

Name	Symbol	Atomic Weight	Principal Valence
Aluminum	Al	26.97	3
Antimony, stibium	Sb	121.76	3 or 5
Argon	A	39.94	0
Arsenic	As	74.93	3 or 5
Barium	Ba	137.36	2
Beryllium, glucinum	Be	9.02	2
Bismuth	Bi	209.0	3 or 5
Boron	B	10.82	3
Bromine	Br	79.92	1
Cadmium	Cd	112.41	2
Cæsium	Cs	132.81	1
Calcium	Ca	40.08	2
Carbon	C	12.00	2 or 4
Cerium	Ce	140.13	4 or 3
Chlorine	Cl	35.46	1
Chromium	Cr	52.0	2, 3 or 6
Cobalt	Co	58.94	2 or 3
Columbium, niobium	Cb	93.3	3 or 5
Copper	Cu	63.57	1 or 2
Dysprosium	Dy	162.5	3
Erbium	Er	167.6	3
Europium	Eu	152.0	3
Fluorine	F	19.0	1
Gadolinium	Gd	157.3	3
Gallium	Ga	69.72	3
Germanium	Ge	72.6	4
Gold, aurum	Au	197.2	1 or 3
Hafnium	Hf	178.6	4
Helium	He	4.00	0
Holmium	Ho	163.5	3
Hydrogen	H	1.008	1
Indium	In	114.8	3
Iodine	I	126.92	1
Iridium	Ir	193.1	3 or 4
Iron, ferrum	Fe	55.84	2 or 3
Krypton	Kr	83.7	0
Lanthanum	La	138.92	3
Lead, plumbum	Pb	207.2	2 or 4
Lithium	Li	6.94	1
Lutecium	Lu	175.0	3
Magnesium	Mg	24.32	2
Manganese	Mn	54.93	2, 4, 6 or 7
Mercury, hydrargyrum	Hg	200.6	1 or 2
Molybdenum	Mo	96.0	2, 4 or 6
Neodymium	Nd	144.3	3
Neon	Ne	20.18	0
Nickel	Ni	58.69	2 or 3
Nitrogen	N	14.008	3 or 5
Osmium	Os	190.8	2, 3, 4 or 8
Oxygen	O	16.00	2
Palladium	Pd	106.7	2 or 4
Phosphorus	P	31.02	3 or 5
Platinum	Pt	195.23	2 or 4
Potassium, kalium	K	39.10	1
Praseodymium	Pr	140.9	3
Radium	Ra	226.0	2
Radon	Rn	222.0	0
Rhenium	Re	186.31	0
Rhodium	Rh	102.9	3
Rubidium	Rb	85.44	1
Ruthenium	Ru	101.7	3, 4, 6 or 8
Samarium	Sa	150.4	3
Scandium	Sc	45.1	3
Selenium	Se	79.2	2, 4 or 6
Silicon	Si	28.06	4
Silver, argentum	Ag	107.88	1
Sodium, natrium	Na	23.00	1
Strontium	Sr	87.63	2
Sulfur	S	32.06	2, 4 or 6
Tantalum	Ta	181.4	5
Tellurium	Te	127.5	2, 4 or 6
Terbium	Tb	159.2	3
Thallium	Tl	204.39	1 or 3
Thorium	Th	232.12	4
Thulium	Tm	169.4	3
Tin, stannum	Sn	118.7	2 or 4
Titanium	Ti	47.90	3 or 4
Tungsten, wolframium	W	184.0	6
Uranium	U	238.14	4 or 6
Vanadium	V	50.95	3 or 5
Xenon	Xe	131.3	0
Ytterbium	Yb	173.5	3
Yttrium	Yt	88.92	3
Zinc	Zn	65.38	2
Zirconium	Zr	91.22	4

LABORATORY MANUAL OF BIOLOGICAL CHEMISTRY

PART I

ACIDIMETRY, ALKALIMETRY, NITROGEN DETERMINATION

Equivalent and Normal Solutions.—Since the molecular weight of sodium hydroxide (NaOH) is 40 and that of hydrochloric acid (HCl) is 36.46, it follows that 40 gm. of the former contain the same number of molecules as 36.46 gm. of the latter. If 40 gm. of sodium hydroxide and 36.46 gm. of hydrochloric acid are each dissolved in pure water sufficient to make one liter of solution, each liter will contain the same number of dissolved molecules.

It will take a little less than one liter of water to make a liter of solution because the dissolved substance takes up some space. A normal sodium hydroxide solution contains four per cent of sodium hydroxide. By per cent in the case of solutions is usually meant the amount (weight) of substance present in 100 cc. of solution. In some cases the term per cent, as often used, is not entirely clear. This is likely to be so when the dissolved substance is either a liquid or a solid containing water—moisture or water of crystallization.

Mixing equal volumes of two such solutions is, therefore, the same as bringing together practically the same number of the two kinds of molecules, and the result is the instantaneous and essentially complete transformation into sodium chloride (and water).

$$NaOH + HCl = NaCl + H_2O$$

If either or both of the solutions should first be diluted with a considerable bulk of pure water, the result on mixing the two

would be the same, for the extra amount of water present takes no part in the reaction (except to the extent of absorbing a part of the heat set free).

The two solutions are equivalent. They also happen to be normal solutions. The hydrochloric acid is normal because it contains 1 gm. of active or replaceable hydrogen per liter of solution, and not because it contains the same number of grams of HCl per liter as there are units in the molecular weight. The sodium hydroxide solution is normal because it is equivalent to a solution containing 1 gm. of replaceable hydrogen per liter.

The molecular weight of sulfuric acid is 98. A sulfuric acid solution containing exactly 98 gm. per liter contains, therefore, the same number of molecules per unit volume as the sodium hydroxide solution containing 40 gm. per liter. But one molecule of sulfuric acid requires two molecules of sodium hydroxide for the formation of the neutral salt, sodium sulfate, because the sulfuric acid molecule has two replaceable hydrogen atoms. The solutions are not equivalent, for the sulfuric acid contains 2 gm. active hydrogen per liter. It is exactly twice as strong as the sodium hydroxide solution; it is a 2 normal solution.

On the basis of the above description of what constitutes a normal solution, calculate the number of grams per liter in tenth normal sulfuric acid (0.1 N H_2SO_4), fifth normal hydrochloric acid (0.2 N HCl), half normal oxalic acid (0.5 N $C_2H_2O_4$, $2H_2O$), fourth normal acetic acid (0.25 N CH_3COOH), half normal sodium hydroxide (0.5 N NaOH), twentieth normal barium hydroxide (0.05 N $Ba(OH)_2$), fifth normal ammonium hydroxide (0.2 N NH_4OH).

The same description of normal solutions applies to other substances than acids and alkalis, as, for example, reducing and oxidizing substances such as potassium permanganate, potassium dichromate, iodine, cupric hydroxide, stannous chloride. A normal solution here is one capable of liberating 1 gm. of reducing hydrogen (or of giving off exactly sufficient oxygen to oxidize 1 gm. of hydrogen) per liter. Potassium permanganate, for example, in the presence of sulfuric acid and some easily oxidizable substance is decomposed as follows:

$$2KMnO_4 + 3H_2SO_4 = K_2SO_4 + 2MnSO_4 + 3H_2O + 5O$$

As the two permanganate molecules liberate oxygen enough for ten hydrogen atoms it takes only one-fiftieth of the molecular

weight expressed in grams (3.161 gm.) to make one liter of tenth normal solution.

The calculation of what constitutes normal or equivalent solutions of any reagent is not very difficult, provided the equation representing the chemical reaction involved is thoroughly clear.

To determine whether a given unknown solution is acid or alkaline it is usually sufficient to dip a piece of delicate violet colored litmus paper into it. (If the solution is acid, the test paper turns red; if alkaline, it turns blue.) Litmus, the substance with which the paper has been impregnated, is a complex organic product, and is one of the most familiar representatives of a most useful class of organic compounds which are so sensitive to acids or alkalis, or both, that they clearly and unmistakably indicate the presence of free acid or alkali even when the amounts present are so small as to be practically unweighable. By means of such *indicators* and accurate measuring instruments (volumetric flasks, burettes, and pipettes), it becomes a simple matter to determine (by titration) the relative concentration or equivalence of acid and alkaline solutions. By their help it is possible to prepare with very little labor normal or tenth normal solutions, even of acids or alkalis which cannot be weighed on the balance, as for example, hydrochloric acid and ammonia, both of which are gases. Before this can be done we must, however, possess one normal or standard solution prepared from some substance which can be weighed.

Volumetric analysis consists of measuring the value of an unknown solution in terms of another, the value of which is known (titration). The known solution is prepared directly or indirectly by the help of the analytical balance, and the first step in any kind of volumetric analysis is the preparation of the standard solution by means of which the values of others are to be determined.

Every student who has had no experience in the use of the analytical balance must consult the instructor before proceeding. He should also ask for instruction as to the proper use of measuring flasks, pipettes, and burettes before using them. He must particularly learn when the presence of unmeasured quantities of water does not interfere with the accuracy of the work and when a single drop of unmeasured water introduces a perceptible error.

All the common mineral acids and strong bases contain so much

water that it is in practice not feasible to weigh out with sufficient accuracy the theoretical quantity required for a standard solution of acid or alkali. The carbonates of sodium or calcium (or the carbonates of sodium or potassium, obtained by ignition of the corresponding oxalates) give exceedingly accurate results. Oxalic acid is very serviceable as starting material for the preparation of standardized solutions of acids and alkalis if it is pure and has lost none of its water of crystallization.

Acids and Bases.—An acid is any substance that dissociates into hydrogen ions; a base is one that dissociates into hydroxyl ions. Accordingly, acetic acid, although it contains a hydroxyl *group,* is an acid, because it ionizes in the following way:

$$CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$$

It is not a base, since it does *not* ionize as follows:

$$CH_3COOH \rightleftharpoons CH_3CO^+ + OH^-$$

Some compounds (*e.g.,* the amino acids) can dissociate into both hydrogen and hydroxyl ions, according to circumstances; they are both acids and bases, and are called amphoteric electrolytes, or ampholytes.

While individual acids have properties peculiar to themselves, the characteristic properties common to all acids are due to the hydrogen ions which their solutions contain. Acids dissolve metals, hydrolyze esters, taste sour, redden blue litmus (and similarly affect the color of other indicators), combine with bases, and so on. But aqueous solutions of various acids, in the same concentration, differ greatly in the *facility* with which they do these things, and they differ because, while they all dissociate into hydrogen ions, they do not all do so to the same degree.

A *strong* acid is one that is almost completely dissociated when dissolved in water; a *weak* acid is one that is only slightly dissociated, and there are all gradations between. The common monobasic mineral acids (HCl, HNO_3) are strong acids. They are considered to be completely dissociated (or ionized) in aqueous solution, but except at very high dilutions some of the positive and negative ions are bound together by electrostatic forces and have no effect on indicators, on the rate of hydrolysis of esters, etc. In a hundredth normal solution of hydrochloric acid,

for example, only 90 per cent of the ions are "active," and in a tenth normal solution only 79 per cent (at 25° C.). Some inorganic acids (H_2S, H_2CO_3) are weak electrolytes, as are most organic acids.

Bases, in a similar way, are strong or weak, according as they are to a large or small extent dissociated into hydroxyl ions. The alkali metal hydroxides (NaOH, KOH, etc.) are strong bases; ammonium hydroxide is a typical weak base.

Hydrogen Ion Concentration.—The complete neutralization of one liter of a normal solution of acid requires one liter of normal alkali, and from that standpoint all acids are alike. But the actual acidity of a normal solution depends entirely upon the nature of the acid, and is determined, not by the amount of replaceable hydrogen per liter, but by the concentration of active *ionized* hydrogen. Thus, in a tenth-normal solution of hydrochloric acid, the hydrogen ion concentration, or activity ($[H^+]$)[1] is 79 per cent of tenth normal, or 0.079N. On the other hand, in a tenth-normal solution of acetic acid, only 1.36 per cent of the acid is ionized, and the $[H^+]$ is only 1.36 per cent of tenth normal, or 0.00136N. Although these two solutions are equivalent as far as titration is concerned, the acidity of one is nearly sixty times that of the other. This illustrates the difference between strong and weak acids, and leads to a distinction between two kinds of acidity, (1) titratable acidity, which is measured by the amount of standard alkali required to neutralize a given solution; and (2) actual acidity, or $[H^+]$—the number of gram-molecules or gram-ions of ionized hydrogen per liter (or, approximately, the number of grams of ionized hydrogen per liter, since the atomic weight of hydrogen is practically 1).

The dividing line between acid and alkaline solutions is water itself, which is ionized to a slight degree, as follows:

$$H_2O \rightleftharpoons H^+ + OH^-$$

Water, in other words, is largely undissociated water (H_2O), in equilibrium with a small proportion of H^+ and OH^- in equivalent quantities, so that the hydrogen ion concentration and the hydroxyl ion concentration, or $[OH^-]$, are equal. That is true

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[1] The symbol for a substance, enclosed in brackets, is the customary method of denoting its concentration.

not only of pure water, but of any neutral aqueous solution. On the other hand, a solution is acid if its $[H^+]$ is greater than its $[OH^-]$, and alkaline if the reverse is the case. The nature of the equilibrium between water and its two ions can be shown by means of the Law of Mass Action.

Law of Mass Action.—If two substances, A and B, when brought together in solution, react with each other to form two other substances, C and D, and if these two can react with each other in such a way that they are converted back into A and B, the reaction is a reversible one, and can be expressed as follows:

$$A+B \rightleftharpoons C+D$$

The two opposing reactions concerned may be called Reaction 1 ($A+B \rightarrow C+D$) and Reaction 2 ($C+D \rightarrow A+B$).

Reaction 1 may first be considered by itself. That is, if the solution at the start contains only A and B, it is the only reaction that can take place at first. The fundamental statement of the Law of Mass Action is that any substance participating in a chemical reaction does so at a rate proportional to its concentration. In the case of Reaction 1, which proceeds with a velocity (v_1) proportional to the concentrations of A and B (that is, $v_1 = k_1 [A][B]$),[2] A and B are used up (that is, their concentrations diminish), and so the velocity of this reaction is greatest at the beginning, and becomes less as time goes on.

However, as soon as A and B have begun to react, the products of their decomposition (C and D) begin to appear, and the concentrations of C and D steadily rise. The velocity (v_2) of Reaction 2, which is proportional to the concentrations of C and D ($v_2 = k_2 [C][D]$), was zero at the start (since C and D were absent), but becomes progressively greater as these products accumulate.

In view of the fact that the rate of one reaction is constantly decreasing, and the rate of the other reaction constantly increasing, the two velocities must at some point become equal. When that point has been reached, the two opposing reactions are taking place at the same rate, and the process will appear to have stopped. It is then said to have come to equilibrium.

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[2] See footnote, p. 9.

Since, at equilibrium, $v_1 = v_2$, it is also true that $k_1[A][B] = k_2[C][D]$, and this equation may be written as follows:

$$\frac{[C][D]}{[A][B]} = \frac{k_1}{k_2} = K$$

The ratio of the two velocity constants $\left(\frac{k_1}{k_2}, \text{ or } K\right)$ is called the "equilibrium constant" of the reaction, and it is always the same, for any given reaction, as long as the temperature does not change.

Ionization of Water.—Ordinary ionization reactions are practically instantaneous, and come to equilibrium immediately. Accordingly, in applying the Law of Mass Action to such a case as the ionization of water, all that need be considered is the situation when equilibrium has been reached. In water, which dissociates into hydrogen and hydroxyl ions ($H_2O \rightleftharpoons H^+ + OH^-$), the condition at equilibrium is shown by the following equation:

$$\frac{[H^+][OH^-]}{[H_2O]} = K, \quad \text{or} \quad [H^+][OH^-] = K[H_2O]$$

Inasmuch as the extent to which water ionizes is very small (corresponding with the fact that it is a poor conductor of electricity), the concentration of undissociated water, or $[H_2O]$, is not appreciably diminished because of the ionization, and the entire expression on the right of the equation ($K[H_2O]$) has a constant value, determined experimentally to be 0.00000000000001, or 10^{-14}, at room temperature (25°). That is,

$$[H^+][OH^-] = 10^{-14}$$

which is the value of the product of the two ion concentrations at that temperature, not only in pure water, but in *any aqueous solution.*

In pure water, there must be equal quantities of positive (hydrogen) and negative (hydroxyl) ions, or $[H^+] = [OH^-]$. Consequently,

$$[H^+][OH^-] = [H^+]^2 = 10^{-14} \text{ and } [H^+] = [OH^-] = 10^{-7} \text{ N}$$

The $[H^+]$ and the $[OH^-]$ of pure water are then both 10^{-7} N, which means that the acidity of water is roughly one ten-millionth as great as that of a normal solution of hydrochloric acid

(and its alkalinity the same small fraction of that of a normal solution of sodium hydroxide).

On this basis, neutral, acid and alkaline solutions may be redefined in more definite terms, as follows:

Neutral solution: $[H^+]=10^{-7}$ N $=[OH^-]$

Acid solution:[3] $[H^+]>10^{-7}$ N $>[OH^-]$

Alkaline solution: $[H^+]<10^{-7}$ N $<[OH^-]$

Hydrogen Exponent.—To avoid the use of unwieldy figures, like that given above (p. 9) for the $[H^+]$ of tenth normal acetic acid (0.00136 N, or 1.36×10^{-3} N), hydrogen ion concentrations are ordinarily expressed as logarithms. The logarithm of $1.36\times10^{-3}=\log\ 1.36+\log\ 10^{-3}=0.13+(-3.00)=-2.87$. This, with the minus sign omitted, is called the hydrogen exponent, or pH, *i.e.*, the pH of tenth normal acetic acid is 2.87. The value for the $[H^+]$ of any solution ($a\times10^{-b}$) may be converted into this system by subtracting log a from b, or, $pH=b-\log a$. Below are given the hydrogen exponents corresponding with a series of hydrogen ion concentrations ranging from 0.01 N to 0.001 N, to illustrate further the relation between the two methods of expression.

$[H^+]$	pH
$0.01=1\times10^{-2}$ N	$2-0.00=2.00$
$0.009=9\times10^{-3}$ N	$3-0.95=2.05$
$0.008=8\times10^{-3}$ N	$3-0.90=2.10$
$0.007=7\times10^{-3}$ N	$3-0.85=2.15$
$0.006=6\times10^{-3}$ N	$3-0.78=2.22$
$0.005=5\times10^{-3}$ N	$3-0.70=2.30$
$0.004=4\times10^{-3}$ N	$3-0.60=2.40$
$0.003=3\times10^{-3}$ N	$3-0.48=2.52$
$0.002=2\times10^{-3}$ N	$3-0.30=2.70$
$0.001=1\times10^{-3}$ N	$3-0.00=3.00$, etc.

[3] The word "acid" is used both as a noun and as an adjective, and these two uses must be sharply distinguished. Water is an acid (*n.*) because it dissociates into hydrogen ions. It is neutral (that is, not acid (*adj.*) or alkaline) because neither the H+ nor the OH— is in excess. Water is both an acid (*n.*) and a base, but nothing can be both acid (*adj.*) and alkaline.

It should be observed that, because of the omission of the minus sign, the pH increases as the $[H^+]$ becomes less. Consequently figures less than 7 signify an acid reaction, while the pH of alkaline solutions is greater than 7.

Ionization of Weak Acids and Bases.—The equilibrium equation for the ionization of any weak acid may be derived from the Law of Mass Action in much the same way that the equation for water was obtained. To take a typical example, acetic acid ($CH_3COOH \rightleftharpoons H^+ + CH_3COO^-$), we may write to begin with, exactly as in the case of water:[4]

$$\frac{[H^+]\cdot[CH_3COO^-]}{[CH_3COOH]} = K$$

K in this case is called the "dissociation constant" or "ionization constant" of acetic acid, and its magnitude is an indication of the strength of the acid. $[CH_3COOH]$ signifies the concentration of undissociated acetic acid. The corresponding term in the equation for water was $[H_2O]$, which was considered to be a constant, since the "concentration" of water, when nothing but water is present, can hardly be altered. But here we are dealing with a substance (acetic acid) dissolved in water, and its concentration may vary within very wide limits.

Since K must remain constant, any change in one of the terms in the above equation must be compensated by a change in the others. If $[CH_3COO^-]$ is increased (*e.g.*, by the addition of sodium acetate, which dissociates into Na^+ and CH_3COO^-), there must be an increase in $[CH_3COOH]$ and a decrease in $[H^+]$. What happens is that the ionization of the acetic acid is repressed by some of the H^+ and CH_3COO^- recombining to form undissociated acetic acid ($H^+ + CH_3COO^- \rightarrow CH_3COOH$).

When alkali (*e.g.*, sodium hydroxide) is added to a solution of acetic acid (as in the process of titration), sodium acetate is immediately formed, and here also the result is a mixture of acetic acid and sodium acetate. The $[H^+]$ at any stage of this titration can be found by means of the equation:

$$\frac{[H^+]\cdot[CH_3COO^-]}{[CH_3COOH]} = K$$

---

[4] It is only with *weak* acids that this sort of equation holds. $\frac{[H^+][Cl^-]}{[HCl]}$ for example, is not a constant.

In a sufficiently dilute solution of acetic acid and sodium acetate, $[CH_3COO^-]$ is practically equal to the concentration of sodium acetate (because the latter is virtually all ionized), and $[CH_3COOH]$ is practically the same as the concentration of free acetic acid (because this is almost completely in the undissociated form). The equation may then be simplified to:

$$[H^+]=K\cdot\frac{[\text{acetic acid}]}{[\text{Na acetate}]}$$

or, in general, for a mixture of any weak acid and its salt:[5]

$$[H^+]=K\cdot\frac{[\text{acid}]}{[\text{salt}]}$$

This shows that the $[H^+]$ of such a mixture of weak acid and salt is determined by the *ratio* of the concentrations of acid and salt.

For example, in a half-neutralized acetic acid solution, containing equivalent amounts of acetic acid and sodium acetate, $[\text{acid}]=[\text{salt}]$, and so $[H^+]=K$. That is, when one-half of one equivalent of a strong alkali has been added to any dilute solution of a weak monobasic acid, the $[H^+]$ is equal to the dissociation constant of the acid.

Similar considerations apply to weak bases. Here the equation becomes:

$$[OH^-]=K\cdot\frac{[\text{base}]}{[\text{salt}]}$$

and this may be used in the same way to find the alkalinity of the solution of a partially neutralized base.

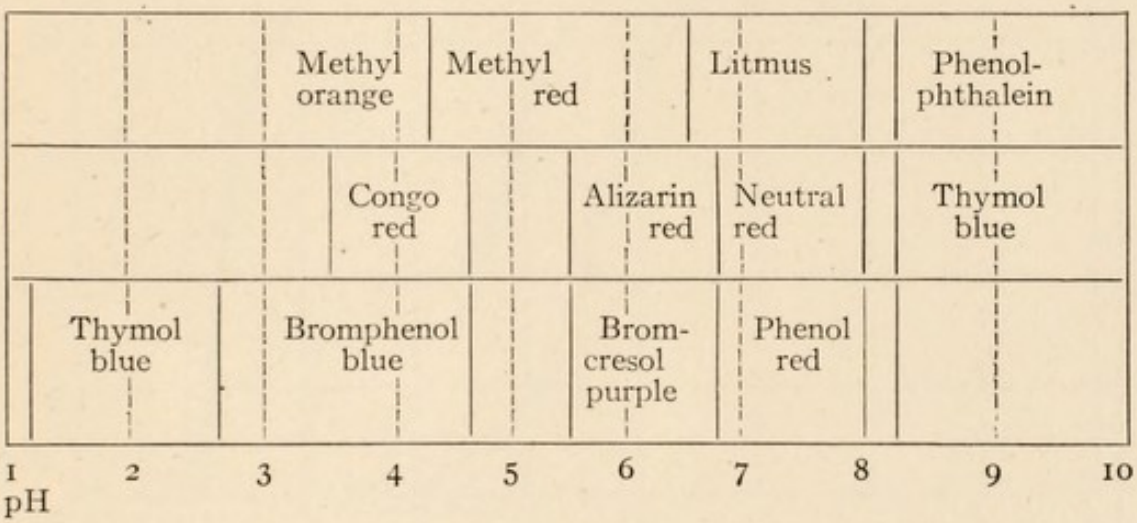
The dissociation constants of a number of important substances are given below:

Hippuric acid	2.2×10^{-4}	Uric acid	1.5×10^{-6}
Acetoacetic acid	1.5×10^{-4}	Carbonic acid	3.0×10^{-7}
Lactic acid	1.4×10^{-4}	Primary phosphate	2.0×10^{-7}
Acid oxalate	3.0×10^{-5}	Boric acid	6.6×10^{-10}
β-Hydroxybutyric acid	2.0×10^{-5}	Ammonia	1.8×10^{-5}
Acetic acid	1.85×10^{-5}		

[5] If the solution is not very dilute, the salt will not be completely ionized, and $[CH_3COO^-]$ will actually be equal to $[\text{salt}]\times\gamma$ where γ is the fraction of the salt present in the form of active ions (*cf.* p. 7). A more exact equation is therefore the following:

$$[H^+]=K\cdot\frac{[\text{acid}]}{[\text{salt}]\cdot\gamma}$$

Indicators.—Indicators are organic substances, themselves either weak acids or weak bases, with the particular characteristic that the conversion of the free acid or base into its salt involves not only the simple change corresponding with the formation of sodium acetate from acetic acid, or of ammonium chloride from ammonium hydroxide, but also a deep-seated alteration in the molecule that modifies the *color* of the substance. An indicator consequently has one color in strongly acid solution, and a different color in strongly alkaline solution. The precise $[H^+]$ at which the change from one color to the other occurs depends upon the individual properties of the indicator, and the number of such substances is sufficiently large so that finding a suitable one for any purpose is in most instances merely a matter of selection. The following diagram shows the turning-points of several commonly used indicators.



Titration of Strong Acid with Strong Base.—The course of the changes in hydrogen ion concentration taking place during the titration of any acid is a convenient guide to the selection of a suitable indicator that will give accurate results. These changes are most clearly shown by plotting the volume of added alkali against the pH. The curves so obtained incidentally reveal, in a graphic manner, certain characteristics of the solutions of partially neutralized weak acids by virtue of which they are able to assist in maintaining the body fluids at a constant degree of alkalinity.

The construction of such a curve for the titration of a strong acid (HCl) with a strong base (NaOH) amounts merely to finding the concentration of acid left unneutralized at each stage of

the titration, making a small allowance for the fact that some of the ions are inactive when the solution is not very dilute, and converting the result into pH terms. Fig. 1 represents the titration of 100 cc. of acid, the volume being assumed to remain unchanged.

In the titration of normal hydrochloric acid (broken line at the left), the $[H^+]$ at the start is somewhat less than normal (since the ionization is not quite complete), and the pH is accord-

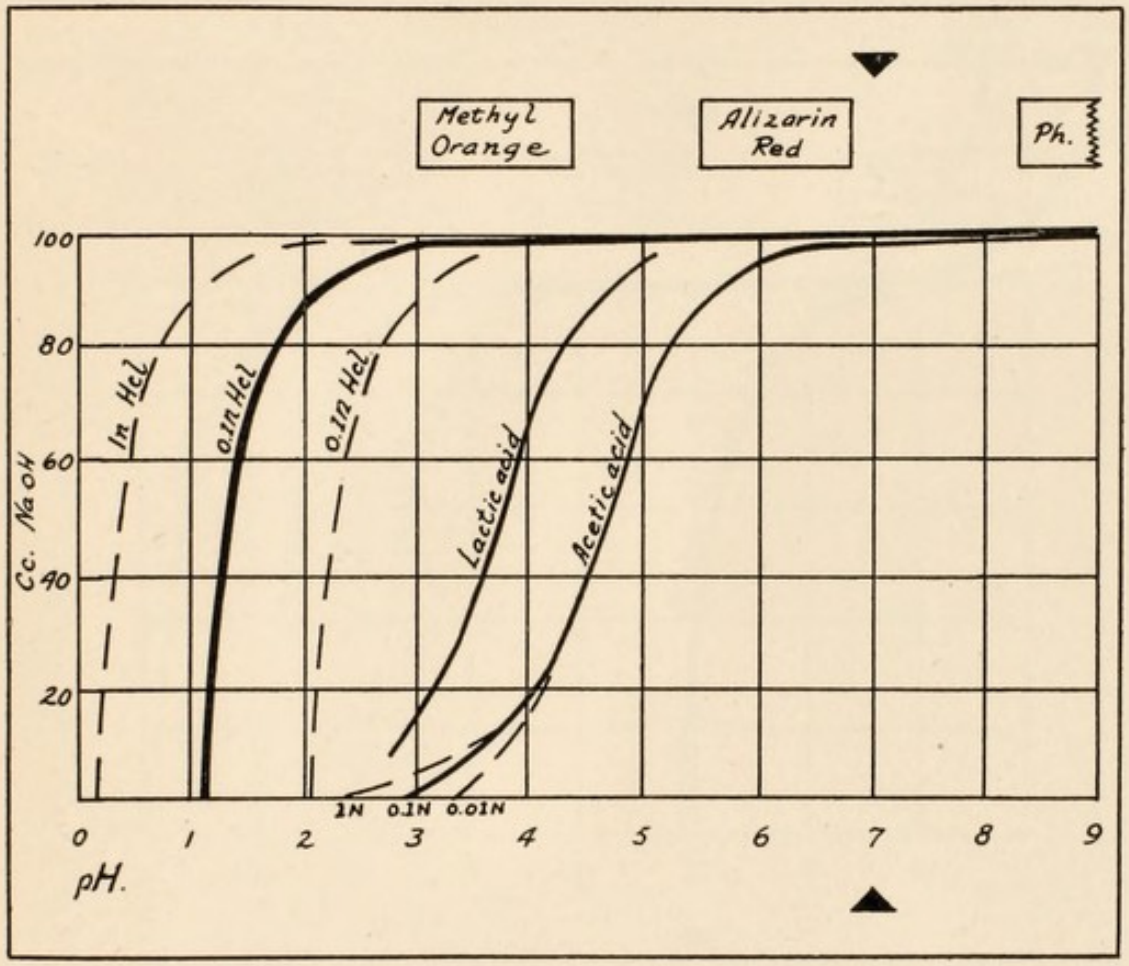


FIG. 1.

ingly a little more than 0. When 90 cc. of alkali (normal) have been added, one-tenth of the acid is left, and its concentration is one-tenth as great as at the beginning (*i.e.*, 0.1 N). The pH at that point is consequently about 1. When 99 cc. of alkali have been added, the concentration of hydrochloric acid remaining is 0.01 N, and the pH is 2. Finally, when only 0.1 cc. of acid is left, the pH has increased by one more unit, to 3. This is more clearly seen from Fig. 2, which shows the end of the titration on a much

larger scale, since it includes only the last cubic centimeter of alkali.

The volume of alkali used so far (99.9 cc.) is within 0.1 per cent of the theoretical amount for complete neutralization, and that is as accurate as any ordinary volumetric titration can be made. Nevertheless, the solution is still very distinctly acid.

The remaining 0.1 cc. of alkali carries the pH through four units to the neutral point (pH 7), since it is then nothing but

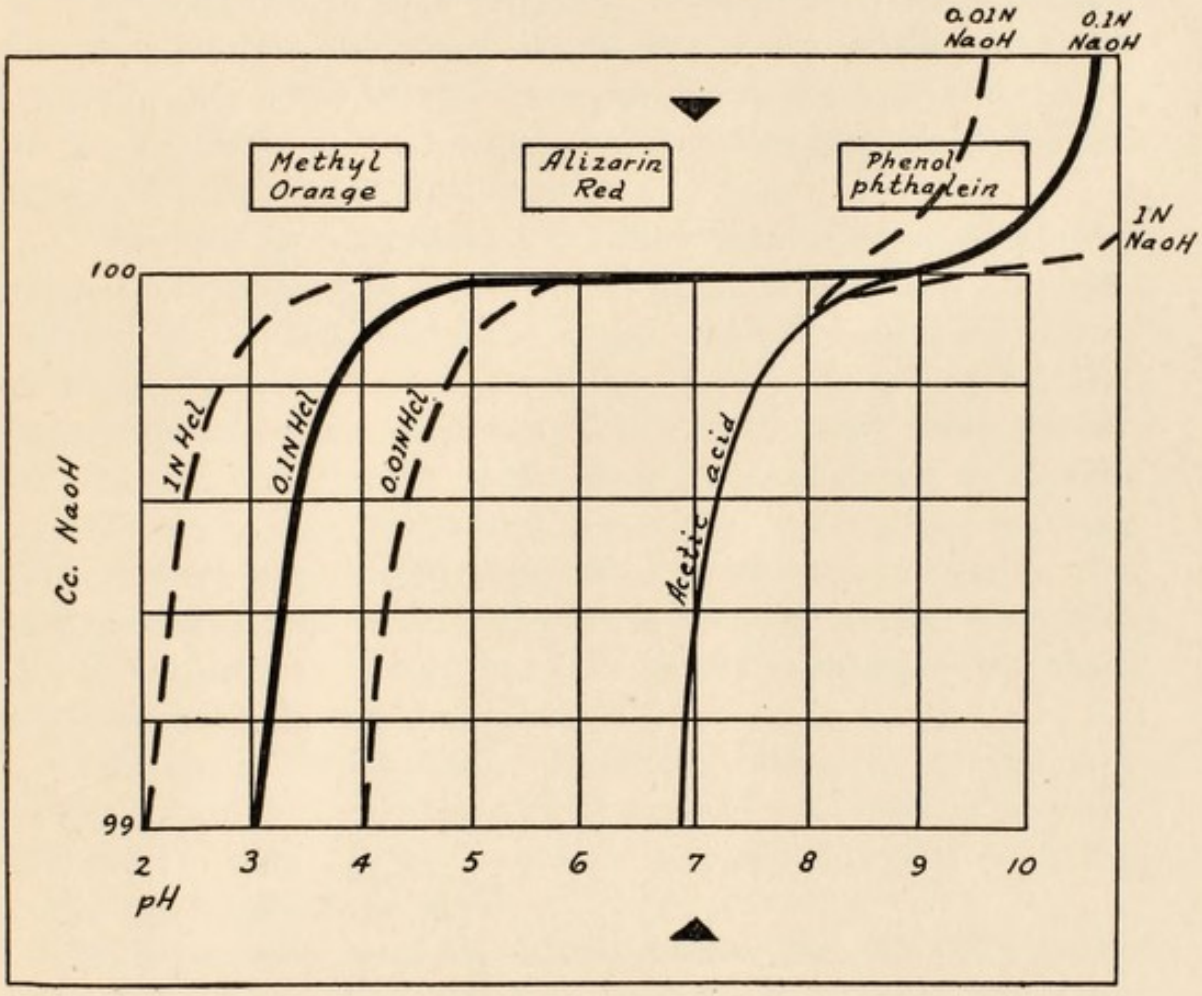


FIG. 2.

a solution of sodium chloride, and an equal quantity (0.1 cc.) of alkali in excess changes the pH by another four units, to 11. This very rapid change in pH at the end of the titration (indicated by the horizontal character of the curve at that stage) is a necessary condition for a sharp end-point in any titration. In this case, titrating to a pH anywhere between 3 and 11 will give a result within 0.1 per cent of the theoretical, and any indicator that changes color within that wide range can be used.

Methyl orange, alizarin red and phenolphthalein (see figure) all turn within these limits, and in fact most common indicators do, so almost any indicator will suffice for this particular titration.

When the acid and alkali are tenth normal instead of normal, the $[H^+]$ at each stage of the titration up to the neutral point is one-tenth as great as before, and the pH one unit higher. The same degree of accuracy as in the preceding case (0.1 per cent) here calls for an indicator turning within limits that are less by one pH unit at either end, *i.e.*, between 4 and 10. Alizarin red and phenolphthalein still meet the requirements, while methyl orange is on the border-line—it will give results of the maximum accuracy only if the titration is continued to the full yellow color of the indicator (methyl orange being red in strongly acid solution, and yellow in alkaline solution).

With 0.01 N solutions, the limits have diminished to the range between 5 and 9, and methyl orange is no longer accurate. Alizarin red is still within the range, and so is phenolphthalein, provided that the alkali is essentially free from carbonate. The absorption of carbon dioxide (itself a weak acid) from the air by standard alkali is one of the most frequent causes of trouble in alkalimetric titrations, and it is a source of error that becomes progressively more troublesome, since a little carbon dioxide gets into the alkali every time the bottle is opened. On that account it is very important to avoid any unnecessary exposure of standard alkali solutions to the atmosphere.

Titration of Weak Acid with Strong Base.—This case may be illustrated by the titration of acetic acid with sodium hydroxide and the curve (Figs. 1 and 2) is totally different from the curve for hydrochloric acid.

To begin with, since the acid is a weak one, the $[H^+]$ at the start, before any alkali has been introduced, is much less (and the pH greater). The addition of the first trace of alkali results in the formation of sodium acetate, and salts of this kind, even of weak acids, are almost completely dissociated. Small as the degree of ionization of the acetic acid is to begin with, it is still further repressed by the acetate ions from the sodium acetate. The fall in acidity at the beginning of the titration is for this reason comparatively rapid, *i.e.*, rapid by comparison with the same stage of the hydrochloric acid titration. But, for the same reason, it presently becomes comparatively very slow, and the

curve correspondingly steep. That is because the repression of the ionization of the acetic acid, brought about in the manner described, soon reaches the point where the acid is, for all practical purposes, not ionized at all. The alkali added at this stage is virtually all used up in neutralizing a large amount of practically undissociated acid, and the change in acidity is much slower than at the same $[H^+]$ in the hydrochloric acid titration, where the alkali neutralizes a small amount of completely dissociated acid.

The acetic acid curve is different also at the end. When exactly one equivalent of alkali has been added, the solution is simply a solution of sodium acetate. Sodium acetate, being a salt of a strong base with a weak acid, is partly decomposed by water, as follows:

$$\begin{array}{ccccc} CH_3COONa & \rightleftharpoons & CH_3COO^- & + & Na^+ \\ & & + & & \\ H_2O & \rightleftharpoons & H^+ & + & OH^- \\ & & \downarrow\uparrow & & \\ & & CH_3COOH & & \end{array}$$

This is called *salt hydrolysis,* and the result here is the formation of acetic acid, which is nearly all undissociated, and sodium hydroxide, which is nearly all dissociated. This provides an excess of hydroxyl ions, and sodium acetate solutions are consequently alkaline.

Because of this salt hydrolysis, the correct end-point in the titration of any weak acid with a strong base is on the alkaline side of the neutral point, and the more so the weaker the acid (Fig. 2). The consequence is that phenolphthalein can be used in such titrations, while methyl orange and alizarin red cannot, for their turning points are passed long before the theoretical quantity of alkali has been added.

The curve for any other weak monobasic acid is similar in form to that for acetic acid, but it will lie as a whole farther to the left or right, depending upon whether the acid is stronger or weaker than acetic. Fig. 1 shows a part of the curve for lactic acid, which is about ten times as strong as acetic acid.

It will be seen from the figure that the acetic acid curve, except at the two ends, is practically the same whether the solution is N, 0.1 N, or 0.01 N. That is so because the $[H^+]$ of a mixture of a weak acid and its salt is determined almost entirely

by the *ratio* of the two constituents, and only to a minor degree by the total concentration of electrolyte.

Titration of Strong Acid with Weak Base.—Strictly speaking, this is a type of titration that is practically never necessary, but all ordinary alkalimetric determinations of nitrogen really amount to the same thing, for they consist, in the final stage, in the titration of hydrochloric acid with sodium hydroxide in the presence of ammonium chloride. The situation is almost the same

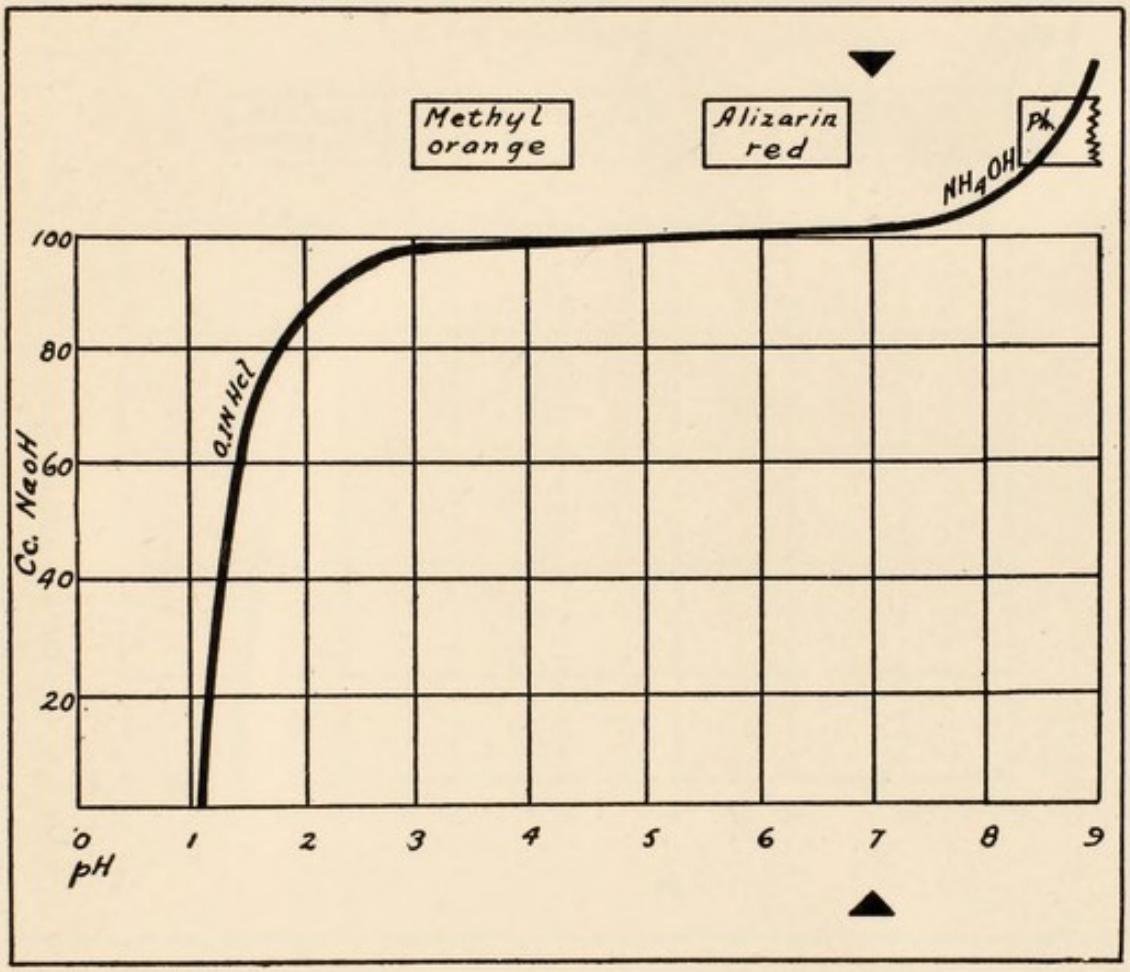


FIG. 3.

as if hydrochloric acid were being titrated with ammonium hydroxide, which is a weak base.

The dissociation constant of ammonium hydroxide happens to be almost equal to that of acetic acid. That is, although one is a base and the other an acid, they are about equally strong. The titration (Figs. 3 and 4) is not affected by the presence of the weak base until the end-point is approached. When that stage has been reached, sodium hydroxide begins to liberate ammo-

nium hydroxide from the ammonium chloride ($NH_4Cl + NaOH = NH_4OH + NaCl$). The hydroxyl ions of the strong alkali combine with NH_4^+, and consequently have less effect on the pH. The curve, as a matter of fact, is the acetic acid curve turned upside down, and becomes very steep soon after the full 100 cc. of sodium hydroxide have been added.

For the same reason that the acetic acid titration requires an indicator turning on the alkaline side, this titration calls for

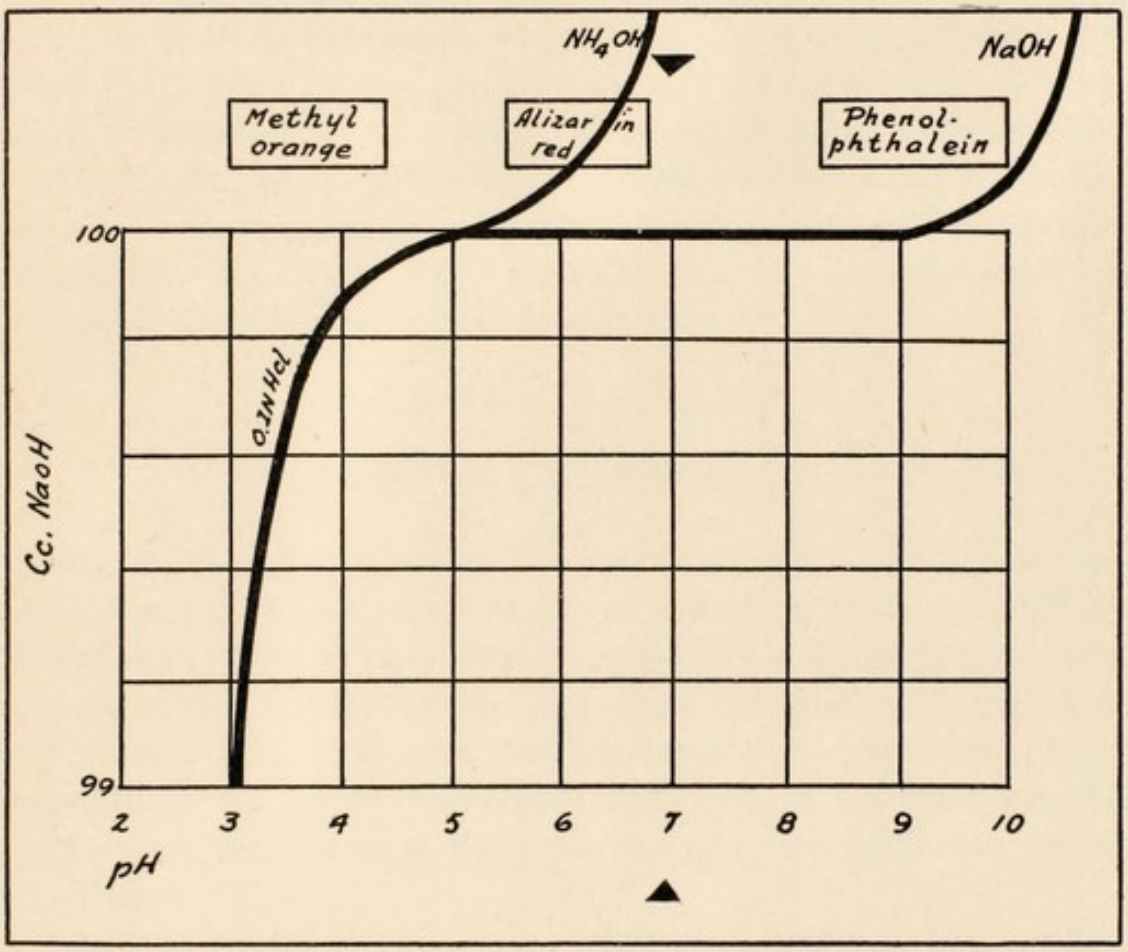


FIG. 4.

one turning on the acid side of the neutral point. Ammonium chloride is hydrolyzed by water, forming a strong acid and a weak base:

$$\begin{array}{c} NH_4Cl \rightleftharpoons NH_4^+ + Cl^- \\ + \\ H_2O \rightleftharpoons OH^- + H^+ \\ \downarrow\uparrow \\ NH_4OH \end{array}$$

The result is an excess of H^+, and ammonium chloride solutions are therefore acid in reaction.

Methyl orange changes color before the effect of the ammonium chloride becomes evident, and alizarin red does so before the effect has become sufficiently marked to influence the results seriously. Phenolphthalein, on the other hand, is quite useless, since its turning point is not reached until about 10 per cent of alkali has been added in excess.

Titration of Polybasic Acids.—Polybasic acids have two or more replaceable hydrogen atoms, and their neutralization by alkali takes place in two or more stages, the completion of each stage corresponding with the formation of some definite salt. Carbonic acid, for example, has two replaceable hydrogen atoms, and so has two series of salts: bicarbonate and carbonate ($NaHCO_3$, Na_2CO_3). The neutralization of carbonic acid by sodium hydroxide takes place in two steps:

$$H_2CO_3 + NaOH = NaHCO_3$$

$$NaHCO_3 + NaOH = Na_2CO_3$$

The titration curve in a case like this is simply a combination of two curves, each representing one of the steps in the neutralization. The first of the two (Fig. 5) is similar in form to the acetic acid curve, but farther to the right, since carbonic acid is a weaker acid than acetic. It is also foreshortened vertically—since only one-half of the total replaceable hydrogen in carbonic acid is involved in this first step, its curve extends only from 0 to 50 cc. of alkali.

Because carbonic acid is a weak acid, sodium bicarbonate solutions are alkaline, and carbonic acid may be titrated through the first half of its neutralization, using the color change of phenolphthalein as the end-point. The end-point is less sharp than in the case of acetic acid, and that is generally the case with polybasic acids, for the second step in neutralization begins before the first is fully completed. On that account the curve at the end-point is never quite as nearly horizontal as it is with monobasic acids.

The turning point of alizarin red comes on a steep part of the carbonic acid curve, and this indicator is useless here. Methyl orange changes color at the very beginning of the curve, and it

can consequently be used when the titration is made in the opposite direction, *i.e.*, by running standard acid into a solution of bicarbonate or carbonate.

In the second step of the neutralization, the acid is sodium bicarbonate (an acid salt). It is so weak an acid that a sharp end-point cannot be obtained under any circumstances; therefore it cannot be titrated with alkali at all.

Since the titration curve of carbonic acid is not a horizontal

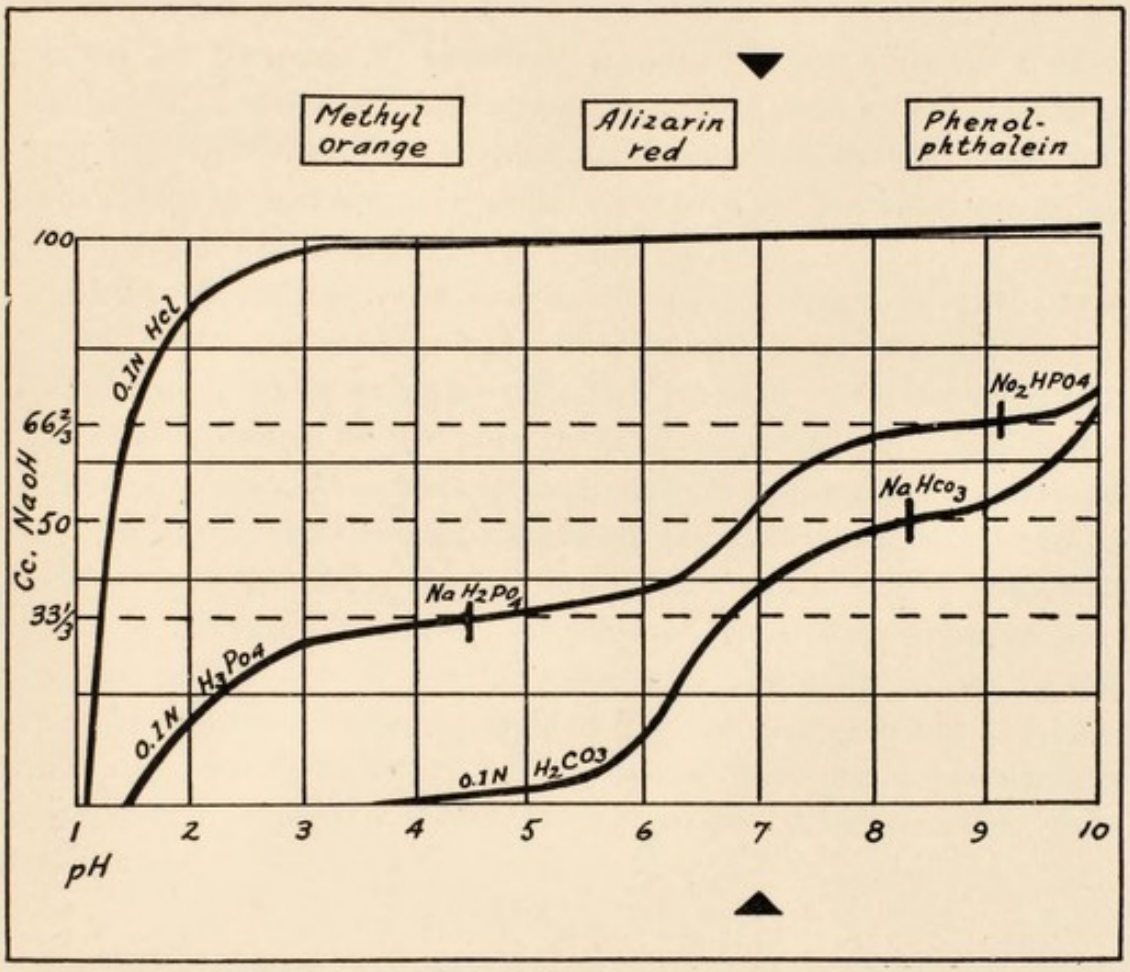


FIG. 5.

line between the turning points of methyl orange and phenolphthalein, the reason why standard sodium hydroxide contaminated with carbonate does not give the same results with all indicators when used for the titration of a strong acid is apparent.

The titration curve of phosphoric acid is also shown in Fig. 5. This acid is tribasic, and has three series of salts: primary phosphate (NaH_2PO_4), secondary phosphate (Na_2HPO_4), and tertiary phosphate (Na_3PO_4). Its curve is accordingly in three parts, of which only the first two are important.

Phosphoric acid may be titrated to the turning point of either methyl orange or phenolphthalein. In the first case, only one-third of the replaceable hydrogen will be neutralized, and in the second case two-thirds. That is because the $[H^+]$ of a solution of primary phosphate happens to coincide approximately with the turning point of methyl orange, while the $[H^+]$ of a solution of secondary phosphate happens to be about where phenolphthalein turns pink.

The accuracy of such titrations as these, where the end-point is not particularly well defined, may often be considerably increased by titrating to match a standard color consisting of a solution of known pH containing the same amount of indicator as in the solution being titrated, and adjusted to the same volume. This is merely a special case of the determination of hydrogen ion concentration (see page 55).

Buffer Mixtures.—In the intermediate stage of the titration of any weak acid or base, the change in pH caused by the addition of a given quantity of alkali or acid is relatively small. This characteristic of partially neutralized weak acids and bases, while often very troublesome in analytical operations because of the resulting indeterminate nature of the end-point, is turned to advantage in the body. Such solutions are called buffer solutions or buffer mixtures, and their presence in the tissues helps to prevent the occurrence of anything but very slight variations in the pH. The principal buffers provided for this purpose are (1) carbonic acid+bicarbonate, (2) primary phosphate+secondary phosphate, (3) protein+sodium proteinate (or some other protein salt).

1. **Calibrations.**—Pipettes, burettes and volumetric flasks are intended for the most accurate measurements of solutions, but these utensils are not always accurate. To determine the true capacity of these instruments of precision weigh on the analytical balance the water which they hold or deliver. For such "calibrations" 1 cc. of water may be assumed to weigh 997 mg. The number 997 is not a perfect constant, but variations due to changes in temperature are disregarded, because it is practically necessary to disregard them in subsequent daily work with the volumetric ware.

The inexperienced student must have learned how to use the

analytical balance and how to use pipettes properly before he can make any useful calibrations—poorly made calibrations are worse than none.

1. Learn to adjust the surface of the water (the bottom of the meniscus) exactly to the graduation mark of a pipette. 2. Be sure that no extra water clings to the tip and that a drop is not shaken out from inside the pipette while moving it from one vessel to another. 3. Drain uniformly and adequately, 20 seconds, then remove the hanging drop by contact between tip of pipette and receiving vessel. 4. Immediately after draining inspect the pipette to make sure that one or more drops have not stuck at some unclean point inside the pipette. The faster a pipette or burette is emptied the greater becomes the tendency to lose drops by adhesion unless the instruments are perfectly clean. Pipettes and burettes are the only glassware which need to be cleansed with "cleaning fluid"—a saturated solution of sodium dichromate in concentrated sulfuric acid. 5. The only vessel in a student's outfit suitable as a receptacle for the water which is to be weighed is a 100 cc. volumetric flask. It is only with such flasks that losses due to evaporation during the weighing become negligible. Even with these flasks it is better, though not really essential, to use clean dry corks.

Because of congestion around the balances the first calibrations should be limited to one 20 cc. pipette and one 25 cc. burette with glass stopcock. The calibration of the burette should not be attempted unless the student can have undisturbed possession of the balance for at least one half hour.

By the help of a small funnel fill the 25 cc. burette with cleaning fluid, put a beaker under it to catch what might leak out, and let it stand thus filled for one half hour or longer. Return the cleaning fluid to its bottle. Rinse the burette with distilled water until the water coming through is as neutral to blue litmus paper as distilled water. Then fill again and adjust the meniscus exactly to the 0 mark. Cover with a test-tube and let stand for 5 minutes to make sure that the burette is not leaking. Weigh a 100 cc. volumetric flask with its clean and absolutely dry cork stopper. The flask must be dry on the outside and the inside near the mouth must also be dry, but below this point the flask need not be dry. The weighing must be accurate to within an error of 1 mg. Record the weight.

Remove the cork carefully so as not to get it wet. With the greatest possible accuracy transfer the contents of the burette from the 0 mark to the 5 cc. mark, taking care not to wet the mouth of the flask and yet not to leave a drop hanging to the tip of the burette. Stopper the flask again with the dry cork and weigh. The difference between the two weights, in mg., divided by 997 will give the correct volume of the first "5 cc." portion of the burette. Remove the cork but leave the water in, and collect and weigh the 2d, 3d, 4th, and 5th "5 cc." portions of the burette in exactly the same manner. It will be found that while most burettes are substantially correct for their entire 25 cc. volume, two or more 5 cc. portions may, nevertheless, be inaccurate, because the inside diameter of the tubing from which they were made is not perfectly uniform.

To calibrate the 20 cc. pipette proceed as follows: Insert the pipette nearly to the bottom of the small bottle holding the cleaning fluid and fill it by suction for some distance above the 20 cc. mark. Some danger is involved in this operation. Close the top of the pipette and hold the cleaning fluid in place for about 2 minutes. Then let the fluid flow back into the bottle, and when the pipette is empty, invert it rather quickly and insert the other end of the pipette in the cleaning fluid, and leave the pipette in the bottle for a few minutes. Then remove the pipette, and by the help of a beaker carry it to a sink without spilling a single drop of the cleaning fluid, and rinse the pipette inside and outside with tap water. Rinse further with distilled water until blue litmus paper shows that all of the acid has been removed.

Next fill the pipette in the ordinary way, by suction, and note whether immediately after being emptied and drained for 20 seconds the inside is free from isolated adhering drops of water. Unless it is free from such drops the cleaning process must be repeated. When perfectly clean, transfer the contents of the accurately filled pipette to a previously weighed 100 cc. flask, as described under the calibration of the burette, and weigh. As before, the weight of the water, in mg., divided by 997, gives the correct volume. Record this volume and take it into account in subsequent work with the pipette.

In careful volumetric work one never uses other than calibrated utensils, and, as opportunity and time permit, the student should calibrate all of the smaller volumetric ware.

After one 20 cc. pipette has been calibrated, the volumetric flasks may be calibrated volumetrically, *i.e.*, without the balance, beginning with a 100 cc. flask, which, *when dry,* should hold exactly five times the volume delivered by the 20 cc. pipette. When this flask is again dry it can be used for volumetrically calibrating 50 cc. and 100 cc. pipettes. If a flask is rinsed with a little wood alcohol and drained, it dries more quickly. In connection with these improvised volumetric calibrations a 1 cc. pipette graduated in 1/100 cc. to the tip is very useful. These can be obtained on temporary orders.

There is no great hurry about completing these calibrations as they become important first in connection with the preparation of the tenth normal solutions.

One calibration is needed, unfortunately, at the very beginning of the volumetric work, namely, that of the 500 cc. flasks used for the preparation of 0.5 N oxalic acid. It is not uncommon to find errors up to 2 cc. or even more in these volumetric flasks. By means of ordinary laboratory scales or a torsion balance and one 500 gm. weight and one set of analytical weights, one can satisfactorily calibrate these flasks. Counterpoise the dry flask on one pan of the special scale by another suitable flask plus water until exact balance is obtained. Fill with distilled water exactly to the 500 cc. mark and weigh. The weight of the water in mg., divided by 997, gives the correct volume in cc.

2. Preparation of 0.5 N Oxalic Acid (500 cc.).—The usefulness of oxalic acid as a starting point for the preparation of standard acids and alkalis is due entirely to the fact that it can be obtained chemically pure and in condition suitable for direct weighing. Oxalic acid is, however, not a strong enough acid to titrate well with all the common indicators, and it is therefore not serviceable for acidimetric titrations in general. But by means of oxalic acid and with phenolphthalein as indicator, standard solutions of a strong alkali, like sodium hydroxide, can be obtained, and by means of the latter standard solutions of the stronger mineral acids can then be prepared.

Weigh accurately (to the fourth decimal) a small, clean, and dry beaker or large crucible. Then add to the weights on the balance pan 15.7560 gm., and add oxalic acid to the vessel on the other side until exact equilibrium is reached. Dissolve in dis-

tilled water this oxalic acid without the loss of a single crystal. The acid dissolves rather slowly. The solution is, therefore, best made in a beaker by the aid of gentle heating with about 250 cc. water. Transfer every drop of the solution to a previously calibrated volumetric flask (500 cc.), carefully rinsing the last traces from the beaker into the flask by means of successive small amounts of cold distilled water. Cool the flask in running tap water until the contents of the flask have reached the room temperature. (If a thermometer is used, it must be rinsed carefully before it is removed from the flask.) Fill up with water until the lower side of the "meniscus" is exactly even with the 500 cc. mark. Stopper the flask, and invert several times (30–40) so that the solution is thoroughly mixed. Transfer to a clean bottle previously rinsed with the solution; label and preserve.

Using a strong base like sodium hydroxide and a sensitive indicator like phenolphthalein for the titration, it is possible to obtain quite reliable and accurate results with oxalic acid. The volumetric determinations involved in metabolism studies and urine analysis are, however, extensively based on titrating ammonia, which is a very weak base. Phenolphthalein is useless in titrations of ammonia. The oxalic acid and the phenolphthalein are therefore used only for the purpose of securing a standard alkali solution.

3. Preparation of Standardized Sodium Hydroxide.—The sodium hydroxide used for titrations must be as free as possible from carbonates, because otherwise the solutions will not have the same titrating value with all the common indicators. Sodium hydroxide rapidly absorbs carbon dioxide from the atmosphere and should therefore not be exposed to the air more than is unavoidable. As the carbonates are insoluble in very strong sodium hydroxide solutions, only clear saturated solutions should be used as the starting point for the preparation of standard solutions.

A satisfactory grade of saturated sodium hydroxide is obtained by dissolving, in an iron kettle, 10 pounds of commercial sodium hydroxide in 5 liters of water. When the turbid solution is cool transfer to large tightly stoppered bottles and let settle. The only drawback is the long time (sometimes months) required to yield perfectly clear supernatant solutions. In some batches one may also find a little ferric hydroxide after dilution, but this does no harm.

With a 50 cc. cylinder transfer about 45 cc. of clear saturated (60–70 per cent) solution of sodium hydroxide to a large bottle, and add by the help of a large cylinder about 1500 cc. of distilled water. Shake quite gently, but continuously, for 5 minutes. To determine the exact value of this solution it is only necessary to find out by titration how much of it is required for the exact neutralization of a known volume of the half normal oxalic acid solution; but it is only by careful work that errors can be limited to about 0.1 per cent.

For these titrations with sodium hydroxide it is advantageous first to transfer (without measuring) about 200 cc. of the alkali to a small flask which previously has been either dried or rinsed with the solution. It is best not to open the large bottle too often. The flask must be stoppered with a well-fitting stopper. To avoid accidental contamination or dilution it is likewise well to transfer about 150 cc. of the 0.5 N oxalic acid solution to a small dry flask or bottle for use in these first titrations. Acid or alkali once taken out of the stock bottle should never be poured back.

Before attempting to do any titrations a student must be sure that he has learned how to use properly pipettes and burettes as described under Calibrations.—Rinse a 20 cc. pipette with the oxalic acid solution. Then fill it exactly so that the lowest part of the curved meniscus just touches the mark. Make sure that there is no hanging drop at the bottom, and then carefully transfer the contents to a thoroughly rinsed, but not necessarily dry, beaker. In connection with this transfer (1) do not shake out a drop from inside the pipette; (2) after the solution has all run out, hold it in a vertical position for a definite time, 20 seconds; (3) remove a part of what has drained into the tip by making contact between the tip and the side of the beaker; (4) inspect the pipette immediately after the draining for isolated drops adhering to the inside. If such drops are found, the pipette is not clean. To the oxalic acid solution in the beaker add 2 drops of indicator (1 per cent alcoholic solution of phenolphthalein). Rinse the burette with the working solution of alkali, fill it a little above the 0 mark, and then adjust the meniscus exactly to the 0 mark. Cover the top with a test-tube and make sure that there is no air bubble in the tip below the stopcock and that there is no hanging drop on the outside.

Run the alkali into the oxalic acid solution, at first fairly

rapidly, then more and more cautiously, with stirring, until finally one single drop (or less than a drop collected by the help of the stirring rod) gives a faint red color which does not disappear on subsequent moderate stirring. The more free from CO_2 the alkali is, the more sharp and definite is the end-point. Note the volume of alkali required (within 0.02–0.05 cc.). Repeat until two successive titrations give exactly the same value.

From the titration figure obtained calculate the normality of the solution and how much of it must be taken for the preparation of 1 liter of tenth normal alkali.

As a check on the work, determine the normality of an unknown hydrochloric acid solution (furnished), using as indicator (a) phenolphthalein, (b) alizarin red (1 drop of 1 per cent aqueous solution). A dated and signed report on the unknown should be handed in before making the tenth normal alkali. Label and preserve the standardized alkali solution.

4. Standardized Hydrochloric Acid.—Concentrated hydrochloric acid is approximately a 10 N solution of HCl. With a cylinder transfer 60 cc. of concentrated hydrochloric acid to a bottle (capacity 1500 cc.); add water, about 1300 cc., and shake very thoroughly.

Titrate this acid in the same way as the oxalic acid solution, but using only alizarin red as indicator. Calculate the normality, and how much of it must be taken for the preparation of 1 liter of tenth normal acid. Label and preserve.

5. Tenth Normal Acid and Alkali.—From the standardized solutions of acid and alkali prepare 1 liter of tenth normal hydrochloric acid and 1 liter of tenth normal alkali. Titrate the acid so prepared (20 cc.) with the tenth normal alkali. The two should be equivalent. Determine the normality of an unknown acid with the tenth normal alkali. Hand in a dated and signed report giving the value obtained for the unknown and giving also the titration figures for the tenth normal acid.

Label and preserve the tenth normal solutions. The two alkali solutions do not always keep their value unchanged because more or less alkali is given off by the glass containers. The hydrochloric acid solutions keep indefinitely. If discrepancies are found later between the acid and the alkali, the acid should be taken as correct.

6. Strong and Weak Acids; the Use of Different Indicators.—(A) Titrate 20 cc. tenth normal hydrochloric acid with the tenth normal alkali, using as indicator (a) phenolphthalein (b) methyl orange (c) alizarin red. Repeat the above mentioned three titrations in the presence of 10 cc. ammonium chloride solution (2 per cent). Repeat the titration with each indicator, using in place of the hydrochloric acid (a) 20 cc. 0.1 N phosphoric acid (b) 20 cc. 0.1 N lactic acid (without addition of NH_4Cl).

Record of titrations in tabular form:

	Methyl Orange		Alizarin Red		Phenolphthalein	
	Cc.	End Point[6]	Cc.	End Point[6]	Cc.	End Point[6]
HCl						
$HCl+NH_4Cl$						
H_3PO_4						
Lactic acid						

(B) Dilute 10 cc. tenth normal hydrochloric acid to 100 cc., making an approximately 0.01 N solution. (Measuring cylinders are accurate enough for the dilutions referred to here.)

From this 0.01 N solution prepare four 100 cc. portions of more dilute acids, viz.: 0.001 N; 0.0004 N; 0.0001 N; 0.00001 N. Arrange in a row four test-tubes, as nearly as possible of the same size, and transfer to each one 5 cc. of one of the four dilute acid solutions. To the contents of each tube add two drops (no more) of a 0.15 per cent alcoholic solution of tetrabromophenolsulfonphthalein ("bromphenol blue"), and compare the colors. The approximate hydrogen ion concentrations of these solutions are as follows:

	C_H	pH
0.001 N	10^{-3}	3.0
0.0004 N	4×10^{-4}	3.4
0.0001 N	10^{-4}	4.0
0.00001 N	10^{-5}	5.0

[6] Sharp (S), fair (F) or indeterminate (I).

Add the same amount of indicator to (a) 5 cc. 0.001 N lactic acid, (b) 5 cc. 0.001 N acetic acid, (c) 5 cc. 0.001 M monopotassium phosphate. Determine the approximate pH of each of these three solutions by comparing their colors with the dilute hydrochloric acid solutions. Although the total acid concentration is the same in the 0.001 N solutions of hydrochloric, lactic, and acetic acids, and monopotassium phosphate (an acid salt), the hydrogen ion concentration (and therefore the degree of dissociation) is obviously different in each case. In any such series of acid solutions of the same total concentration (0.001 N in this instance), the hydrogen ion concentration is less (and the pH greater) the weaker the acid.

7. Acidity of Gastric Contents.—The acidity of the normal stomach contents is due almost wholly to hydrochloric acid. In pure gastric juice, the concentration of hydrochloric acid is about 0.15 N, but the acidity of the material usually found in the stomach is less, as a result of dilution and partial neutralization. When, under abnormal conditions, the concentration of hydrochloric acid becomes very low, certain micro-organisms are able to grow in the stomach contents, producing lactic acid. It is nevertheless an easy matter to distinguish between a relatively low concentration of hydrochloric acid and a relatively high concentration of lactic acid, since the latter is a much weaker acid.

To 5 cc. 0.01 N hydrochloric acid in a test-tube add just 5 drops of a 0.04 per cent aqueous solution of thymol blue. Add the same amount of indicator to (a) 5 cc. 0.001 N hydrochloric acid, and (b) 5 cc. 0.1 N lactic acid. Compare the colors. The hydrogen ion concentration of the 0.1 N lactic acid should be less than that of the 0.01 N hydrochloric acid.

8. Colorimetric Determination of Hydrogen Ion Concentration.—(Clark, *The Determination of Hydrogen Ions.*)—The hydrogen ion concentration of most biological fluids is considerably less than in the solutions tested in the preceding experiments, and dilute hydrochloric acid solutions cannot be used here as standards, owing to the ease with which the pH is changed by slight contamination. Instead, it is necessary to have a series of standard buffer mixtures whose pH is not readily altered.

A suitable set of stock solutions from which to prepare such standards is: 0.2 M monopotassium phosphate, 0.2 M acetic acid, 0.2 M boric acid (containing also 0.2 M potassium chloride), and 0.2 M sodium hydroxide. The sodium hydroxide solution must be practically free from carbonate, and should not contain calcium or barium. The compositions of the standard mixtures (diluted to 200 cc. in each case) are given in the table below. These mixtures, once made up, can be relied upon for only about one week, but the stock solutions from which they are prepared should keep indefinitely in receptacles of resistance glass, except the sodium hydroxide solution, which will gradually increase in strength unless kept in a paraffined bottle.

The determination is carried out as follows: Measure 5 cc. of water[7] into a test-tube, and add 4 or 5 drops of the unknown solution, followed by 3 drops (no more) of phenol red solution. Compare the color with the set of standards.[8] In case the color is beyond the limits for phenol red on either side, repeat with the next indicator in order (see table), until the unknown has been correctly matched against one of the standards. Do not try more than one indicator at a time. The pH reading may be made to one-tenth unit by adding or subtracting 0.1 in case the color lies definitely between two consecutive standards.

For very accurate work, the standard and the unknown must contain about the same salt concentration.

Determine, in the manner described, the hydrogen ion concentration of two unknowns (supplied). The same method will later be applied to urine. In determining the hydrogen ion concentration of urine, precautions must be taken to prevent loss of carbon dioxide.

9. Special Test for Hydrochloric Acid.—Günzberg's reagent (2 gm. phloroglucin and 1 gm. vanillin in 100 cc. alcohol) is very reliable as a means of distinguishing between hydrochloric acid and lactic or other organic acids. The reaction is best carried out as follows:

Transfer 5–6 drops of the reagent to a shallow evaporating dish, and evaporate to dryness over a water bath consisting

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[7] In this work, pipettes should never be blown out, and water should not be taken from a wash bottle that has been blown into, since a small amount of carbon dioxide readily spoils the result.

[8] A series of diluted standard mixtures, with the indicators already added will keep for a short time, and offers the most convenient arrangement when a great many pH determinations are being made simultaneously. Pyrex test-tubes must be used.

Composition of Standard Buffer Mixtures

(Diluted in each case to 200 cc.)

Indicator: METHYL RED *

				pH
50 cc. 0.2 M CH_3COOH	and	23.0 cc. 0.2 M NaOH		4.6
50 "		29.0 "		4.8
50 "		34.5 "		5.0
50 "		38.5 "		5.2
50 "		42.5 "		5.4
50 "		45.0 "		5.6

* Dimethylaminoazobenzene-*o*-carboxylic acid (0.4 per cent alcoholic solution; use 2 drops).

Indicator: BROMCRESOL PURPLE †

50 cc. 0.2 M KH_2PO_4	and	3.7 cc. 0.2 M NaOH		5.8
50 "		5.7 "		6.0
50 "		8.6 "		6.2
50 "		12.6 "		6.4
50 "		17.8 "		6.6

† Dibromo-*o*-cresolsulfonphthalein (0.04 per cent aqueous solution of monosodium salt; use 2 drops).

Indicator: PHENOL RED *

50 cc. 0.2 M KH_2PO_4	and	23.6 cc. 0.2 M NaOH		6.8
50 "		29.6 "		7.0
50 "		35.0 "		7.2
50 "		39.5 "		7.4
50 "		42.8 "		7.6
50 "		45.2 "		7.8
50 "		46.8 "		8.0

* Phenolsulfonphthalein (0.02 per cent aqueous solution of monosodium salt; use 3 drops).

Indicator: THYMOL BLUE †

50 cc. 0.2 M H_3BO_3,KCl	and	5.9 cc. 0.2 M NaOH		8.2
50 "		8.5 "		8.4
50 "		12.0 "		8.6
50 "		16.3 "		8.8
50 "		21.3 "		9.0

† Thymolsulfonphthalein (0.04 per cent aqueous solution of monosodium salt; use 4 drops).

simply of a beaker of boiling water. The alcoholic solution spreads all over the dish, leaving a thin coating of the dry reagent. By means of pipettes, or glass tubes drawn out like pipettes, transfer one drop of 0.01 N hydrochloric acid to one

side of the dish, and on another side deposit one drop of 0.1 N lactic acid, and again place the dish on the water bath. A purplish ring is quickly formed around the hydrochloric acid drop while the lactic acid remains colorless.

Heating over the flame may be substituted for the water bath, but the least overheating tends to obscure the reaction by charring. This reaction is extensively used in the examination of stomach contents.

10. Special Test for Lactic Acid.—More or less specific tests for lactic acid are known and are considered important because of the frequency with which hydrochloric acid is absent and lactic acid present in the stomach. A convenient yet reliable method is the following:

To 5–10 cc. of 0.1 N lactic acid (or filtered stomach contents) in a large test-tube add a few drops (0.5 cc.) of normal hydrochloric acid and about 10 cc. of ether. By cautiously inverting the test-tube during 3 to 4 minutes (taking care to avoid explosions due to expanding ether vapors) the lactic acid is in part taken up by the ether. By means of a 25 cc. or 50 cc. pipette and suction, remove the lower aqueous layer as completely as possible. Decant the remaining ether into another test-tube so as to free it from the few drops of aqueous solution not taken out by the pipette. Then add to the ether solution 0.2 per cent ferric chloride solution, a little at a time with shaking, until the maximum yellow color is obtained. The amount of solution added and the depth of the color obtained give a rough index as to the amount of lactic acid present.

Old, deep colored, ferric chloride solutions frequently fail to give the test for lactic acid. By the addition of hydrochloric acid, in the proportion of 1 cc. concentrated acid to 5 cc. of 10 per cent ferric chloride solution, the pale color of the latter and its value as a reagent for lactic acid are restored.

One cubic centimeter of acidified 10 per cent ferric chloride solution diluted with 40–50 cc. of tap water gives a suitable solution for the test.

11. Nitrogen Determination in Ammonium Salts.—The most convenient and useful analysis of nitrogenous products of physiological significance is the determination of the nitrogen. The nitrogen of such products can be split off by hydrolysis in

the form of ammonia, which can then be determined by distillation and subsequent titration.

In a small beaker weigh out exactly 2.3580 gm. of pure ammonium sulfate. The salt contains traces of water (0.5 to 1 per cent), unless it has been dried by heating 1 to 2 hours at about 110° C.; it should be kept in a desiccator over sulfuric acid. Dissolve the salt without the loss of a single crystal in a 500 cc. volumetric flask, add 1 to 2 cc. concentrated hydrochloric acid, and fill up to the mark with water. The acid is added to keep out moulds. Mix thoroughly and transfer to a dry bottle, or to a bottle freshly rinsed twice with about 25 cc. of the solution.

This solution should be stoppered, labeled, and preserved as a standard solution. It is used to check up the accuracy of ammonia determinations and sulfate determinations, and later for colorimetric nitrogen determinations.

By means of a pipette transfer 25 cc. of the ammonium sulfate solution to a 300 cc. Kjeldahl flask. Add with a cylinder 75 cc. of water, and add also a small pinch of talcum powder (to prevent bumping during the boiling). Put the flask in a clamp so that the bottom is about 1 cm. above the top of a micro burner.

Transfer 25 cc. of tenth normal hydrochloric acid to a 500 cc. Florence flask; add water enough to make a volume of about 200 cc. and add two or three drops of indicator (alizarin red).

The indicator is added at the beginning so that if by any chance the ammonia distilled over is more than enough to neutralize the acid, that fact as at once revealed. When this happens add more standard acid (10 *or* 25 *cc.*).

Add about 5 cc. of saturated sodium hydroxide solution to the contents in the Kjeldahl flask and connect immediately by means of a rubber stopper and glass tubes with the receiver containing the acid. Light the burner without delay, to prevent back suction, and boil *vigorously* for not less than 7 minutes, counting from the time the boiling begins. Withdraw the receiver so that the delivery tube is well above the liquid before removing the flame. Cool the receiver in running tap water. Titrate the remaining uncombined acid with tenth normal alkali. From the figures obtained calculate the amount of nitrogen recovered (in

milligrams) and compare with the theoretical figure which the amount of ammonium sulfate taken should give.

In calculating the nitrogen from the titration figures, the amount of acid combined with the ammonia can be regarded as a tenth normal nitrogen solution, each cubic centimeter of which accordingly represents 1.4, or more accurately 1.401, milligram nitrogen. Example: 25 cc. 0.1 N HCl was the original amount of acid in the receiver. After the distillation the titration of the distillate required 2.1 cc. of 0.1 N NaOH. The ammonia had therefore neutralized 25 — 2.1 or 22.9 cc. of the tenth normal acid, $22.9 \times 1.4 = 32.06$ (milligrams nitrogen).

12. Kjeldahl's Method for Determining Nitrogen.—(*J. Biol. Chem.*, 38: 461.)—In a 100 cc. volumetric flask dissolve 1 to 1.5 gm. accurately weighed urea, and make the volume up to 100 cc., mix, and transfer to a clean and dry bottle, label, and preserve by means of a few drops of toluene or chloroform.

When organic substances are boiled with strong sulfuric acid both oxidation and hydrolysis take place. The oxidation occurs at the expense of the oxygen in the sulfuric acid, and the latter is consequently reduced. The sulfurous fumes thus produced are very irritating to the mucous membranes of the nose and throat. The digestion must, therefore, be made in a hood having a reasonably good draft.

Instead of a hood a "fume absorber" can be used. By the help of an ordinary water pump (of glass) the fumes are then partly aspirated directly into the drain pipes, and the remainder is collected in the lower part of the fume absorber.

In Kjeldahl's method sulfuric acid is used for the destructive digestion, but other substances are added to hasten the process. These accessory substances act either as catalyzers (copper sulfate or mercury) or, when added in large quantities, they raise the temperature and thus hasten the digestion. Potassium sulfate (5 to 20 gm.) is most commonly used for the purpose of raising the temperature. In the modification here described, a mixture of sulfuric acid (1 volume) with phosphoric acid (3 volumes) is substituted for sulfuric acid. This mixture gives a very high temperature, but it acts on glass much more rapidly than sulfuric acid alone and can not, therefore, be used except in connection with digestions which can be completed in a few minutes. It is probably the best for the destructive digestion of urine. Also, only 5 cc. are taken for each digestion instead of 15 to 20 cc., the amount required in the case of sulfuric acid.

The acid mixture is prepared as follows: To 50 cc. of a 5 per

cent copper sulfate solution add 300 cc. of 85 per cent phosphoric acid and mix. Add 100 cc. of concentrated sulfuric acid (free from the least trace of ammonia), mix, and cover well, to prevent absorption of ammonia from the air.

A 10 per cent solution of ferric chloride ($FeCl_3 \cdot 6H_2O$) is also required.

When urea is decomposed by means of boiling concentrated sulfuric acid, it is simply hydrolyzed into carbonic acid and ammonia and a solution of ammonia in a very large excess of acid is obtained. The presence of all this acid must be taken into account when preparing to remove the ammonia by distillation. (The amount of alkali required should be determined by a rough titration of 5 cc. of the acid dissolved in 500 to 700 cc. of tap water, adding the saturated alkali with a measuring cylinder.) A 20 to 30 per cent excess of alkali should be added for the distillation.

With a pipette transfer 5 cc. of the urea solution (or urine) to a Kjeldahl flask (capacity 300 cc.); add 5 cc. of the phosphoric-sulfuric acid mixture and 2 cc. of ferric chloride solution; add also a pyrex antibumping tube to prevent bumping. (See p. 277.) Fix the flask in a clamp (in a hood) so that the bottom is only about 1 cm. above the top of a micro burner. Boil with a full flame until all the water is driven off and the flask becomes filled with white, dense fumes. At this point cover the mouth of the flask with a watch glass and note the time. Continue the heating (without changing the flame) for two minutes. At the end of two minutes reduce the flame; the white fumes should now be confined within the flask. With the small flame the heating is continued for two minutes, making a total boiling period of four minutes, counting from the time the mouth of the flask was closed. Remove the flame and let cool for not less than four, nor more than five, minutes and then add, with a cylinder, 50 cc. of water. If the cooling process is made too long the contents in the flask, now chiefly metaphosphoric acid, solidify and then do not mix well with water.

The ammonia is to be distilled into a receiver. The latter should contain from 25 to 75 cc. of tenth normal acid, the amount depending on how much ammonia is expected. The receiver should also contain water enough to make a volume of about 200 cc. Without adding more water to the hot acid solu-

tion in the Kjeldahl flask, introduce the necessary alkali, usually 15 cc. of saturated sodium hydroxide, and connect promptly with the receiver. With the bottom of the flask only about 1 cm. from the top of the micro burner, boil vigorously for five minutes, counting from the time the solution begins to boil hard. At the end of five minutes, withdraw the receiver, allowing it first to rinse itself with steam for a few seconds. Beginners can advantageously replace the receiver with another flask or a beaker containing 100 cc. of water, indicator, and a drop of tenth normal acid, and continue distillation for two or three minutes, so as to be sure that none of the ammonia failed to get into the first receiver.

Note. For some more or less obscure reasons the final distillation in this determination sometimes miscarries in the hands of students, because of a combination of bumping and vacuum with consequent back suction. One or more of the following suggestions should prove helpful: (1) Without any preliminary shaking of the contents in the Kjeldahl flask, apply at first only a moderate flame, not under the center of the flask but somewhat to one side, until the contents are mixed and are boiling; then, but not before, apply the full flame under the center so as to secure brisk boiling. (2) Some students have found it helpful to have available a second flame from an ordinary Bunsen burner for intermittent application to the flask. (3) The mishap is less apt to occur with fresh dry flasks, probably because these contain microscopic air pockets in the glass which are only gradually replaced by liquid. If one determination has been ruined by back suction it is therefore usually best not to use immediately the same flask for a second determination. (4) All ordinary antibumping contrivances have failed in this distillation, but the special antibumping tube shown in the illustration on page 275 does seem to provide almost unfailing protection against the disastrous back suction due to excessive bumping.

Cool the first distillate, titrate, and compare the nitrogen value obtained with the theoretical figure which the urea should give.

The process for the determination of the total nitrogen in urine (5 cc.) is exactly the same as the process described above for urea. In urine there is, however, considerable complex organic matter to be oxidized and some charring and foaming is

encountered. With urine it is usually necessary to have not less than 50 cc. of acid in the receiver to begin with.

13. **Determination of Nitrogen in Uric Acid.**—Transfer 50 to 70 mg. of pure uric acid to a clean, dry test-tube. Weigh the test-tube and uric acid (to the fourth decimal). Shake most of the uric acid into a Kjeldahl flask, and again weigh accurately the empty test-tube. The difference between the two weighings is the amount of uric acid taken. In the same way, charge another Kjeldahl flask with 50 to 60 mg. of uric acid.

To each flask add 5 cc. of the phosphoric sulfuric acid mixture described in the preceding section. Digest, distill, and titrate, as in the case of urea. Calculate the absolute and percentage amount of nitrogen and compare with the theoretical figures.

PART II

CATALYSIS, CATALYZERS, ENZYMES

1. **Hydrogen Ion.**—In each of two test-tubes place about 5 cc. of 2 per cent cane sugar solution. To one add 5 cc. of half normal hydrochloric acid, to the other 5 cc. of water. Heat both in a beaker of boiling water for ten minutes. Cool. Transfer 5 cc. of a sugar reagent (alkaline copper solution) to a test-tube, add about one-half cc. of one of the heated cane sugar solutions and boil 1 to 2 minutes. Repeat with the other cane sugar solution.

Cane sugar when split by hydrolysis yields reducing sugars.

2. **Hydroxyl Ion.**—Fill a small test-tube up to within about 1 cm. from the top with 1 per cent tannic acid solution. Add a few drops of 10 per cent sodium hydroxide solution, mix quickly, and let stand for a few minutes. Condensations as well as oxidations probably take place in this reaction.

3. **Metallic Salts.**—To 1 cc. of urine in each of two Kjeldahl flasks add 5 cc. concentrated sulfuric acid. To one add a crystal of copper sulfate. Heat to gentle boiling for 10 minutes to 20 minutes. Compare the rate of disappearance of the brown color.

4. **Pepsin.**—Prepare a pepsin solution from the mucous membrane of a pig's stomach as follows: strip off the mucous membrane from a pig's stomach, mix with 300 cc. of approximately 0.1 N hydrochloric acid (the "concentrated hydrochloric acid" is approximately a 10 N solution) in a wide-mouth bottle capacity 900 to 1,000 cc.), and let stand over night. Remove by decantation 75 cc. of the stomach extract. To the remaining mixture in the bottle add 5 to 10 cc. of chloroform, cork tightly, shake vigorously for a few seconds, label, and place in an incu-

bator. The stomach will digest itself and give a solution suitable for the later study of peptones.

Suspend a piece of egg albumen, or a Mett tube, in the top of each of the following solutions:

(a) 5 cc. consisting of one part of juice to 3 parts 0.1 N hydrochloric acid.

(b) 5 cc. of juice diluted as in (a) and heated in a water bath at a temperature of 75° C. for 15 minutes.

Put the solutions in an incubator (the warm room) over night. Note the results and explain.

5. Trypsin.—Free a beef pancreas from fat, cut up fine, and weigh; transfer to wide-mouth bottle and add 3 cc. 10 per cent alcohol for each gram of pancreas. Add 5 cc. of chloroform, about 2 gm. of sodium bicarbonate and a short strip of intestine (5 cm.), cork tightly, shake, and set aside for two or three days.

Then take out 25 to 35 cc. of the clear liquid and pour on a filter. Stopper tightly again, and put the bottle in the warm room (or incubator) to be preserved for later experiments on "amino acids." Test the filtered portion of the pancreatic extract for its digestive power on egg albumen (Mett's tubes).

6. Urease.—1. (a) Rinse out a 100 cc. volumetric flask with a few cc. of dilute nitric acid, and wash thoroughly with water. To the clean flask transfer 10 cc. of the urea solution which was used for nitrogen determinations (p. 65), 5 cc. of neutral phosphate mixture (pH 7) and 40 cc. of water. Place a thermometer inside the flask, and immerse it in a boiling water bath; shake gently until the temperature of the contents has reached 55° C. From a pipette, and *without delay*, add 2 cc. of 5 per cent alcoholic jack bean extract. With the help of the water bath, now keep the temperature between 50° and 55° C. When exactly 10 minutes have elapsed since the addition of the extract, add 10 cc. of approximately 0.5 N hydrochloric acid. Cool under the tap, dilute to the mark, and mix.

(b) Repeat the above experiment, heating this time to 90° C. before adding the enzyme, and then keeping the temperature between 85° C. and 90° C. for 10 minutes.

(c) Repeat at room temperature.

Transfer 5 cc. of the contents of each of the three volumetric flasks to a separate 250 cc. bottle. To each add 85 cc. of water, 2 or 3 drops of 2 per cent gum ghatti solution (see p. 269) and 10 cc. of Nessler's reagent. This reagent gives a color with solutions containing ammonia, and the intensity of the color is a measure of the amount of urea that has been hydrolyzed by the enzyme.

2. Repeat 1 (c), substituting for the neutral phosphate (a), 5 cc. of acid phosphate (pH 5), (b), 5 cc. of alkaline phosphate (pH 9) and (c) 1 cc. of acidified acetate (pH 5.5). Compare the results with 1 (c).

3. Add 2 cc. of mercuric chloride solution or of Nessler's reagent to a 100 cc. volumetric flask. Shake for one or two minutes. Pour out the mercury solution and rinse the flask three or four times with water. With the apparently clean flask repeat 1 (c) (without rinsing first with nitric acid). Very small traces of mercury inhibit the action of urease and other enzymes.

7. Reversible Reactions (Mass Law).—Mix 5 cc. methyl acetate, 100 cc. water, and 1 drop concentrated sulfuric acid in a small flask (200 to 300 cc.).

(a) Titrate 5 cc.

(b) Boil for about 5 minutes, using reflux condenser. Cool, remove 5 cc., titrate the acidity (what indicator?), and calculate the acidity for 100 cc.

Continue the boiling for one hour, and repeat the titration.

(c) Mix 5 cc. of glacial acetic acid with 100 cc. of methyl alcohol, and add one drop of concentrated sulfuric acid. Remove 5 cc., dilute this with water, and titrate the acidity. Introduce the preparation into a 250 cc. flask attached to a reflux condenser, and boil for two hours. Cool, remove 5 cc., dilute, and titrate as before.

Considerable methyl alcohol is apt to be lost in these experiments. These losses can be much reduced by boiling only *very slowly* and by running a good stream of water through the condenser.

PART III

FATS

1. Solubility of Fats.—Test the solubility of tallow in water, ether, chloroform, and alcohol, carefully avoiding the vicinity of a flame.

Let a drop of the ether solution fall on paper, and note the result.

Dissolve in 3 cc. of warm benzene enough tallow to give a very slight precipitate on cooling. Place some of the precipitate on a slide under a cover glass, examine, and describe. Note especially the shape of the ends of the individual crystals.

2. "Iodine Number." Degree of Unsaturation of Fats; Wys' Method.—Start simultaneously the determination of the iodine number of cottonseed oil and beef tallow. Weigh about 0.3 gm. of cottonseed oil, or about 1 gm. of beef tallow, into a 250 cc. flask, and dissolve in chloroform (10 cc.). Add 25 cc. Wys' iodine solution with a pipette, stopper, and put in a dark place for half an hour. Add 15 cc. of 10 per cent potassium iodide, and dilute with 100 cc. of water, titrate the excess of iodine (partly in solution in the water, partly in the chloroform) with 0.1 N sodium thiosulfate, by running the latter into the flask until, after repeated shaking, both the chloroform and the watery solution are but faintly straw colored. Then add a few drops of 1 per cent starch solution, and continue the titration to the disappearance of the blue color. For dependable results a 50 per cent excess of iodine must be present.

While waiting for absorption to take place, the value of the iodine solution may be determined in terms of 0.1 N thiosulfate by adding KI and titrating in the same way as above. The difference between the two values represents the amount of iodine absorbed by the fat, and is calculated in grams of iodine per 100 gm. of fat.

Example:

0.3 gm. cottonseed oil, when treated as above, required 35 cc. of 0.1 N thiosulfate for back titration.

25 cc. of the iodine solution required 60 cc. of 0.1 N thiosulfate.

The oil therefore absorbed iodine corresponding to $60-35=25$ cc. 0.1 N thiosulfate, i.e., 25 cc. 0.1 N iodine or 25×0.0127 gm. iodine $=0.317$ gm. I. The iodine number is, therefore, $\frac{100}{0.3}\times 0.317=105.6$.

Wys' Iodine Solution.—Dissolve 13 gm. of iodine in 1 liter of glacial acetic acid. Titrate the iodine content of the solution, and then pass washed and dried chlorine gas into the solution until the titration number is doubled. A very distinct change in the color of the solution indicates when this has taken place.

The thiosulfate solution is prepared by dissolving 24 gm. of the crystallized salt in 1 liter of water and standardizing it in the usual way (*see* page 203).

3. Saponification and Preparation of Fatty Acids.—Heat about 20 gm. of beef tallow with 100 cc. of saturated alcoholic solution of sodium hydroxide on a water bath over night, or until the residue is dry. To the mixture add about 300 cc. water and heat to boiling. To the hot solution add a few drops of methyl orange; while continuing the heating (and stirring), acidify with dilute sulfuric acid, and filter. Save the filtrate, which contains glycerine, then wash the fatty acid residue several times with hot water. Throw away the washings.

Transfer the "glycerine filtrate" to an evaporating dish, label, and place on the water bath for evaporation to dryness.

4. Solubility of Fatty Acids and Soap.—Test the solubility of the fatty acid mixture prepared from tallow in water, 2 per cent NaOH, ether, alcohol, and benzene. Compare the results with those obtained with fat.

Let a drop of the ether solution fall on paper, and note the result.

As a test for fat or fatty acid the "grease spot" is often elusive or misleading when applied to dilute solutions in ether. The test may be greatly improved by merely inserting one end of a narrow strip of filter paper in the ether solution. The strip functions as a wick and leaves a highly concentrated deposit at

one narrow cross section. The strip should be cut from quantitative paper, for otherwise much of the "spot" may represent only extractions from the paper.

Dissolve enough of the fatty acid mixture in warm alcohol to give a very slight precipitate on cooling. Examine the crystals under the microscope, and describe as in the case of the fat crystals. Study also the solubilities of soap.

5. Spontaneous Saponification of Fats.—Dissolve about 0.5 gm. tallow, or a few drops of oil, in 10 cc. warm alcohol in a test-tube (avoid fire!). Add 3 to 4 drops phenolphthalein solution, and then, drop by drop, tenth normal sodium hydroxide solution (alcoholic sodium hydroxide solution is best) until the indicator reveals a distinctly alkaline reaction. Let the mixture stand in a warm room over night, and again add alkali (drop by drop) until the alkaline reaction reappears.

6. Titration of Higher Fatty Acids.—Dissolve about 0.2 gm. fatty acid mixture in 10 cc. warm alcohol or benzene (avoid fire!). Add 3 to 4 drops phenolphthalein, and titrate with tenth normal alcoholic sodium hydroxide solution until an alkaline reaction is obtained. One cubic centimeter of the alkali corresponds to how much fatty acid? Why does this titration produce turbidity or a precipitate?

7. Glycerine and Acrolein Test.—This test is usually made, as described below, by heating in crucibles. There is some danger because of the spattering. This danger is avoided if a small porcelain dish and a funnel be substituted for the crucible. The acrolein fumes will then come out through the stem of the funnel.

To about 5 gm. acid potassium sulfate ($KHSO_4$) in a porcelain crucible add one drop of glycerine, heat over a direct flame, and note the pungent odor and tear-begetting quality of the fumes. Note how much heat must be supplied to secure an unmistakable test.

When the filtrate (saved from the saponification of beef tallow) has evaporated to dryness, the residue obtained is sodium sulfate; mixed with it there should be some glycerine. (How much glycerine might be there?)

Mix with a glass rod 2 to 3 gm. of this residue with 5 to 6

drops concentrated sulfuric acid in a dry crucible, and apply heat. If an unmistakable acrolein test is not obtained, repeat with more of the residue.

The acrolein reaction does not work out well as a test for fat.

8. **Emulsification.**—Put 1 to 2 cc. of a solution of sodium carbonate (0.2 per cent) in a watch glass, and place in the center a drop of rancid oil. The oil soon shows a white rim, and a milky opacity spreads over the solution. Note with the microscope the active movements in the vicinity of the fat drop, due to the separation of minute particles of oil.

Examine a sample of milk under the microscope. The fat should be in a state of fine emulsion.

9. **Cholesterol; Liebermann's Reaction.**—Dissolve a crystal of cholesterol in 2 cc. of dry chloroform, and to this solution add 10 drops of acetic anhydride (formula?) and 4 drops of concentrated H_2SO_4. Shake. The liquid becomes rose red, blue, then dark green on standing.

10. **Problem.**—On the basis of the solubilities and other properties of fats, fatty acids and soaps, work out a scheme for their separation and identification. Apply the scheme to two unknowns furnished. Hand in a dated and signed report on the same.

PART IV

CARBOHYDRATES

It is often of clinical importance to determine with certainty whether a person's urine does or does not contain glucose. A great many different methods bearing on this problem have been in use at different periods, but nearly all of these old methods of testing for sugar, including Trommer's and Fehling's, are now only of historical interest, and are omitted here. The problem is complicated by uncertainty as to whether normal urines may not contain traces of glucose, and by the presence in urine of other reducing materials than glucose.

1. **Benedict's Test.**—One of the best qualitative tests for sugar in urine by means of copper solutions is the one proposed by S. R. Benedict. Benedict's reagent is so adjusted that it is rather more sensitive to glucose than Fehling's solution, yet is not reduced by creatinine or uric acid, and little, if at all, by chloroform (which is often added as a preservative to urine). Unlike Fehling's reagent it consists of a single solution. The reagent is made as follows:

Dissolve 85 gm. sodium citrate and 50 gm. anhydrous sodium carbonate in 400 cc. of water. Dissolve 8.5 gm. copper sulfate in 50 cc. of hot water. Pour the copper sulfate solution slowly, and with stirring, into the alkaline citrate solution. Filter if necessary. Label and preserve.

Heat to boiling about 5 cc. of Benedict's reagent in a test-tube together with a pebble or two, to prevent bumping. Add about 8 drops of sugar solution (or urine) and boil for two minutes. If more than two- or three-tenths per cent of sugar is present, the solution will be filled with a colloidal (greenish, yellow, or reddish) precipitate. With smaller amounts of sugar the precipitate will usually appear only on cooling. (The cooling should not be hastened by immersion in cold water.)

2. Folin-McEllroy's Test for Sugar.—The reagent in this test is made as follows: Dissolve 100 gm. of sodium pyrophosphate, 30 gm. of disodium phosphate and 50 gm. of dry sodium carbonate in 1 liter of water by the aid of a little heat. Dissolve separately 13 gm. of copper sulfate in about 200 cc. of water. Pour the copper sulfate solution into the phosphate-carbonate solution and shake.

To 5 cc. of the solution in a test-tube add 5 to 8 drops of urine (never add more than 0.5 cc.) and boil for 1 to 2 minutes, or heat in a beaker of boiling water for 3 minutes. If more than the normal traces of sugar be present, the hot solution will be filled with a colloidal (greenish-yellow or reddish) precipitate as in Benedict's test. This test is a trifle more sensitive than Benedict's; therefore when working with urine, only a distinctly positive test obtained with the solution still *hot* is to be regarded as positive.

3. Phenylhydrazine Test (Osazone Test).—To 5 to 10 cc. of 2 per cent glucose solution in a test-tube add 5 cc. of a phenylhydrazine solution (containing 5 per cent phenylhydrazine hydrochloride, 20 per cent sodium acetate, and 10 per cent acetic acid). Heat in a beaker of boiling water for half an hour. Let the test-tube remain in the beaker until the water has cooled, and examine the glucose osazone crystals under the microscope.

Write the reaction involved in the formation of osazone.

4. Glucose Reactions versus other Carbohydrates.—Apply one of the copper reducing tests, and the phenylhydrazine test to 0.2 per cent solutions of arabinose, fructose, cane sugar, maltose, and lactose.

5. Selivanoff's Test for Ketose-Sugars.—Selivanoff's reagent contains 0.05 per cent of resorcin and about 12 per cent of hydrochloric acid.

To 5 to 10 cc. of the reagent in a test-tube add about 1 cc. of 0.1 per cent solution of fructose, boil for one minute, set aside to cool, and note the development of the color.

Repeat the test with glucose and with cane sugar (and, if desirable, with other dilute sugar or carbohydrate solutions). Tabulate the results.

6. Test for Ketose Sugars (Fructose?) in Urine.—Collect urine for one hour (preferably just before the noon hour). Then take 50 gm. of cane sugar and collect urine for another hour. Dilute the smaller volume of urine to that of the larger, or both to a convenient small volume. To 10 cc. of each in a test-tube add 5 cc. of 10 per cent lead acetate solution, shake, and filter. Apply Selivanoff's fructose test to 2 cc. of each filtrate. Record the results obtained.

7. Orcin Test for Pentoses.—The pentose reagent is made by dissolving 1 gm. of orcin in 500 cc. 30 per cent hydrochloric acid and adding 1 cc. of 10 per cent ferric chloride solution. Protected from light, as in brown bottles, the reagent keeps a very long time.

To 5 cc. of the orcin reagent in a test-tube add 1 cc. of 1 per cent pentose solution. Heat to boiling and remove from flame.

Repeat with 1 cc. of 0.2 per cent pentose solution.

Note the colors obtained—green, blue, violet, depending on the amount of pentose present. Also the formation of turbidity or a precipitate.

Repeat with 1 per cent pentose solution previously diluted (a) with 4 volumes of urine, (b) with 4 volumes 1 per cent glucose solution.

Repeat (a) with urine alone, (b) with 1 per cent glucose solution, (c) with 1 per cent cane sugar solution.

8. Fermentation Test for Sugar.—Certain sugars (which?) are decomposed by yeast into carbon dioxide and alcohol. The formation of CO_2 in fermentation has been extensively used both for qualitative studies and for quantitative determinations of sugar. Except among physicians who have not the facilities for making other tests, the fermentation method in this form is now seldom used.

A. To some 0.5 per cent sugar solution (glucose, pentose, cane sugar, lactose) in a test-tube add a small piece of yeast. Shake to make uniform mixtures, and with each fill a "fermentation tube." Substitute water for sugar solution in a control test, and set the tubes aside in a warm place for 2–14 hours. Record the results.

These long drawn out fermentation tests have come down to us from a comparatively remote period, when no one had ever

heard of such a thing as a yeast cake. Fully as convincing and dependable fermentation tests can now be made in the course of a few minutes. See below under B.

B. Dilute 10 cc. of each sugar solution used in A (except the cane sugar) with an equal volume of water so as to give solutions which do not contain more than 0.25 per cent of sugar. With each of these solutions make the following experiment:

Transfer 10 cc. to a test-tube and add one quarter of a fresh yeast cake. Shake well so as to secure a uniform suspension, and then immerse the test-tubes with some additional shaking into a 500 cc. beaker nearly filled with water, having an initial temperature of about 45° C. At the end of 10 to 12 minutes remove the test-tubes, cool, add 2 to 3 gm. of kaolin, shake well and filter. Unless kaolin is used the filtrates will be turbid.

Apply a suitable qualitative test to each solution and to its fermented counterpart, so as to show, roughly, whether loss of sugar has occurred in the fermented portions.

This quick fermentation test is capable of a high degree of perfection both for selective qualitative work and for quantitative analysis of mixtures involving the determination or the preliminary removal of fermentable sugars.

9. Benedict's Method for the Determination of Sugar.—(*J. Am. M. Ass.*, 57: 1193.)—Prepare 500 cc. of Benedict's solution as follows: Dissolve 9 gm. pure copper sulfate in a 500 cc. volumetric flask with about 100 cc. distilled water. Dissolve 50 gm. anhydrous sodium carbonate, 100 gm. sodium citrate, and 50 gm. sodium thiocyanate in 250 cc. distilled water. The copper sulfate must be weighed accurately on the analytical balance. Pour the copper solution, slowly, with stirring and without loss of a single drop, into the alkaline citrate solution. Then pour the mixed solution back into the measuring flask without loss, add 5 cc. 5 per cent potassium ferrocyanide solution, and with the rinsings make the total volume up to 500 cc. Mix, transfer to a clean, dry bottle, label, and preserve. Twenty-five cubic centimeters of the solution corresponds to 50 mg. of glucose, 52 mg. of fructose, 67 of lactose, or 74 of maltose.

The determination is carried out as follows:

Measure 25 cc. of Benedict's solution into a porcelain dish, add 5 to 10 gm. of solid sodium carbonate, heat to boiling, and

while boiling, run in the sugar solution (or urine) fairly rapidly until a white precipitate begins to form. Then add the solution more slowly (with slower boiling) until the last trace of the blue color disappears. The addition of the sugar solution should be done at such a speed that the boiling solution is kept nearly constant in volume during the operation. The original sugar solution (or urine), if concentrated, should be diluted so that not less than 10 cc. will be required to give the amount of sugar which the 25 cc. of reagent is capable of oxidizing.

Five divided by the volume of sugar solution taken gives the per cent of sugar. Check the value of the reagent by determining the sugar in 0.5 per cent glucose solution.

10. Alternative Method for Titration of Sugar.—(*See J. Biol. Chem.*, 1918, 33: 513; 1919, 38: 287.)

1. Alkaline phosphate mixture. Powder in a large mortar 200 gm. of crystallized disodium phosphate (HNa_2PO_4, $12H_2O$), sprinkle over it about 50 gm. of sodium thiocyanate (or 60 gm. of potassium thiocyanate). Mix with pestle and spoon for about ten minutes. A uniform semi-liquid paste is obtained. To this paste add 120 gm. of dry, granular sodium carbonate, and mix with pestle and spoon until a rather fluffy granular powder is obtained. Leave in mortar covered with paper over night, then mix once more. This reagent keeps indefinitely but should be kept in stoppered bottles so as not to lose too much moisture.

2. A saturated solution of sodium carbonate containing 14 to 20 per cent Na_2CO_3.

3. A copper sulfate solution containing 60.5 gm. of $CuSO_4$, $5H_2O$ and 2 cc. of concentrated sulfuric acid per liter. The sulfuric acid in this reagent is added only to prevent precipitation of copper hydroxide by the traces of alkali gradually given off by glass. The solution keeps indefinitely.

Five cc. of the copper solution is reduced by 25 mg. of glucose or fructose, by 40.4 mg. of anhydrous lactose, or by 45 mg. of anhydrous maltose. The normal reduction time is 5 minutes, except in the case of fructose, which is reduced within 2 minutes. In working with lactose it should be noted that crystallized lactose contains 1 molecule of water of crystallization.

The titration is best made in large, hard glass test-tubes. Urines or sugar solutions up to a concentration of 7 or 8 per

cent are titrated directly, that is, without any preliminary dilution.

A special sugar burette, total capacity 5 cc., divided in 0.02 cc., is used for measuring. This burette should have an accessory tip very fine and about 5 cm. long; it should also have a rubber tube attachment above for filling by suction.

Transfer 5 cc. of the copper solution to the large test-tube; add 1 cc. of sodium carbonate solution, thereby precipitating the copper and rendering the solution alkaline. Add 5 gm. (not less than 4.5 nor more than 5.5 gm.) of the solid phosphate mixture. Heat gently, with shaking, until all the salts, except for a few isolated particles of sodium carbonate, have dissolved. A practically clear solution is usually obtained in less than 1 minute, and temperature need not exceed 60° C. Use only a micro burner as the source of heat and use only extremely mild heat for securing the preliminary solution of the salt mixture.

From the sugar burette, filled by suction with the urine or sugar solution, add 0.4 cc. to 1.0 cc. to the warm, clear copper solution. With watch in hand (or clearly visible), heat the mixture rapidly to unmistakable boiling. Note, on the second hand, when the boiling begins; from that moment keep track of the time, and thereafter heat only enough to keep the contents just to boiling—by moving the test-tube back and forth, through the flame. When bumping begins, add a pebble to promote even, gentle boiling.

If the contents of the test-tube do not suddenly become turbid from precipitated cuprous thiocyanate within the first 15 seconds of boiling, then less than one-half the required amount of sugar has been added and more should be introduced at once. When the full amount of sugar (25 mg.) is present, the turbidity appears within 5 seconds after the boiling has begun. The boiling should normally be continued for 3 minutes, counting from the time that the boiling point was reached, before any more sugar is added. Boil 1 minute after each subsequent addition of sugar. The total boiling period for a correct titration must not be less than 4 or more than 6 to 7 minutes and should not require more than three or four separate additions of the sugar solution. But the preliminary titration may last for 8 to 9 minutes, and if the boiling process has been gentle, the result will then be only about 1 per cent too high.

In the preliminary titration it will frequently happen that the first sugar addition contains more than 25 mg. of glucose, and the greater the excess the more quickly will decolorization of the copper take place.

Time of boiling for complete reduction of copper solution by an excess of glucose.

Glucose	Boiling Time	
Mg.	Min.	Sec.
50	0	25
40	0	40
35	0	55
30	1	20 to 30
27.5	1	30 to 55
25.5	3	

By noting the boiling time in which complete reduction has occurred a valuable guide to the amount of sugar solution to be taken for the next titration is obtained.

After some experience has been gained it should very seldom be necessary to make more than two titrations, a preliminary and a final, for any one sugar determination. Note. The most common errors made by students are: 1. that they use too much heat to dissolve the salt mixture whereby a blue sediment is produced which does not react with sugar and obscures the end-point, and 2. that they boil too fast during the reduction. The total volume in the test-tube should be nearly the same at the end of the titration as it was just before any sugar solution had been added. This means that when a strong sugar solution is titrated one must boil more slowly than when a weak solution is used.

Calculation: 0.025 times 100, or 2.5 divided by the titration figure in cc., whether this be several cc. or a fraction of 1 cc., gives the per cent of glucose present.

Prepare 100 cc. of 1, 1.5 or 2 per cent solution of pure glucose, and learn the titration on the basis of that solution. Without some preservative, moulds are apt to develop in this solution in the course of 3 or 4 days. It is not a bad plan to substitute tenth normal hydrochloric acid, or, still better, saturated benzoic acid solution for one-half of the water used in making the solution. Preservatives, such as chloroform or toluene, are not very satis-

factory in this case because they contaminate the burette so that drops of sugar solution soon begin to stick to the sides.

11. Polariscope Method for the Determination of Sugar.— The specific rotation of a substance is the angle through which the plane of polarized light is turned by 1 dm. of a solution containing 1 gm. of the optically active substance per cc.

A definite temperature and light of a definite wave length (sodium light) must be used in determining specific rotations.

The angle of rotation is determined by means of some form of "polariscope" (polarimeter, saccharimeter, etc.); and the specific rotation is calculated according to the following formula:

$$\text{Specific Rotation} = \frac{\text{Observed Rotation} \times 100}{\text{Percentage} \times \text{Length of Observation Tube (dm.)}}$$

If the specific rotation is known, as in the case of the common sugars, the per cent of sugar is obtained by the following transposition of the above formula:

$$\text{Percentage} = \frac{\text{Observed Rotation} \times 100}{\text{Specific Rotation} \times \text{Length of Tube (dm.)}}$$

Following are the specific rotations (yellow light) of some common sugars:

Glucose, 52.8; Fructose, −93; Cane Sugar, 66.5; Lactose, 55; Maltose, 137.

The determination of sugar by means of the polariscope is as follows:

Rinse the polariscope tube (length usually 1 or 2 dm.) with the sugar solution, and fill almost to overflowing. Place the glass plate over the open end in such a way that the tube does not contain any air bubble, and screw on the cap. Place the tube in the groove of the polariscope. Light the lamp, and move the eyepiece back and forth until the lines which divide the field are sharp. Then turn the screw until the several divisions of the field are equally illuminated, and take a reading by means of the vernier. The circle upon the disk of the apparatus is divided into quarter degrees; 24 divisions of the vernier correspond in length to 0.25°. Consequently every division of the vernier corresponds to 0.01°. Ascertain whether the disk, starting from its middle point, has been moved to the right or to the left of the zero point of the vernier. Read off the number of whole degrees and

hundredths. Take several readings by moving the lever and coming back again to the point where the different parts of the field are equal. Correct for the zero point of the instrument by taking readings with the tube filled with water. This value is added to, or subtracted from, the reading found with the sugar solution.

If a saccharimeter is used instead of the general circular polariscope, the reading on the scale is converted into angular degrees of rotation by multiplying by the factor 0.345. The percentage of sugar in the solution is then calculated by using the formula given above.

12. **Cane Sugar.**—With the help of the balance, prepare 100 cc. of a known cane sugar solution (8 to 12 per cent). So-called lump sugar is better for this purpose than granulated sugar, because of freedom from dust. Determine the concentration of the solution with the polariscope according to the directions given in the preceding section.

Transform a part of the solution into "invert sugar" and determine the sugar content by titration. The inversion is made as follows:

Transfer 10 cc. of the cane sugar solution to a 100 cc. volumetric flask and add 1 cc. of concentrated hydrochloric acid. Add no extra water. Heat a large beaker or porcelain dish to 80° C. When the water has reached 80° C., immerse the flask in water, and, without allowing the temperature of the water bath to sink below 80° C., rotate and shake the flask continuously, but gently, for 10 minutes. At the end of this time cool and dilute to the 100 cc. mark with water, and mix.

Instead of heating to 80° C., complete inversion can also be accomplished by allowing the mixture of sugar and acid to stand at room temperatures over night. The over night process is, of course, inapplicable in "practical examinations."

In connection with titrations of invert sugar, due note must be taken of the 5 per cent increase in the weight of the sugar accompanying the inversion. In titrating unknown solutions of invert sugar it must not be forgotten that one-half of the sugar is fructose, and that fructose reduces alkaline copper solutions much more rapidly than does glucose. Turbidity within the first 5 seconds of boiling does, therefore, not necessarily indicate that almost enough or too much sugar has been introduced.

13. **Problems.**—Determine by the polariscope the sugar concentration of one unknown solution of glucose and one of lactose. Determine, without the polariscope, the glucose and cane sugar content of one unknown mixture of these two sugars.

Hand in dated, signed reports on these unknowns; each report to represent two unknowns.

14. **Preparation of Maltose.**—Mix 10 gm. of starch with 30 cc. of cold water until a smooth paste is obtained. Pour this slowly, and with stirring, into 250 cc. of boiling water in a large beaker, continue the boiling 1 to 2 minutes, let cool to 45° C., stir in 1 teaspoonful of malt, and keep at this temperature for 30 minutes. Boil, cool, transfer to a 250 cc. volumetric flask, make up to volume. Determine the maltose by titration in a filtered sample, and calculate the amount of maltose obtained. Taste the solution.

15. **Preparation of Milk Sugar.**—To 300 cc. of skimmed milk diluted with 800 cc. of water, add cautiously 2 per cent acetic acid to precipitate the casein. When enough has been added the liquid is nearly clear. Filter. Boil the filtrate, and filter off the coagulated albumin. Evaporate the filtrate on the water bath to a thin syrup, and allow it to stand until the sugar has crystallized out.

A more expensive, but otherwise much better, method for preparing crystallized lactose from milk is as follows: Transfer 100 cc. of skim milk to a 200 cc. flask or bottle, add 5 gm. of solid picric acid. Shake for 10 minutes, let stand for half an hour, and filter into a 300 cc. flask or bottle. Add to the filtrate 200 cc. of acetone and let stand for 24, or better for 48, hours. Examine and test the crystalline deposit. It is all milk sugar.

16. **Starch and Dextrin.**—Heat 500 cc. of water to boiling in a beaker. Transfer 5 gm. of finely powdered starch to a test-tube, add 5 cc. of cold water, shake well and pour the resulting starch suspension, a little at a time, into the boiling water. Boil gently for about 10 minutes. Transfer starch solution to a flask and cool.

Prepare also about 50 cc. of 2 per cent dextrin solution, using heat if necessary to get a clear solution.

Commercial dextrin sometimes contains unaltered starch and

usually contains reducing sugar. If appreciable traces of starch are present, the gradual addition of diluted iodine solution gives at one stage a pure blue color. If no starch is present, only red, brown or violet colors appear.

To each of 2 test-tubes add 5 cc. of water. To one add about 2 cc. of starch solution, and to the other, about 2 cc. of dextrin solution. Add diluted iodine solution drop by drop (by means of a pipette) to each test-tube until the maximum color is obtained. Note the color obtained in each case.

Dilute 5 cc. of the starch solution to 100 cc. Add varying amounts of this diluted starch solution to 2 cc. of dextrin solution and apply the iodine test. Note at which stage of iodine addition the pure blue color is obtained. Note also that a positive starch reaction may easily be missed by adding too much iodine. The iodine solution should be very dilute; the ordinary 1 per cent solutions need be diluted 20 times. If the original dextrin solution is free from starch, what is the minimum quantity of added starch required to give a positive pure blue reaction?

To 5 cc. of starch solution add about 10 cc. of saturated ammonium sulfate solution and shake. Note that starch is precipitated. Repeat with the dextrin solution; if the dextrin is free from starch according to the iodine test, it will not give a precipitate with ammonium sulfate solution. The starch precipitation is not strictly quantitative; a faint starch reaction can usually be obtained with the ammonium sulfate filtrate from starch solutions. Try this (a) with starch filtrates (b) with mixtures of starch and dextrin. A fairly satisfactory separation of starch from dextrin is attained by the help of ammonium sulfate.

Test for reducing sugar in the solutions of starch and dextrin.

Mix 2 to 5 gm. of brown crackers with about 50 cc. of cold water. Filter. Test the filtrate for starch, dextrin, and reducing sugar.

To 5 cc. of saliva in a small flask add 200 cc. of the starch solution. (Fresh starch solutions are best for this experiment.) Heat in a beaker of warm water (40 to 42° C.). Test at the end of ½, 2, 3 and 5 minutes for reducing sugar, and repeat until a positive test is obtained.

Continue the digestion at 40 to 42° C., and at 5 minute intervals transfer 5 cc. portions to each of 2 test-tubes. To one add

just enough iodine to give an unmistakable color; to the other add enough iodine to give the maximum color. Save the series of test-tubes for comparison, and note the gradual disappearance of the starch and the formation and disappearance of dextrin.

17. **Glycogen.**—Cut four fresh (Why fresh?) oysters into small pieces, and throw into four times their weight of boiling water slightly acidulated with acetic acid. After boiling for a short time, remove the pieces, grind in a mortar with some sand, return to the water, and continue the boiling for several minutes. Filter while hot. The opalescent solution thus obtained is an aqueous solution of glycogen and other substances.

With the solution of glycogen thus obtained, make the following tests:

Add iodine solution drop by drop to a portion of the glycogen solution. The liquid will assume a dark red color.

Test the glycogen solution for reducing sugar.

Add some saliva to a portion of the glycogen solution, and put in the warm room until the next day. Remove, and divide into two portions. Test one with iodine solution for glycogen, the other for sugar. Report the result.

PART V

PROTEINS

1. Test for Nitrogen, Sulfur, and Phosphorus in Protein.—Put a little dry protein [1] into the *bottom* of a dry, cheap test-tube. Add a piece of metallic sodium the size of a pea, and heat strongly for at least 5 minutes. Cool. Carefully and without handling the material, break into a dry evaporating dish. Cover the substance with a wet filter paper. After five minutes, cautiously add 25 cc. water. Stir well. Filter into a test-tube. The filtrate should contain (a) cyanide, (b) phosphate, (c) sulfide—as sodium salts, besides NaOH.

(a) To 5 cc. add a few drops of ferrous sulfate and a drop of ferric chloride solution. Warm, and acidify with concentrated hydrochloric acid, noting the result. The blue precipitate, Prussian blue, is a nitrogenous product.

(b) Acidify 5 cc. with nitric acid, and add a few cc. of ammonium molybdate solution. Let stand, and look for a yellow crystalline precipitate. Ammonium phosphomolybdate has a strong yellow color and is not very soluble.

(c) To another 5 cc. add a few drops concentrated sulfuric acid, and suspend over the mouth of the test-tube a piece of filter paper previously moistened with lead acetate solution.

Consult some textbook on inorganic chemistry about the tests (a), (b) and (c).

If too much protein is taken for the sodium fusion failure is likely to result, but the most common cause of failure is inadequate heating.

2. Simple Test for "Amide" Nitrogen and for Sulfur.—Put a little protein in a test-tube with a few cubic centimeters of

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[1] A mixture of casein and dry egg albumin.

strong sodium hydroxide solution and three drops lead acetate solution. Heat to boiling, and suspend a piece of litmus paper over the mouth of the test-tube. Amide nitrogen and a part of the cystine sulfur are set free by the action of the hot alkali in the form of NH_3 and H_2S.

3. **Albumins.**—Preparation of an albumin solution. Considering "egg white" to contain 12 per cent of albumin, prepare a 2 per cent solution by suitable dilution with distilled water. Shake thoroughly, and filter through a plug of cotton.

4. **Coagulation by Heating.**—Heat a little of the albumin solution in a test-tube. Compare the coagulation so obtained with that obtained when the solution is diluted (a) 20 times with distilled water, (b) with a solution containing 0.01 N acetic acid and 0.01 N sodium acetate.

5. **Sulfosalicylic Acid Test.**—To some albumin solution in a test-tube add a few drops of sulfosalicylic acid solution (25 per cent). This very delicate and dependable test is often used for the quantitative determination of albumin in urine. See pp. 221–223.

6. **Nitric Acid Test.**—Put 5 cc. of the solution in a test-tube, and introduce 5 cc. of concentrated nitric acid very carefully with a pipette to the bottom, forming an under layer. Determine the lowest protein concentration at which the test is unmistakable. Allow 10 minutes if the reaction is slow in appearing.

7. **Picric Acid Test.**—Add to a portion of the albumin solution a few drops of a solution of picric acid (1 per cent) and citric acid (2 per cent)—Esbach's reagent. Determine the lowest protein concentration at which this test is unmistakable.

8. **Action of Ammonium Sulfate.**—Add some powdered ammonium sulfate to 10 cc. of the albumin solution in a test-tube, shaking frequently until the solution is thoroughly saturated. Allow to stand for a while, occasionally shaking, filter, and test the filtrate for albumin by the heat test. Test the solubility in water of the precipitate on the filter paper. Pour some water on the precipitate and apply the coagulation test to the filtrate.

9. Action of Magnesium Sulfate.—Perform a similar experiment, using solid magnesium sulfate instead of ammonium sulfate. To a portion of the filtrate add one or two drops acetic acid.

10. Biuret Test.—To a portion of the albumin solution add a little sodium hydroxide, then, drop by drop, very dilute copper sulfate. The solution becomes violet. Study the delicacy of the reaction. After adding to the albumin solution some solid ammonium sulfate, repeat the test with (a) the same amount of alkali (b) a large amount of 40 per cent alkali.

11. Millon's Test.—To a portion of the albumin solution add a few drops of Millon's reagent. A precipitate forms, which, on heating, becomes brick red. Repeat, using a dilute solution of phenol instead of albumin. On what group in the protein molecule does this test depend? Add sodium chloride and repeat the test. The failure of the reaction in the second case is possibly due to the action of chlorine.

12. Xanthoproteic Test.—To a few cc. of the solution add one-third of its volume of concentrated nitric acid; a white precipitate may or may not be produced (according to the concentration and the nature of the protein). Boil. The precipitate or liquid turns yellow. Allow the solution to cool, and add an excess of ammonia. It is to be noted than in Experiment 6 nitric acid is used only as a precipitant (Heller's test), while here nitric acid is used to give a color reaction.

13. The Glyoxylic Acid Reaction (Hopkins and Cole).—Treat 2 or 3 cc. of the solution with the same volume of "reduced oxalic acid." Mix and add an equal volume of concentrated sulfuric acid, pouring down the side of the tube. A purple ring forms at the junction of the fluids. Mix the fluids by shaking the tube gently from side to side. The purple color spreads through the whole fluid. Repeat in the presence of nitrates, chlorates, nitrites, excess of chlorides, and carbohydrates, respectively.

"Reduced oxalic acid" is prepared by Benedict's method as follows:

To 10 gm. powdered magnesium in a flask add a little water, and then add slowly, with shaking and cooling, 250 cc. of cold saturated oxalic acid solution. Filter, acidify the filtrate with acetic acid, and dilute to one liter.

14. **Alcohol.**—Add an excess of alcohol (one or two volumes) to some albumin solution. If the precipitate is small, add a little dilute sodium chloride solution.

15. **Tannic Acid.**—Make some protein solution slightly acid with 0.1 per cent of acetic acid, and add a few drops of tannic acid solution.

16. **Phosphotungstic Acid.**—Make a protein solution acid with dilute hydrochloric acid, and add a few drops of the reagent.

Globulins.—The tests are made upon blood serum.

17. **Action of Carbon Dioxide.**—Dilute 5 cc. of clear serum with 45 cc. of ice-cold water. Place the mixture in a cylinder or large test-tube, and pass through it a stream of carbon dioxide. What is the effect of too much carbon dioxide?

18. **Precipitation by Dialysis.**—Pour 20 cc. of serum into a parchment or cellophane dialyzing tube previously soaked in distilled water. Suspend the tube, with its contents, in a large volume of water. Explain the precipitation.

Pour serum, drop by drop, into a large volume of distilled water (in a beaker). What takes place? Explain.

19. **Precipitation by Magnesium Sulfate.**—Saturate about 5 cc. of the serum with magnesium sulfate. A heavy precipitate will be formed. Compare this with the action of the same salt on the egg-albumin solution. By the help of magnesium sulfate it is possible to separate globulins from albumins.

20. **Precipitation with Ammonium Sulfate.**—To 30 cc. of serum add an equal volume of a saturated solution of ammonium sulfate, thus obtaining a half-saturated solution. Filter off the precipitate, wash two or three times with a half-saturated ammonium sulfate solution, and dissolve in about 60 cc. of water. This yields a clear solution of globulin. Apply 5 protein tests to this solution.

21. **Keratin.**—Show that keratin (hair or horn) is a protein. Keratin may be dissolved by heating with 10 per cent NaOH.

22. **Gelatin.**—Make dilute gelatin solution, and with it make six tests for protein (including Millon's). Test for sulfur.

23. **Phosphoproteins.**—Test the solubility of casein in water, dilute acid, dilute alkali, and dilute salt solution.

Make six protein tests on a solution of casein. How would you test for albumin and casein when both are present? Apply to milk and to unknown furnished.

24. **Peptones (Proteoses).**—For the following experiments use the peptic digestion mixture obtained with the pig stomach (p. 73). Neutralize, heat to boiling and filter. Use the filtrate.

Apply protein tests described under "albumin," and record the results obtained.

Dialyze about 10 cc. of the peptone solution against about 100 cc. of distilled water in a beaker. After 24 hours test the outside water for peptones. Explain.

How would you test for proteoses and albumin in a solution which might contain either or both? The quantitative determination of each is more laborious, but can be done fairly well by means of nitrogen determinations before and after the removal of the coagulable albumins. 1 mg. of N corresponds to 6.25 mg. of protein.

25. **Amino Acids, Tyrosine and Leucine.**—For this experiment use the pancreatic digestion mixture prepared for the study of ferment reactions (p. 75).

With a pipette take out 10 cc. of clear supernatant liquid. Filter this portion if necessary; dilute it with 2 volumes of water, and by means of Mett's tubes determine whether the proteolytic ferment has been destroyed or is still active.

Pour the rest of the digestion mixture without filtering into a good-sized beaker or flask. With continuous stirring or gentle shaking to prevent burning and bumping, heat the digestion mixture until it begins to boil. Some care is needed in this operation because of the presence of alcohol. When the mixture is boiling remove the flame. Note approximately the volume of the mixture, and measure into a test-tube some "Merck's dialyzed iron," 8 to 10 cc. for each 100 cc. of digestion mixture. Dump the colloidal iron into the digestion mixture, and shake or stir vigorously. Filter.

To the filtrate add a few drops of ammonia and 5 to 10 gm. of bone-black. Boil for a few minutes and again filter. A clear, faintly colored solution should be obtained.

Pour this last filtrate into an evaporating dish, acidify with a little acetic acid, and boil down to about one-sixth of the original volume.

Transfer the concentrated liquid to a flask or beaker, and set aside in a cool place for a day or two. Tyrosine and leucine crystallize out, the former first and in much greater abundance. Examine the sediment under the microscope.

The isolation of other amino acids from the mother liquor is much more difficult.

26. Preparation of Cystine (from Wool).—Heat 50 gm. of wool in a 500 cc. flask with 100 cc. concentrated hydrochloric acid on a water bath until dissolved. A 3-foot glass tube should be inserted to prevent the loss of too much acid liquid. When dissolved, boil very gently over a *small* flame for 3 to 4 hours. Add solid sodium acetate (100 to 130 gm.) until no free mineral acid can be detected in the solution by means of Congo red paper. Allow the mixture to stand from 3 to 5 days. The longer the mixture is allowed to stand, up to 3 weeks, the more cystine is obtained. Filter on a Büchner funnel and wash with cold water. Then dissolve the precipitate in water (150 cc.) plus 5 to 10 cc. concentrated hydrochloric acid, add about 20 gm. purified bone-black, and boil 5 to 10 minutes.

To prepare pure bone-black, let the impure sample stand in an excess of dilute hydrochloric acid over night, filter, and wash with cold water until the filtrate is neutral.

Filter again with suction, heat the filtrate to boiling, and neutralize the hot hydrochloric acid by adding very slowly hot concentrated sodium acetate solution (avoid an excess, test with Congo red paper). The precipitate formed consists of cystine, and should be very white and pure. If it is dark colored, re-dissolve in water and a little hydrochloric acid, and repeat the bone-black treatment.

Keep the mixture boiling, and add very slowly the hot sodium acetate solution until the crystallization begins; keep hot, and after a few minutes add cautiously a little more acetate. Well-formed, large, and characteristic crystals should be obtained.

Examine the crystals under the microscope.

Test for sulfur. Test also for tyrosine. (Ordinarily the crystals are quite free from tyrosine.)

Tyrosine can be prepared from the original mother liquor by decolorizing with bone-black and letting stand in a cold place.

PART VI

URINE ANALYSIS AND METABOLISM

Quantitative urine analysis has no value except in connection with known volumes of urine representing a definite known metabolism period. Even in student exercises invoiving the learning of methods, only urines which represent a definite metabolism period should be used, and from the analytical figures obtained the total value for the whole urine (and metabolism period) should be calculated.

The standard common metabolism period is twenty-four hours. The only correct way to collect twenty-four hour urines is to begin the metabolism period immediately after passing the night urine in the morning. Note the time, and then collect all the urine passed up to the same hour the following morning. The reason for this rule is that during the night much of the waste products corresponding to the food intake of the preceding day is passed, and in the early morning, before any food has been taken, the excretion is at its lowest level.

Sometimes it is impracticable to collect twenty-four hour urines, and sometimes it is desirable to study the urine representing shorter metabolism periods such as three, four, or six hours. Formerly it was not practicable to make use of such short periods, because the analytical procedures require too much urine; the uric acid determination alone required 150 cc. By the help of the modern colorimetric methods, nearly complete analysis can be made on the basis of three hour urines. For such short metabolism periods it is necessary to drink not less than 200 cc. of water at the beginning of the period. The period, whether consisting of three hours or twenty-four hours, should begin immediately after passing the night urine.

It is necessary to use some preservative for the urine unless the analysis can be completed within twenty-four hours. Chloro-

form (2 cc.), or toluene, or xylene, should be introduced into the empty bottle in which the urine is to be collected so as to exclude from the beginning any possibility of bacterial decomposition.

In systematic urine analysis some determinations should be made as soon as possible (within twenty-four hours) while others can be postponed as long as desirable. The uric acid determination should be made the first day, because the uric acid may either fall out as a sediment or may be decomposed as the result of long standing. The creatinine determination should be made within forty-eight hours, because in some urines it is gradually in part converted into creatine. From the standpoint of spontaneous decomposition in well preserved urine the ammonia determination can be postponed indefinitely, but it is usually better not to delay the ammonia determination more than two or three days. In turbid urines there is more or less danger of precipitation of a part of the ammonia as ammonium magnesium phosphate.

The acidity titration should be made within twenty-four hours. Most urines darken very much on standing, and the deepened color makes it difficult to see the faint color which marks the correct end-point of the titration.

Standard figures for the composition of normal twenty-four hour urines are abundant in the literature. (See *Am. J. Physiol.*, 13:45–115, 1905.) Standard figures for three hour urines are as yet not numerous. The figures recorded below for the first three hour morning urines were obtained in 1918 by G. Youngburg. The subjects were medical students.

Aeration Method for the Determination of Ammonia.—Measure 25 cc. of the ammonium sulfate solution previously used for nitrogen determinations into a tall aerometer cylinder. The cylinder is fitted with a two-hole rubber stopper and glass connections so arranged that compressed (outside) air (or laboratory air previously freed from ammonia) is passed to the bottom of the cylinder and out through a calcium chloride tube filled with dry cotton, and then through a special absorption tube (see *J. Biol. Chem.*, *97*, 143) into a receiver containing water and a known amount of acid. The cotton serves the purpose of holding back traces of solid sodium carbonate, formed by evaporation on the sides of the cylinder.

Add to the ammonium sulfate solution about 10 gm. sodium chloride, about 2 gm. sodium carbonate, and a few drops of kero-

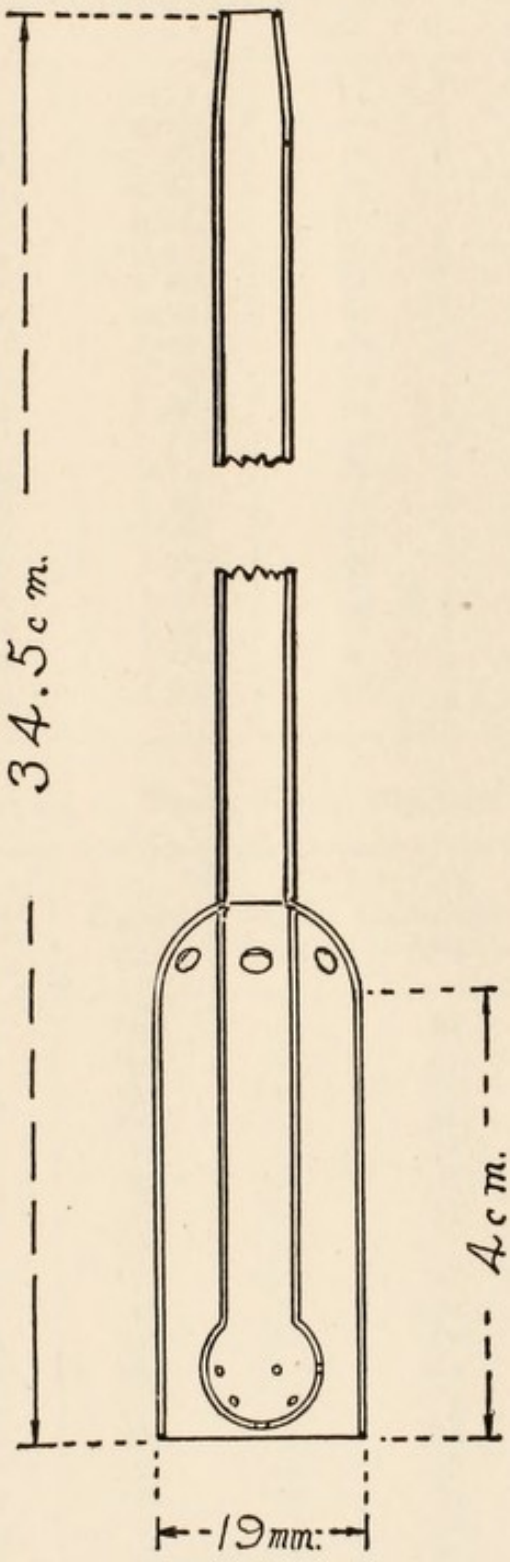

FIG. 6.

sene or antifoaming mixture (p. 277). Do not add any water; the greater the volume the longer it takes to drive off all the ammo-

COMPOSITION OF THREE HOUR MORNING URINE

No.	Total N gm.	NH_3—N mg.	Urea—N gm.	Uric Acid mg.	Creatinine mg.	Body wt., K.
1	0.87	42	0.72	73	176	58
2	1.00	47	0.85	66	192	56
3	1.02	68	0.82	56	176	59
4	1.02	79	0.82	57	224	66
5	1.12	43	0.94	48	154	60
6	1.14	74	0.97	72	163	64
7	1.18	51	1.00	71	192	66
8	1.23	107	0.99	88	204	64
9	1.26	100	1.02	85	240	80
10	1.26	41	1.04	65	222	79
11	1.33	79	1.08	64	220	77
12	1.37	77	1.16	65	190	70
13	1.42	46	1.13	88	203	54
14	1.46	88	1.23	91	183	67
15	1.48	58	1.29	88	229	65
16	1.51	40	1.27	69	190	64
17	1.54	34	1.35	77	212	58
18	1.55	81	1.34	67	186	61
19	1.88	69	1.66	89	247	73
20	2.18	49	1.85	105	236	68

COMPOSITION OF THREE HOUR MORNING URINE

No.	Volume, cc.	Acidity in cc. 0.1 N	H_3PO_4 mg.	Total S mg.	Inorganic S mg.	Ethereal S mg.	Cl gm.
1	52	34	159	46	30	4	1.64
2	96	6	142	54	31	7	1.08
3	207	30	293	113	39	28	1.11
4	61	38	247	69	51	4	0.54
5	204	5	12	51	40	0	0.90
6	81	24	86	62	38	2	1.00
7	111	40	317	96	73	6	1.15
8	185	52	381	99	74	7	1.74
9	90	44	374	107	77	5	0.62
10	138	6	19	86	50	4	1.15
11	84	35	232	86	60	11	0.75
12	66	48	369	114	93	6	0.27
13	203	26	241	76	57	4	1.21
14	102	42	283	107	38	8	1.00
15	142	6	193	95	59	22	1.28
16	107	33	142	105	59	24	0.76
17	514	13	92	78	56	10	1.64
18	745	3	165	105	81	7	2.62
19	520	17	237	65	47	1	1.57
20	139	10	261	142	93	6	1.29

nia. Pass a very strong air current through the mixture for not less than one hour, and collect the ammonia, which the air carries off, in a receiver containing 25 cc. 0.1 N acid and about 200 cc. water. Titrate, and compare the result with the figures obtained by distillation (p. 61). If the results are too low, the air current has been too slow, or the aeration process has not been continued long enough to drive off all the ammonia.

In a similar manner determine the ammonia in urine (25 cc.) and calculate the 24-hour amount. When working with urine it is desirable, though not absolutely necessary, to substitute 10 gm. of potassium oxalate for the 10 gm. of sodium chloride. Salts hasten the removal of the ammonia, and oxalate incidentally prevents the (possible) formation of insoluble ammonium magnesium phosphate.

Save the remainder of the ammonium sulfate solution for other nitrogen determinations and for sulfate determinations.

On the Use of the Colorimeter.—Colorimetric estimations are becoming increasingly numerous and important and beginners should not fail to make themselves reasonably expert in the use of the colorimeter. The directions given here are based on the Bausch and Lomb type of colorimeter, having two movable cups, each with its own rack and pinion. These cups differ from others in that the glass part is enclosed in a metal case with an adjustable bottom. By virtue of this arrangement it is easy to adjust the cups so that the instrument reads exactly zero when the plungers are moved down to the bottom of the cups. In connection with this preliminary adjustment of the zero point the space between the glass cylinder and the top part of the metal jacket should be packed with "stop cock grease" (1 part of vaseline to 3 parts of paraffin). One such packing will last for several months and will completely exclude the corrosive fluids from entering the space between the metal jacket and the glass. It is also advantageous to apply a little grease to the threaded lower part of the metal cylinder to prevent the metal bottom from sticking too hard, as may happen unless rusting is prevented. After the cups have been thus adjusted they should not be taken apart and should merely be rinsed with cold water after each determination just as one does with cups made entirely of glass. Never omit to rinse the cups and also the plungers of the

instrument immediately after a colorimetric comparison has been finished. The plungers are best rinsed by the help of a small beaker of water. When wiping the plungers, do no rubbing; unless the plungers are handled very gently they are apt to become loose. Do not omit to turn off the light from the colorimeter lamp immediately after having finished a color comparison.

The most serviceable colorimeter lamp is probably the Chalet microscope lamp with one "daylite glass" window and a 150 watt Mazda bulb. For most colorimetric comparisons a colorimetric lamp of some sort is better than ordinary day light. One special drawback to the use of day light through a window is that the extra light renders the eye too sensitive. For this and for other reasons the colorimeter lamp should be located in the darkest available space in a laboratory.

Sources of error in colorimetry are plentiful and in many cases are so obscure that only specialists know them. The most serious errors do not occur during the actual color comparison, but in the preparation of the colored solutions which are to be compared. But certain simple precautions need to be taken in making the comparisons.

1. Before attempting to determine the value of the unknown colored solution in terms of the known standard it is essential to compare the standard against itself, since one thereby obtains a check on the instrument, on the light conditions, and on the eye. Rinse the colorimeter cups with a little of the standard solution, and then half fill the cups with the solution. It is always best not to put more solution into a cup than is really needed.

With each half filled cup in place, lower both of the plungers to the 20 mm. mark. The bottom of the plungers should then not be much more than 1 cm. below the surface of the liquid. Make sure by inspection through the opening in the metal jacket that no air bubble has been lodged under the plunger. Turn on the colorimeter light and adjust the position of the colorimeter until the two fields as seen through the eye piece look alike. Do not stare too long at a time and close the eye frequently to avoid fatigue. It is sometimes necessary to move the position of the instrument as much as 30° or more in order to secure the necessary equality of the two fields.

2. When the two fields are equal the beginner should move one of the plungers without disturbing the position of the instru-

ment and then bring it back until the two fields are again equal and note on the colorimeter scale with vernier how near he comes to 20 mm. Two or three readings should be made at this stage in order to acquire facility in making the comparison. If these readings are not substantially correct, set the moved plunger again at 20 mm., so as to see the fields alike, and repeat the process.

3. After certainty and confidence in the matching of the colorimetric fields have been attained, the repeated preliminary readings of the standard against itself may be omitted. The preliminary comparison may then be limited to seeing that the two fields look alike when the plungers are set at 20 mm. with the standard in both cups. The standard in one of the cups is then replaced by the unknown and one careful matching of colors is made, and this one reading is accepted as final.

Note. When replacing one solution by another in a colorimeter cup, as when replacing the standard by an unknown, it is of course essential first to rinse both cup and plunger with the unknown solution. In many cases the total volume of solution available is only 25 cc., and the rinsing must then be done without any unnecessary waste.

Colorimetric Method for the Determination of Ammonia. —*The several colorimetric methods described in this part of the Manual are in principle identical with the corresponding methods used in blood analysis.*

With a Folin-Ostwald pipette transfer 1 cc. of the ammonium sulfate solution into a large test-tube (200×25 mm.). *The usual 20 seconds draining must be followed by careful blowing in the use of these special pipettes.* Fit the test-tube with a two hole rubber stopper carrying an inlet tube, reaching to the bottom, and an outlet tube. Connect the former with the compressed air jet, and the latter with the special ammonia absorption tube used in the macro aeration method (see p. 129). Insert the absorption tube into a clean 100 cc. measuring cylinder. Add to the cylinder 2 cc. of 0.1 N acid and about 15 cc. of water.

Add 2 drops of kerosene or other antifoaming mixture and a few drops of a solution containing 10 per cent of potassium carbonate and 15 per cent of potassium oxalate. Insert promptly and firmly the rubber stopper and start the air current. The

optimum speed for this aeration is 6 to 7 liters per minute (less than one-half of the maximum obtainable speed) and at this speed every trace of the ammonia should be obtained in the course of 10 to 15 minutes.

As a check on the aeration dilute the residue in the test-tube to about 10 cc., add 2 or 3 drops of gum ghatti solution and about 2 cc. of Nessler's reagent.

At the end of the aeration period, disconnect, add 2 or 3 drops of gum ghatti solution, dilute to 60 or 70 cc., and stir with the absorption tube (up and down movement).

Transfer the contents of the cylinder to a 100 cc. volumetric flask together with a little rinsing water. Pipette 10 cc. of standard ammonium sulfate solution (see below) into another 100 cc. volumetric flask, add 2 or 3 drops of gum ghatti solution, and dilute to approximately the same volume as the unknown.

Add, as nearly simultaneously as practicable, 10 cc. of Nessler's solution to each flask, mix, but do not shake hard, dilute to volume, mix by a few inversions, and make the color comparison as described above.

Calculation.—If the standard containing 1 mg. of ammonia nitrogen is set at 20 mm., the unknown contains $\frac{20}{x}$ mg. x is the colorimetric reading of the ammonia solution obtained by aeration.

Note. If the 100 cc. cylinder has been calibrated and is found to be reasonably accurate, the Nesslerization of the unknown can be made in the cylinder. When this is done the absorption tube should not be removed until it has been used for stirring *after* the Nessler reagent has been added. It may be worth while to try this modification.

Standard Ammonium Sulfate.—As a standard ammonia nitrogen solution for the colorimetric nitrogen determinations described in this section, it is best to use, in diluted form, the same ammonium sulfate solution as is used for the determinations on page 63. Dilute 10 cc. to 100 cc. in a volumetric flask and mix. This should give a solution containing 1 mg. of nitrogen in 10 cc.

Alternative Colorimetric Method for Determination of Ammonia.—(*J. Biol. Chem.*, 29: 329.)—In this method the ammonia is extracted from the urine by gentle shaking with a synthetic aluminate silicate powder sold under the trade name *permutit.* Only such preparations as have passed through a 60 mesh sieve and do not pass through an 80 mesh sieve should be used. Powders of any desired degree of fineness are obtainable.

Before applying this method to urine, use it for the determination of ammonia in the ammonium sulfate employed for nitrogen determinations and in the preceding aeration process. Compare the results obtained by permutit with those obtained (a) by distillation (p. 61); (b) by the macro aeration process (p. 127).

The essential mechanical feature of this reagent for absorbing ammonia is that it is a clean, moderately fine, insoluble powder which gives off very little dust or turbid material to water, and settles, like sea sand, from water in the course of a few seconds. By virtue of this novel feature the (absorbed) ammonia can be separated by decantation from the solution (or urine) which contained it.

The reagent is a complex insoluble sodium salt containing active, i.e., easily replaceable, sodium, and the adsorption of ammonia involves the replacement of a part of this sodium by ammonia. The chemical affinity of the active group in the reagent for ammonia is remarkably strong so that under suitable conditions the exchange becomes quantitative as far as the ammonia is concerned.

While the chemical reaction involved in the absorption of ammonia by this reagent is apparently a reaction between a solid and a solution, it remains to be said that the solid powder contains about 20 per cent of water, and if this water of hydration is removed by heat the activity of the reagent is lost. Even gentle dry heat (100° C.) greatly reduces its activity, so that a freshly purified and rapidly dried product is less active than the same product allowed to dry at ordinary temperatures, or than the same product dried rapidly at 100° C., and allowed to "weather" for a day or two.

An important characteristic of this reagent for the absorption of ammonia is that it does not appreciably deteriorate by being used. After washing away the Nesslerized ammonia and

surplus alkali first with water, then with one portion of 2 per cent acetic acid, then once more with water, the powder remaining is just as efficient as before for the absorption of more ammonia.

The process for the colorimetric determination of ammonia in urine by the help of the synthetic zeolite powder is as follows:

Transfer about 2 gm. of the powder to a 100 cc. volumetric flask. Add about 5 cc. of water (no more), and with an Ostwald pipette introduce 1 or 2 cc. of urine, or with 5 cc. pipette introduce 5 cc. of previously diluted urine (corresponding to 1 or 2 cc. of the original urine). With urines extraordinarily poor in ammonia it may be necessary to use more urine (5 cc.), but, in so far as it is practicable, it is better not to use more than 2 cc. Our reason for not wishing to use more than 2 cc. of urine is based partly on practical experience, and partly on the recognition of the fact that the salts in the urine tend to prevent the ammonia absorption from being quantitative. Rinse down the added urine by means of a little water (1 to 5 cc.), and shake gently but continuously for 5 minutes. Rinse the powder to the bottom of the flask by the addition of water (25 to 40 cc.), and decant. Add water once more and decant. (In the case of urines rich in bile it is advisable to wash once or twice more.) Add a little water to the powder, introduce 1 cc. of 10 per cent sodium hydroxide, shake for a few moments, and set aside, while preparing the standard ammonium sulfate solution as follows:

Transfer 5 cc. of the standard ammonium sulfate solution (p. 139), containing 0.5 mg. of nitrogen, to another 100 cc. volumetric flask and add 1 cc. of 10 per cent sodium hydroxide (to balance the alkali added to the permutit mixture in the other flask). Add also 0.2 cc. of gum ghatti solution (see p. 269). Dilute to about 75 cc. and mix. Transfer 10 cc. of Nessler's solution (see p. 337) to a measuring cylinder. Now give the volumetric flask a vigorous whirl so as to set the solution spinning within the flask and add at once the whole of the Nessler solution in the cylinder. With another whirling movement secure the complete mixing of the contents in the flask. If the process of Nesslerization has been successful, a deep red but *crystal clear* solution is obtained. If it is not perfectly clear, throw it away and prepare a fresh standard. With a little experience no trouble is encountered in getting clear solutions. When the standard solution is

thus satisfactorily Nesslerized, add 0.2 cc. gum ghatti solution to the contents in the flask containing the permutit and the urinary ammonia, dilute to about 75 cc., whirl the mixture and add the Nessler reagent (10 cc.) exactly as in the case of the standard solution. Dilute the contents of both flasks to volume (100 cc.) and make a quantitative color comparison by means of the colorimeter.

The ammonia content is inversely proportional to the colorimeter reading, provided that one is not more than one and one-half times as strong as the other. Ten, divided by the reading of the unknown, in mm., gives the ammonia nitrogen, in mg., in the volume of urine (or ammonium sulfate solution) taken for the analysis.

Calculate the 24-hour quantity of the ammonia as NH_3 and as ammonia-N, and compare with the figures given by the macro aeration method.

Note. Concerning the direct "formol titration" of ammonia in urine see comment on p. 255.

Total Nitrogen Determination. — A. *Modified Kjeldahl Method.* This standard old method, as applied here to urine, is described and explained in a preceding section. See page 65.

B. *Colorimetric Method.* — *Micro Kjeldahl Method.* — The regular macro Kjeldahl method is used for the determination of from 5 to over 50 mg. of nitrogen, while the micro method is used for the determination of 0.7 to 1.5 mg. in urine (or 0.03 to 0.7 mg. in blood). The preliminary digestion process is in principle the same in the micro method as in the macro method, in that all the nitrogen present is first converted into ammonium sulfate.

Transfer 5 cc. of concentrated urine, or 10 cc. of dilute urine, to a 100 cc. volumetric flask, dilute to volume and mix well. Rinse a clean small flask with the diluted urine and then pour into it 20 to 30 cc. The Folin-Ostwald pipettes can not be inserted in most small volumetric flasks. With such a pipette transfer 1 cc. to a large pyrex test-tube, not forgetting that the last step in this transfer is to blow out what is left in the tip of the pipette. With an ordinary 1 cc. pipette add 1 cc. of the concentrated mixture of sulfuric acid and phosphoric acid described on pp. 65–67. Add a pebble to prevent bumping and heat over a

micro burner until the water is driven off and the white fumes become abundant within the tube. This should take place in about two minutes. When filled with fumes close the mouth of the tube with a watch glass and continue the heating with a small flame, at such a rate that the tube remains nearly filled with the fumes, yet almost none escape. Within two minutes after the mouth of the test-tube was closed the liquid contents should become clear and bluish or light green in color. The clearness of the solution does not necessarily signify that all the organic nitrogen has been transformed into ammonium sulfate, and the total heating period after closing the mouth of the tube should not be less than two minutes. Excessive heating is undesirable, especially the use of too strong a flame, because it etches the glass and yields insoluble silica. Remove the flame and let cool for a little less than two minutes. Then add water and rinse the hot digestion mixture into a 200 or 250 cc. volumetric flask with about 150 cc. of water.

Introduce into another 200 or 250 cc. flask 10 cc. of the diluted standard ammonium sulfate solution (page 139) and add 1 cc. of the same sulfuric acid-phosphoric acid mixture as was used for the digestion, together with about 140 cc. of water. Add to each flask a few drops of gum ghatti solution, mix, and with a 50 cc. cylinder add 30 cc. of Nessler's reagent. It is desirable that each flask be given a whirl just before pouring in the Nessler solution so as to secure a rapid mixing. Shake just a little more before diluting to volume and making the final mixing.

The resulting red solutions should be crystal clear unless the digested unknown may be turbid because of excessive heating. If it is turbid, a portion of it must be centrifuged in two clean centrifuge tubes before it is used for the color comparison. In connection with this centrifuging, do not fail to inspect the sediment. If it is reddish, instead of white, it has carried down some of the ammonia and the results obtained will tend to be too low. Unless the conditions have been grossly unsuitable, such a predicament can not occur when the Nesslerization is made in the presence of a little gum ghatti (see page 269).

Calculation.—$\frac{20}{x}$ = the nitrogen, expressed as mg., present in the fraction of a cubic centimeter of undiluted urine used for the determination.

Determine the nitrogen in 5 cc. of the same (undiluted)

urine by the macro Kjeldahl method (page 65). The two methods should give substantially identical values, in grams per liter.

Reactions of Urea.—Put a crystal of urea on a glass slide or a watch-glass and cover it with a drop of water. With a glass rod, put a drop of nitric acid next to this. Let the two drops run together, and notice the precipitation of urea nitrate at the junction. Examine under the microscope, and sketch the crystals.

Put a few crystals of urea into a dry test-tube, and heat till they melt. With moist litmus paper test the reaction of the fumes given off. Explain.

Dissolve a few crystals of urea in 5 cc. water in a test-tube. Test its reaction with litmus paper. Heat the solution to boiling and test the steam with moist litmus paper. Cool the liquid and test with litmus paper. Explain.

To a solution of urea in a test-tube add an equal volume of sodium hypobromite solution. Make this by mixing and cooling equal volumes of bromine solution and 40 per cent sodium hydroxide solution.

The reaction with sodium hypobromite has been used for the quantitative determination of urea, but as ordinarily used for this purpose the method has very little value.

Colorimetric Method for Determination of Urea.—Urea, as indicated in qualitative tests given above, is easily decomposed to a certain extent when its solutions are heated. The decomposition with ammonia formation begins at about 60° C. But the quantitative decomposition of urea can be attained only by boiling for a long time (hours) or by heating to very high temperatures (150° C.) under pressure. Since the discovery that soy beans and particularly jack beans are very rich in a urea splitting enzyme, urease, the older chemical methods for hydrolyzing urea have been almost completely abandoned and replaced by urease methods. Jack bean powder is much better than soy bean meal, not only because it contains more urease, but also because it contains less oil.

A rather unstable but temporarily very serviceable urease preparation is made as follows:

Transfer 84 cc. of water and 16 cc. of ordinary 96 per cent alcohol to a 250 cc. flask, add 5 gm. of jack bean meal, shake gently but continuously for 10 to 15 minutes, and filter. The

filtrate contains nearly all of the urease and very little other material. The solution will remain serviceable for a few days at room temperatures, and for about a month in a refrigerator. For the making of more stable urease preparations (urease paper) see pp. 277–279.

Buffer Mixtures for Urease Decompositions.—The speed with which urease decomposes urea depends to a very great extent upon the reaction, pH, of the solution, but probably also upon some other factors. The most efficient buffer mixture is probably that obtained by dissolving 14 gm. of sodium pyrophosphate ($Na_4P_2O_7$, $10H_2O$) in 100 cc. of one-third molar solution of phosphoric acid.

The most commonly used buffer is that obtained by dissolving 6.9 gm. of monosodium phosphate and 17.9 gm. of crystallized disodium phosphate in 50 cc. of warm water and diluting to 100 cc.

The most efficient buffer mixture, next to that obtained from pyrophosphate and phosphoric acid, and the most easily prepared, contains 15 gm. of crystallized sodium acetate and 1 cc. of glacial (99 per cent) acetic acid in 100 cc. of solution.

The Method.—Transfer with a Folin-Ostwald pipette 1 cc. of diluted urea solution or urine, diluted as for the total nitrogen determination, to a *chemically* clean test-tube; add 1 or at the most 2 drops of buffer solution and 1 cc. of urease solution. Digest in a beaker of warm tap water (40° to 55° C.) for 5 minutes, or at room temperature for 15 minutes. Then rinse the contents into a 200 or 250 cc. volumetric flask with about 150 cc. of water.

Transfer 1 mg. of nitrogen in the form of ammonium sulfate to another similar volumetric flask; to this standard add 1 cc. of urease solution and dilute to about 150 cc. Add to each flask a few drops of gum ghatti solution and shake gently for a few seconds. Then add with shaking (with a cylinder) 20 cc. of Nessler solution to each. Dilute to volume and make the color comparison, never omitting first to read the standard against itself.

The height of the standard (usually 20 mm.) divided by the height of the unknown gives the nitrogen, in mg., present in the fraction of a cc. of undiluted urine contained in the 1 cc. of diluted urine taken for the analysis.

Unless the colorimetric reading is between 14 mm. and 30 mm., the determination should be repeated with 1 cc. of urine so diluted as to give readings coming within those limits. Calculate the nitrogen (urea plus ammonia) and subtract the preformed ammonia-N.

A few explanatory remarks may be added. Many kinds of biological nitrogenous materials, particularly amino acids, peptones, and proteins, prevent the development of the color reaction given by ammonia and Nessler's reagent. This was first discovered in attempts to determine by direct Nesslerization the ammonia formed in pancreatic digestion mixtures. If very little such nitrogenous material is present the result obtained is deceptive, for then the color is merely diminished and the error will not be detected. The careful observer will find, however, that in such cases the color obtained tends to be visibly more greenish and less distinctly red than the standard. Because of the serious interference caused by albuminous materials it may be thought that the procedure described above is not applicable to albuminous urines, but a series of determinations have shown that even urines very rich in albumin have, in fact, so little in comparison with the amount of urea present that correct results are invariably obtained by direct Nesslerization.

Because of the extremely low nitrogen content of our urease preparation it is not really essential that the urease should also be added to the standard ammonia solution, but we have thought it best to recommend that it be added simply as a precaution against the possible occurrence of less good urease preparations.

The reason why the urease decomposition had best be made in test-tubes rather than in volumetric flasks is to avoid failure due to the use of flasks which have been used for Nesslerization purposes. Such flasks may look perfectly clean, but, unless they have been rinsed with nitric acid, they will contain enough mercury compounds to destroy entirely the urease, and scarcely a trace of ammonia will be obtained.

Compare the urea-N value of the urea solution with that obtained by the macro Kjeldahl process.

Colorimetric Determination of Uric Acid in Urine.—Practically all modern methods for the determination of uric acid in urine (and in blood) are based on the color reaction first used by Folin for such determinations, in 1912 (*J. Biol. Chem.*, 12:239; 13:363; 1912–1913). Many modifications and improvements have been worked out during the intervening 20 years, and some unsuspected sources of error have been discovered and corrected. The uric acid reagent, a phosphotungstic acid (phospho-18-tungstic acid) is really an oxidizing reagent which, when reduced, yields a deep blue solution suitable for

quantitative measurements. It was inevitable that such a reaction could not be entirely specific for one particular reducing substance, uric acid, and it was recognized from the beginning that urine contains other substances (poly-phenols) capable of reducing the uric acid reagent. The reaction is nevertheless fairly selective and many reducing substances which might interfere, such as glucose, fructose, creatinine and creatine, do not spoil the determination. In addition to positive sources of error, as exemplified by the poly-phenols, there are negative sources of error, exemplified by the amino acids, which for some as yet unknown reason greatly cut down the color obtainable from uric acid. It is probably more than a coincidence that these sources of loss in color in the uric acid determination are to a large extent the same materials which diminish the color reaction between ammonia and Nessler's reagent (see page 153). In the light of these various sources of error it is clear that strictly dependable uric acid values can be obtained only by first precipitating the uric acid by some more or less specific precipitant and thus separate it from the disturbing materials. On the other hand, by a series of happy innovations, the color obtainable from a given minute quantity of uric acid has been increased so much that the disturbing effects of the interfering substances have been much diminished, though by no means entirely eliminated.

The methods described below were published in 1933 (*J. Biol. Chem.*, 101 : 111).

The second, "direct," method in which the uric acid is not separated from the interfering materials is given, partly so that each worker can determine for himself how much of an error is involved, and partly because that determination represents exactly the method recommended in the same paper for the determination of the uric acid in unlaked blood filtrates, where it does yield correct values.

CAUTION.—A very strong and very dangerous sodium cyanide reagent is used in these determinations. Do not spill. Pour all solutions containing the cyanide directly into the drains of the sinks, and flush the sinks with cold water immediately.

Indirect Method.—Two solutions, in addition to those used in blood analysis, are required; both keep indefinitely.

1. A 5 per cent solution of silver nitrate. This solution, even if perfectly clear when first prepared, may develop a slight color on standing. This color is most quickly produced by heating to 100° C. for 2 hours in a flask covered with a beaker. After cooling, add a few cc. of a solution containing 50 mg. of sodium chloride, shake thoroughly, and filter through a double layer of quantitative filter paper until crystal clear. Thereafter the solution will remain perfectly colorless and need not be kept in brown bottles.

2. A solution containing 1 per cent of sodium chloride, 2 per cent of crystallized sodium acetate and 1 volume per cent of concentrated acetic acid (99 per cent).

Half fill a 100 cc. volumetric flask with water. With a Folin-Ostwald pipette introduce 1 cc. of the urine. Add 10 cc. of the chloride-acetate solution and then, without shaking so as to avoid foaming, dilute to the mark with water and mix.

From this diluted urine transfer 5 cc. and 3 cc., plus 2 cc. of water, to 15 cc. centrifuge tubes. Add to each 3 cc. of the silver nitrate solution and centrifuge at once fairly rapidly for 4 to 5 minutes so as to get perfectly clear supernatant solutions. A few tiny flakes may float on the surface, but these contain no uric acid. Decant and drain over a sink. It is permissible to let cold tap water rinse the mouths of the tubes during the draining. With a 25 cc. cylinder or a burette add to each tube 10 cc. of the urea-cyanide solution described on page 295. Stir immediately (and simultaneously) with glass rods until the two sediments have completely dissolved. Transfer the silver cyanide solutions to test-tubes graduated at 25 cc., and rinse with exactly 5 cc. of water. Mix by whirling at an angle of about 60° until the solutions are visibly uniform. In another graduated test-tube place 5 cc. of the standard uric acid solution containing 0.02 mg. of uric acid (see page 289) together with 10 cc. of the urea-cyanide solutions and mix.

With a 10 cc. blood pipette add to each of the three tubes 4 cc. of the uric acid reagent described on pp. 291–293, and let stand for 15 to 25 minutes. Dilute to volume, mix, and make the color comparison between the standard and the unknown which is nearest to it in depth of color. When the standard is set at 20 mm., colorimetric readings between 35 mm. and 10 mm. are acceptable.

Direct Method.—Half fill a 100 cc. volumetric flask with water. With a Folin-Ostwald pipette introduce 1 cc. of urine, dilute to volume and mix. Introduce into test-tubes graduated at 25 cc., 5 cc. of the diluted urine and 3 cc. of the undiluted urine plus 2 cc. of water. To another graduated test-tube add 5 cc. of the standard uric acid solution. Add 10 cc. of the urea-cyanide solution to each, mix, and add 4 cc. of the uric acid reagent. Let stand for 15 to 25 minutes. Dilute to volume, mix, and make the color comparison between the standard and the unknown which is nearest to it in depth of color.

Calculation.— $\frac{20}{x}$ times 0.02 gives the quantity of uric acid, in milligrams, present in the volume of urine (0.03 cc. or 0.05 cc.) used for the analysis.

Note.—No method is known for the accurate determination of the uric acid in urines which contain much bile. If uric acid determinations must be made on such urines the direct method is apt to be as nearly correct as the indirect method.

Colorimetric Determination of Creatinine.—This is the only known method for the determination of creatinine in urine. It is based on the fact that so far as is known no other substance in normal human urine or in ordinary pathological urines, except creatinine, reacts with picrates in alkaline solutions to give, at room temperatures and in a few minutes, an intense red color which is stable and therefore can be measured with a colorimeter. As the method was formerly used, acetoacetic acid, and to a lesser extent acetone, interfered with the determination, partly by giving a color and partly by inducing rapid fading. These disturbing side reactions arose from the fact that the original method was based on the use of about 10 mg. of creatinine, and 10 mg. gave a maximum color only in the presence of a large excess of alkali. When the reaction was adapted to the use of only 1 to 2 mg., or less, together with a minimum excess of alkali, the difficulties disappeared.

The unique specificity of this color reaction seemed always somewhat remarkable on the basis of the old interpretation of the reaction as one of reduction and oxidation. But if Greenwald's recent interpretation of the reaction is correct, the specificity becomes understandable. The color, according to Greenwald, is due to the formation of a deep red tautomeric form of

creatinine picrate and involves no reduction of picrate or oxidation of the creatinine (*J. Biol. Chem.*, 1924, 59: 601). This color reaction suffers from one minor practical drawback; the color is so brilliant that it is rather difficult to read with precision by the kind of artificial light which is suitable for other color comparisons.

The method as described here provides for the use of either picric acid (in 1 per cent solution) or sodium picrate (in 1 per cent solution). The reasons for giving such a choice are given on pp. 285–287.

With a Folin-Ostwald pipette transfer 1 cc. of standard creatinine solution (containing 1.61 mg. creatinine zinc chloride or 1 mg. creatinine per cc.) to a 100 cc. volumetric flask. Rinse the pipette first with water, then with the urine, and then transfer with the same pipette 1 cc. (or in the case of very dilute urine 2 cc.) to another 100 cc. flask. Add to each flask 1.5 cc. of 10 per cent sodium hydroxide and 20 cc. of 1 per cent picric acid solution; or add 1 cc. of the alkali and 20 cc. of 1 per cent sodium picrate solution. Shake a moment. Let stand for 10 to 15 minutes, dilute to volume, mix and make the color comparison in the usual manner, not forgetting first to read the standard against itself.

As already indicated, this color comparison is more difficult than any other in common use. Diffuse, ordinary day light is best.

Instead of using 10 per cent sodium hydroxide, equivalent amounts of a weaker alkali, such as half normal sodium hydroxide, may be used.

$\frac{20}{x}$ = the creatinine, in mg., in the volume of urine taken for the analysis.

The administration of relatively large amounts of creatinine has come into vogue in recent years as a renal function test. In these circumstances the urines may be very rich in creatinine, and the method for its determination must be modified to a certain extent. Several different modifications may be used, but when sodium picrate is used, the simplest modification is to rinse the deep colored unknown into a larger volumetric flask (200, 250 or even 500 cc.) and in connection with this dilution, to add more picrate solution before adjusting to volume. No extra

alkali is called for. 5 mg. of creatinine in a 500 cc. flask, on adding 80 cc. of sodium picrate solution and diluting to volume, give exactly the same depth of color as the 1 mg. standard in a 100 cc. flask.

Creatine.—(*J. Biol. Chem.*, 17:469)—Unless considerable meat or fish has been eaten, the urine of normal adults contains only traces of creatine. Urines of children and of sick persons, particularly fever patients, appear, on the other hand, to contain relatively considerable quantities of creatine (0.2 gm. to 1 gm. or more per day in fever patients).

Creatine is determined in such urines by first converting it into creatinine. This is done as follows:

Measure the urine (usually 1 cc.) into a flask (capacity 300 cc.) and add 20 cc. saturated picric acid solution (or 20 cc. of sodium picrate solution plus 2 cc. of normal hydrochloric acid). Weigh flask and contents and add about 150 cc. water. Boil gently for 45 minutes, then more rapidly until the original volume (determined by weighing) is obtained (a variation of 3 or 4 gm. makes no appreciable difference). Cool. Add 1.5 cc. 10 per cent sodium hydroxide, let stand 10 minutes, rinse the solution into a 100 cc. volumetric flask, dilute to volume, and compare, as in the case of preformed creatinine, with the color obtained from 1 mg. creatinine.

Twenty divided by the reading gives the sum of the creatine and creatinine present.

Calculate the total quantity and subtract the preformed creatinine.

If an autoclave is available, the conversion into creatine can be made more rapidly.

Measure the urine (1 cc.) into a 100 cc. volumetric flask, and add 20 cc. saturated picric acid solution (or 20 cc. of sodium picrate solution plus 2 cc. of normal hydrochloric acid). Cover the mouth of the flask with tinfoil, and heat in the autoclave at 115° C. to 120° C. for 20 minutes. Cool, add 1.5 cc. sodium hydroxide, and finish the determination in the usual manner.

Hippuric Acid.—Take with the evening meal 2 gm. of sodium benzoate, and collect the urine until next morning.

Evaporate to small volume and transfer with a little wash water to a small flask. Acidify strongly with sulfuric acid and

put away for twenty-four hours. Filter and dry the precipitate, consisting of hippuric acid, uric acid, and other substances. Extract the hippuric acid with acetic ether. Set aside for spontaneous evaporation. Examine microscopically. Heat the dry substance in a dry tube, and note the odor of bitter almonds (benzaldehyde).

Determination of Sulfur in Urine by Titration.—(Fiske, *J. Biol. Chem.*, 47: 59.)—Transfer to a 100 cc. volumetric flask sufficient urine to contain between 10 and 20 mg. of sulfur in the form of inorganic sulfate, and dilute to about 50 cc. with water. (Ordinarily, an amount of urine corresponding to a 30-minute period will be suitable. When smaller volumetric flasks are available, proportionately less urine may be used.) Add 1 drop of phenolphthalein solution, followed by concentrated ammonium hydroxide, drop by drop, until the contents become faintly pink. Now add 10 cc. of a 5 per cent solution of ammonium chloride and about 1.5 gm. of finely powdered basic magnesium carbonate (to remove the phosphate). Make up to the mark, stopper, shake for 1 minute, and let stand for half an hour. Filter, through a dry paper, into a dry flask or bottle. This filtrate is used for all three sulfur determinations.

Inorganic Sulfate.—Pipette 5 cc. of the filtrate into a large lipped pyrex test-tube. Add 2 drops of a 0.04 per cent alcoholic solution of bromphenol blue and 5 cc. of water. Then add approximately N HCl, drop by drop, until the solution is yellow without a trace of blue. Run in 2 cc. of benzidine reagent,[1] and let stand for 2 minutes. Finally, add 4 cc. of 95 per cent acetone, and let stand for 10 minutes more. Meanwhile prepare a thin mat of paper pulp in the bottom of the special filtration tube provided for the purpose (Fig. 7, p. 247), wash with water, and suck dry. On this mat, filter the benzidine sulfate precipitate with very gentle suction. Wash by rinsing down the sides of the pyrex test-tube with 1 cc. of 95 per cent acetone and transferring to the filtration tube; wash twice more with 1 cc. of acetone, and once with 5 cc.

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[1] Suspend 4 gm. of benzidine in about 150 cc. of water in a 250 cc. volumetric flask. Add 50 cc. of N HCl. Shake until dissolved, and dilute to the mark. Filter if necessary.

With the aid of a wire and a little water, poke the precipitate and mat through the hole in the bottom of the filtration tube back into the pyrex test-tube. Rinse off the wire with a few drops of water, and titrate the contents of the test-tube (hot) with 0.02 N NaOH,[2] after adding 2–4 drops of a 0.05 per cent aqueous solution of phenol red. In order to be sure that all the precipitate is removed from the filtration tube, the latter should be left suspended in the mouth of the test-tube until about 1 cc. of alkali has been run in (through the filtration tube). The tube can then be rinsed with 2 or 3 cc. of water from a wash bottle, and further washed by boiling the solution until steam escapes from the mouth. After another rinsing with a few cc. of water, the filtration tube may be removed, and the titration completed by running the alkali directly into the test-tube, until 0.02 cc. produces a definite pink color that remains after boiling again.

Benzidine is an extremely weak base, and benzidine sulfate behaves in this titration as if it were an equivalent amount of sulfuric acid. The amount of inorganic sulfur in the 5 cc. of filtrate analyzed is obtained (in mg.) by multiplying the titration figure by 0.32.

Total Sulfate.—To 5 cc. of the same filtrate in a 100 cc. beaker add 1 cc. of 3 N HCl (approximate). Heat on a water bath until the solution has evaporated to dryness, and for 10 minutes longer. Transfer the contents of the beaker to a lipped pyrex test-tube with five 2 cc. portions of water, add 2 cc. of benzidine reagent, and proceed as in the method for inorganic sulfate. The calculation is the same as before.

Total Sulfur.—Transfer about 0.25 cc. of Benedict's total sulfur reagent[3] to a small evaporating dish (6 cm.), and add 5 cc. of urine filtrate. Evaporate cautiously to dryness on a wire gauze or hot plate. Gradually increase the heat, as in the gravimetric method, and finally ignite for 2 minutes directly over the flame (at red heat). Cool for 5 minutes. Add 1 cc. of 3 N HCl and evaporate to dryness (until the green color has changed to

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[2] Made by diluting 20 cc. of 0.1 N NaOH to 100 cc. A micro-burette should be used for the titration.

[3] 20 gm. of copper nitrate crystals and 5 gm. of potassium chlorate per 100 cc.

brown). Transfer to a lipped pyrex test-tube with five 2 cc. portions of water, add 1 drop of N HCl and 2 cc. of benzidine reagent. The rest of the determination is the same as in the two other methods, except that the first cc. of acetone for washing should be replaced by 2 cc. of 50 per cent acetone.

Gravimetric Determination of Inorganic Sulfate. — (*J. Biol. Chem.*, 1 : 131.)—Transfer to a 250 cc. beaker 25 cc. of the ammonium sulfate solution in which the ammonia was determined (p. 63). Dilute with water to about 100 cc.; add 10 cc. of 20 per cent sodium chloride solution and 5 cc. of concentrated hydrochloric acid.

The addition of sodium chloride is necessary only when the sulfate determination is to be made in ammonium or potassium sulfate solutions. Such solutions give too low sulfate values unless sodium chloride is added. The reason is rather obscure, but in general it may be stated that the sulfate precipitate obtained is practically never pure $BaSO_4$, but by adding sodium chloride the precipitate obtained has been found, empirically, to give the weight corresponding to $BaSO_4$. Urine contains so much more of sodium than of ammonium and potassium salts that in urine analysis the addition of sodium chloride is not required.

Fill a burette with 5 per cent barium chloride solution and place the beaker containing the sulfate solution under it so that the burette will deliver on the side (the spout) of the beaker. Add thus, drop by drop, 10 to 15 cc. of barium chloride. The contents in the beaker must not be stirred or agitated to any degree while the barium is added. If $BaSO_4$ is formed too rapidly in the cold, much "occlusion" takes place, and, in addition, the precipitate is apt to be so fine that it will pass through when filtering. After the beaker has stood for 10 to 30 minutes mixing the contents of the beaker will do no harm and must be done to complete the precipitation. Let stand for 1 to 24 hours, according to convenience, before filtering. (The ethereal sulfates are not hydrolyzed in the cold.)

Prepare an asbestos mat as follows: Fill the Gooch crucible with the freshly shaken asbestos suspension once, or at the most twice. Pack the asbestos down by vigorous suction (water pump). The mat should be about 1 mm. thick. Wash the asbestos with water and moderate suction until the filtrate is free from asbestos particles, and water-clear. Make as dry as possible by suction. Heat very gently at first (not over 100° C.) to drive off

the steam without mechanical disturbance of the mat; then ignite; cool for 15 to 20 minutes, and weigh. The same mat can be used for several successive sulfate determinations, provided that adequate care is taken not to disturb the mat either by too rapid use of water when filtering or by steam during the heating.

Transfer the barium sulfate to the Gooch crucible and wash 6 to 10 times with distilled water. Heat to dryness, ignite, cool, and weigh. From the weight of the $BaSO_4$ obtained, calculate the ammonium sulfate and compare with the weight known to be present. Repeat with 25 cc. of urine to which has been added about 75 cc. of water and 5 cc. of hydrochloric acid. (No sodium chloride need be added when working with urine.) Calculate the result as S.

Gravimetric Determination of Total Sulfate.—In this determination the ethereal sulfates must be hydrolyzed by hydrochloric acid and heat before barium chloride is added. Transfer 25 cc. of urine to a beaker, add about 25 cc. of water and 5 cc. of concentrated hydrochloric acid. Cover with a watch glass and boil gently for 20 to 30 minutes. Then dilute to about 100 cc., heat to boiling, and with a pipette add 10 cc. of 5 per cent barium chloride solution. Let stand for an hour, or as much longer as may be convenient. Filter, wash, ignite, cool, and weigh, as in the case of the inorganic sulfate determination.

Calculate the S and, by subtraction of the corresponding values obtained for inorganic sulfates, calculate the S present in the form of ethereal sulfates.

Gravimetric Determination of Total Sulfur.—(Benedict, *J. Biol. Chem.*, 6: 363; Denis, *J. Biol. Chem.*, 8: 401.)—Transfer 25 cc. of urine to a porcelain dish (diam. 10 to 12 cm.); add 5 cc. of a solution containing 25 per cent of copper nitrate and 25 per cent of sodium chloride, and 10 per cent of ammonium nitrate. Evaporate to dryness on the water bath. Then heat over a flame, preferably over a Fletcher burner; the burners attached to the copper condensers used in Kjeldahl determinations are also good. The heat should be very moderate at first and should then be gradually increased until the dish becomes almost red hot; continue the vigorous heating for 10 minutes so as to decompose and drive off all of the nitric acid fumes. The organic

matter, including the sulfur compounds, is thus oxidized, but the sulfates formed do not escape; they are held back as copper sulfate. The sodium chloride present in the oxidizing nitrate mixture serves to prevent mechanical losses due to the explosive violence with which the oxidations are apt to occur without the presence of the sodium chloride. Allow the dish to cool. Add 20 cc. of 10 per cent hydrochloric acid and warm quickly. Filter the dissolved contents into a beaker, using for this purpose 75 to 100 cc. of hot water. Heat to boiling and add slowly 15 cc. of 5 per cent barium chloride solution. Let stand an hour, or longer, filter on the asbestos mat, wash, ignite, cool, and weigh.

Calculate the S, and by subtraction of the S found as total sulfates, calculate the values corresponding to the "neutral" or "unoxidized" sulfur.

Colorimetric Method for the Determination of Inorganic Phosphate in Urine.—(Fiske and Subbarow, *J. Biol. Chem.*, 66: 375.)—This method depends upon the selective reduction of phosphomolybdic acid with the formation of a blue color. If molybdic acid is added to a solution of phosphoric acid (i.e., an acidified solution of phosphate), phosphomolybdic acid is formed. For accurate results, the reagent used to produce the blue color must reduce phosphomolybdic acid rapidly, and yet give no trace of color with molybdic acid alone. Wide range of proportionality between color intensity and phosphate content, and lack of sensitiveness to variations in acidity and to certain interfering impurities (nitrate, nitrite, iron, silicate, etc.) are other important considerations. The most satisfactory reducing agent is aminonaphtholsulfonic acid.

Method.—Measure 1 to 5 cc. of urine into a 100 cc. volumetric flask; to another similar flask transfer 10 cc. of standard monopotassium phosphate solution containing 0.4 mg. of phosphorus (0.1755 gm. KH_2PO_4 per liter). To each flask add water enough to make the total volume 70 cc., followed by 10 cc. of molybdic acid solution (2.5 per cent ammonium molybdate in 5 N sulfuric acid) and 4 cc. of 0.25 per cent aminonaphtholsulfonic acid (see below). (Mix by gentle shaking after the addition of each reagent.) Dilute to the mark, mix, and read in the colorimeter after 3 or 4 minutes, setting the standard at 20 mm.

AMINONAPHTHOLSULFONIC ACID.—This is the 1,2,4-acid, the preparation of which is described on pages 323–327 (Nos. 1 to 8); when made by that process for use in phosphate determinations, it should finally be washed with alcohol as long as any color is removed. The purified product is now on the market (Eastman Kodak Co.), and is also readily obtained by recrystallization of "technical" aminonaphtholsulfonic acid from the same source, as follows: Heat 1000 cc. of water to about 90° C., and dissolve in it 150 gm. of sodium bisulfite and 5 gm. of sodium sulfite. To this mixture add 15 gm. of the crude sulfonic acid, and shake until all but the amorphous impurity has gone into solution. Filter the hot solution through a paper large enough to hold it all, cool the filtrate thoroughly under the tap, and add to it 10 cc. of concentrated hydrochloric acid. Filter with suction, wash with about 300 cc. of water, and then with alcohol until the washings are colorless. The product, when dry, should be kept in a brown bottle.

To prepare the reagent, mix in a 100 cc. Erlenmeyer flask 14.25 gm. of Merck's Reagent sodium bisulfite, 0.25 gm. of aminonaphtholsulfonic acid, and 80 cc. of water. Add 10 cc. of 5 per cent sodium sulfite (anhydrous, Merck's Reagent), and shake. In case the sulfonic acid does not all go into solution (disregarding any traces of amorphous impurity that may be present), add more of the sulfite solution, 0.2 cc. at a time, until the reagent dissolves completely after thorough shaking. Dilute to 100 cc.

Once the proper proportions of sulfite and bisulfite have been determined in this manner, a large quantity of dry reagent mixture may be prepared in a few minutes: In a *large* mortar mix 142.5 gm. of sodium bisulfite, 2.5 gm. of powdered aminonaphtholsulfonic acid, and 5 gm. of anhydrous sodium sulfite (or a proportionately larger quantity if more than 10 cc. of 5 per cent solution was required in the preliminary test). The constituents must be thoroughly mixed together with a pestle, but should not be ground. The dry mixture keeps indefinitely in a brown bottle.

50 cc. of the reagent solution may now be readily prepared by dissolving 7.5 gm. of this dry mixture in 47 cc. of water. Since exposure to the air is the chief cause of deterioration, the solution is best kept in a stoppered 50 cc. cylinder. A fresh supply should be made each week.

Acidity of Urine.—(See *Am. J. Physiol.*, 1903, 9: 265, and 1905, 13: 102.)

Nearly all of the titratable acidity of urine is due to acid phosphate. The end-point of this titration is not very sharp, partly on account of the color of the urine (compare p. 127) and partly because of the presence of ammonium salts. The titration is further complicated by the presence of calcium, for when alkali is added to a mixture of phosphate and calcium some tribasic phosphate is precipitated. In the presence of sodium or potassium oxalate the premature formation of tribasic salt is prevented. But, on account of the ammonium salts, there is still danger of over-titrating and the first distinguishable coloration should be taken as the end-point. With phenolphthalein as indicator titrate the acidity of 1 per cent monopotassium phosphate solution (20 cc.).

Repeat after having added 5 cc. of neutral calcium chloride solution (2 per cent).

Repeat after having added 5 cc. of the calcium chloride solution and 5 cc. of saturated neutralized potassium oxalate solution.

Note.—For accurate work 15 gm. of solid neutral potassium oxalate should be used instead of the oxalate solution prescribed, but it is rather difficult to obtain strictly neutral oxalate.

Transfer 20 cc. of undiluted urine to a small flask or beaker, add 5 cc. of the oxalate solution. With another sample of the same urine in another flask or beaker as a guide, and with phenolphthalein as indicator, titrate until the oxalated urine becomes a shade darker than the other.

Calculate the acidity for the whole urine in terms of tenth normal acid.

Calculate the phosphate of the same urine in terms of tenth normal phosphoric acid, but considering the H_3PO_4 as a monobasic acid. Compare the two values.

Compare also the acidity and the phosphate with the ammonia, expressed in cc. of tenth normal solution. The ammonia usually varies more or less directly with the acid phosphate (and the acidity).

Determination of Chloride.—STANDARD SILVER NITRATE SOLUTION.—This is prepared by dissolving 23.94 gm. silver nitrate per liter of solution (or 5.99 gm. in 250 cc.).

Standard Ammonium Thiocyanate Solution.—Dissolve 6 gm. of the salt in 800 to 900 cc. water. Transfer 10 cc. of the silver solution to a beaker or flask; add 50 cc. water, 5 cc. concentrated nitric acid, and 2 cc. of saturated ferric ammonium sulfate solution. By means of a burette, titrate the acidified silver solution with the thiocyanate solution. On the basis of the result, dilute a part of the thiocyanate solution so as to give 500 cc. (or a liter) of a solution, 20 cc. of which is exactly equivalent to 10 cc. of the silver solution.

Each cc. of the silver solution corresponds to 5 mg. chlorine (or to 8.23 mg. sodium chloride).

The chlorine determination in urine is carried out as follows:

Pipette 10 cc. of urine into a 100 cc. volumetric flask, add 50 cc. distilled water, 5 cc. saturated ferric alum solution, and 5 cc. concentrated nitric acid. Add 20 cc. standard silver nitrate solution, fill up to the mark with distilled water, and shake. Filter through a dry filter into a dry beaker or flask. With a clean, dry pipette transfer 50 cc. of the filtrate to another beaker, flask, or evaporating dish, and titrate in the same manner as when standardizing the silver solution.

Since the thiocyanate solution is half as strong as the silver solution, and since only one-half of the surplus silver was taken for titration, 20 minus the thiocyanate titration figure represents the silver nitrate which has combined with the chlorine of the urine to form silver chloride. This figure multiplied by 5, or by 8.23, gives the chlorine, or sodium chloride, (in milligrams) present in 10 cc. of urine.

Calculate the twenty-four hour quantity.

Simplified Chloride Determination.—The removal of the silver chloride precipitate in the preceding method is generally conceded to be necessary, because of the fact that a part of the silver chloride is converted into silver thiocyanate during the titration, unless thus removed. The error due to this side reaction when the chloride is not removed is a loss of about 0.05 cc. of thiocyanate for 5 cc. of urine, a loss amounting to a deficit of about 0.1 gm. of sodium chloride per liter of urine.

Standard Silver Nitrate.—Dissolve 7.28 gm. of silver nitrate and dilute to a volume of 250 cc. One cc. is equivalent to 10 mg. of NaCl.

INDICATOR.—To 100 gm. of ferric ammonium sulfate add 100 cc. of water and 200 cc. of concentrated nitric acid. Five cc. of the resulting solution is taken for each titration.

STANDARD AMMONIUM THIOCYANATE.—Dissolve 4 gm. of ammonium thiocyanate in 200 cc. of water and mix. Transfer 10 cc. of the standard silver nitrate solution to a beaker, add 20 cc. of distilled water and 5 cc. of the indicator. Fill a burette with the thiocyanate solution, and titrate with constant stirring until the characteristic reddish end-point is reached. On the basis of the figure obtained, prepare 200 or 250 cc. of ammonium thiocyanate solution, which is equivalent to the standard silver nitrate solution.

With a pipette transfer 5 cc. of urine to a beaker, add 20 cc. of water, 5 cc. of indicator, and finally (with a pipette) 10 cc. of silver nitrate solution. While stirring with a glass rod, titrate the surplus silver with the standard thiocyanate solution until the first faint but unmistakable brown or reddish coloration is obtained. On standing or continued stirring the color would disappear, so the very first end-point must be taken.

Subtract the ammonium thiocyanate used (in cc.) from 10 and multiply by 10. This gives, in mg., the amount of sodium chloride present in 5 cc. of urine. Calculate the twenty-four hour quantity.

Indican.—To 10 cc. of urine add 2 cc. of copper sulfate soiution, 5 cc. chloroform, and an equal volume (12 cc.) of strong hydrochloric acid. Close the mouth of the tube with the thumb, and cautiously invert a few times.

The amount of indican present is proportional to the depth of color of the chloroform extract. This qualitative test is often made roughly quantitative by using the color of Fehling's solution as a standard.

Metabolism Experiments. — Weigh accurately a small, clean, dry flask. Pipette into it 25 cc. of urine and weigh again. From the data obtained calculate the specific gravity of the urine.

Determine the specific gravity of the same sample of urine by means of an ordinary clinical areometer. Compare the results obtained, and explain how to standardize a clinical areometer.

Collect a full twenty-four hour quantity of urine, and in it determine the following: Volume, specific gravity, acidity, pH, total nitrogen, urea, ammonia, uric acid, creatinine, chloride, phosphate, inorganic sulfate, ethereal sulfate. Test qualitatively for indican.

For two days eat no meat, fish, eggs, milk, cheese, peas, or beans, and only a little bread. Eat much butter, potatoes, vegetables, starch preparations, fruit, and candy. Repeat all the determinations with the second twenty-four hour quantity.

For two days eat all the meat products you can, and collect the two twenty-four hour quantities of urine. In the second twenty-four hour quantity determine all the factors enumerated above. Tabulate and compare the results obtained in the three series of analyses.

Eat much sweetbread, kidney, or liver, for one day; collect the urine for the whole twenty-four hours, and estimate the uric acid. Compare with the uric acid figures previously obtained. Explain.

PART VII

BLOOD

Hemoglobin Crystals.—Place a drop of defibrinated rat blood on a slide, add a drop or two of water, mix, and cover with a cover-glass. Sketch the crystals which separate after a few minutes.

Hemoglobin (Reduced Hemoglobin).—Add to dilute blood a few drops of strong ammonium sulfide, or one or two drops of freshly prepared Stokes' reagent.

Examine spectroscopically.

Stokes' reagent is a 2 per cent solution of ferrous ammonium sulfate in 3 per cent tartaric acid, to which is added ammonia until a clear solution is obtained. The ammonia should be added only to the amount of reagent immediately needed.

Shake the solution of hemoglobin with air, and note the rapid change to oxyhemoglobin. Change the same solution of oxyhemoglobin to hemoglobin, and reverse two or three times, and note the facility with which hemoglobin takes up and loses oxygen.

Carbon Monoxide Hemoglobin.—Pass a current of illuminating gas through a dilute oxyhemoglobin solution for a minute, and filter. Note the change of color. Try the effect on the solution of (1) ammonium sulfide, (2) Stokes' reagent, (3) potassium ferrocyanide, (4) shaking with air. Note the stability of the compound.

Examine spectroscopically.

Methemoglobin.—Add to dilute defibrinated blood (1 : 5) two drops of a freshly prepared solution of sodium nitrite. Note

the change. What is the effect produced by the addition of reducing agents?

Hematin.—Hemolyze a small quantity of blood and add dilute hydrochloric acid cautiously till a precipitate occurs.

Acidify strongly with hydrochloric acid. Note color (acid hematin). Then add sodium hydroxide till strongly alkaline. Note color (alkaline hematin). To the alkaline solution add a few drops of ammonium sulfide and warm gently. Note color (reduced hematin or hemochromogen).

Hemin Crystals—Teichmann's Test.—Place a bit of powdered dried blood on a glass slide, add a minute crystal of sodium iodide and two drops of glacial acetic acid. Cover with a coverglass and warm gently over a flame until bubbles appear. Describe the crystals which separate.

Fibrinogen.—Allow about 6 to 8 volumes of fresh blood to run from the animal into 1 volume of a 1 per cent potassium oxalate solution (why?). Allow to stand over night in the cold room, and siphon off the clear plasma. With the solution so obtained make the following experiments:

Dilute 10 cc. with 20 cc. of distilled water and divide into 3 equal portions. To one add a little dilute (1 per cent) calcium chloride solution. To the second add a few drops of blood serum (why?). Place the three tubes in a beaker of water heated to 40° C. and observe the time of clotting.

PART VIII

MILK

Determine the specific gravity as in the case of urine.

Determination of Total Nitrogen.—Transfer 10 cc. milk to a 100 cc. volumetric flask. Fill to the mark with water, mix, and determine the total nitrogen in 1 cc. Calculate the total protein content of the milk by multiplying its nitrogen with the factor 6.25.

Determination of Casein.—Transfer 50 cc. of the diluted milk to another 100 cc. flask, and carefully precipitate the casein by the addition of dilute acetic acid and gentle shaking. Make up to volume (100 cc.) with water. Centrifuge a portion, and determine the nitrogen in 1 cc. of the clear liquid.

Calculate the total nitrogen (and protein), making due allowance for the dilutions, and subtract from the total protein found in the preceding experiment. The difference represents casein.

Determination of Milk Sugar.—Transfer 5 cc. of milk to a small flask or beaker. Add 20 cc. of water and mix well. Fill the special 5 cc. sugar burette used in glucose titrations with the diluted milk. Transfer to a large test-tube 5 cc. of 6.05 per cent copper sulfate solution and 1 cc. of saturated sodium carbonate solution. Shake for a moment, then add about 5 gm. of the salt mixture used in glucose titrations (p. 95). Add 4.2 cc. of diluted cow's milk, or 2.8 cc. of diluted mother's milk, and boil gently for four minutes counting from the time of actual beginning boiling. At the end of four minutes add more milk (0.02 to 0.3 cc.), unless it is apparent that the initial addition is enough. Boil one minute after each fresh addition.

In the sugar titration in the case of milk one can assume that cow's milk will contain a little less than 5 per cent and that

mother's milk may contain no more, but may go as high as 7 per cent; hence with mother's milk it is not advisable to start with more than 2.8 cc. for the first boiling period.

Calculation: 5 cc. of the copper sulfate solution is reduced in about 5 minutes by 40.4 mg. of lactose. 4.04 times 5, the degree of dilution, or 20.0, divided by the volume of diluted milk used gives the per cent of lactose in the milk.

Determination of Fat.—Measure out 17.6 cc. of thoroughly mixed milk into a Babcock flask. Add 17 cc. sulfuric acid (sp. gr. 1.82) and mix thoroughly, with gentle turning and shaking, until all the precipitated proteins have dissolved. Rotate in the centrifuge for 3 minutes. Add hot water up to the beginning of the graduations in the neck of the flask, and rotate for 1 minute. The graduations read in per cent of fat.

PART IX

BONE

Weigh a piece of clean, raw bone on the laboratory scales. Immerse in about 10 times its weight of 10 per cent hydrochloric acid in a flask. If any gas is evolved, determine what it is.

After 48 hours dilute the volume of the solution and what remains of the bone to a definite volume in a cylinder. Mix so as to get the solution uniform in composition.

Pipette out 25 cc. of the solution, neutralize with sodium hydroxide, using Congo red paper as indicator, and determine the phosphates. Repeat.

Calculate the tricalcium phosphate corresponding to the phosphoric acid found.

Taking other portions of the original solution, demonstrate experimentally that all the calcium in the bone can not be precipitated together with the phosphoric acid present.

In what form is this excess of calcium present in bone?

Examine the insoluble substance left in the hydrochloric acid solution. What is the substance? Prepare a "gelatin" solution from it.

PART X

BILE

Character of Bile.—Determine the specific gravity, taste, odor, color, consistency, reaction, of the bile supplied.

Test for coagulable protein.

Bile Salts.—Mix 250 cc. ox-bile with one-fourth its volume of bone-black, and evaporate nearly to dryness on the water bath. Cool, transfer the residue to a flask, and extract with 200 cc. of alcohol over night. Filter, and evaporate the filtrate to dryness on the water bath. Dissolve the residue in absolute alcohol, and filter into a dry flask. Add anhydrous ether till permanent cloudiness develops. Place in the cold room to crystallize. Filter. Describe the crystals.

Pettenkofer's Test for Bile Salts.—Mix a little bile with 2 or 3 drops of 10 per cent solution of cane sugar. Place in a test-tube some concentrated sulfuric acid. Incline the tube containing the sulfuric acid, and pour the bile solution slowly down the side of the tube so that it forms a layer above the sulfuric acid.

Gmelin's Test for Bile Pigments.—Put 5 cc. of nitric acid, containing some nitrous acid, in a test-tube, and introduce on top of it (pipette) about 5 cc. of diluted bile. Note what occurs. Study the delicacy of the reaction with very dilute solution of bile.

Test for Bile in Urine.—The presence of bile in human urine is usually indicated by its color and the color of the foam. In making the nitric acid test for albumin, the presence of bile is also revealed by a series of colored rings (green, blue, violet, red, and yellowish-red).

A similar series of colors is occasionally obtained from urines which have been preserved with thymol. This is one of the objections to this otherwise excellent preservative.

To 10 cc. of urine add a few drops of calcium chloride solution and a few drops of 10 per cent sodium hydroxide. Filter. Remove the filter paper from the funnel, open it, and drop 1 or 2 drops of concentrated nitric acid on the sediment. In the presence of (human) bile the usual, characteristic series of colored rings is obtained.

SUPPLEMENT

Qualitative Test for Acetone in Urine.—Clinicians seldom differentiate between acetone and acetoacetic acid, and the "acetone tests" which they use are tests for acetoacetic acid rather than for acetone. (*See* p. 211.)

In the qualitative test for acetone, as for its quantitative determination, the acetone is first removed from the urine by means of an air current, just as in corresponding determinations (and tests) for ammonia.

In the large test-tube used for the colorimetric determination of ammonia place first 5 cc. of urine and 1 to 2 drops dilute acid (HCl or H_2SO_4). Then insert the rubber stopper carrying the absorption tube, etc. Place the test-tube in a beaker of lukewarm water (35 to 40° C.), and aspirate the volatile acetone by means of a moderately rapid air current into a test-tube containing 5 cc. distilled water and 5 cc. Scott-Wilson reagent. If acetone is present, even if only in minute traces, the solution becomes turbid. If the amount of acetone obtained is extremely small, the turbidity may not appear for 5 to 10 minutes.

The Scott-Wilson reagent for acetone, which is used for qualitative tests as well as for quantitative determinations, is most conveniently prepared as follows:

To 10 gm. of mercuric cyanide dissolved in 600 cc. of water add a cooled solution of 180 gm. of sodium hydroxide in 600 cc. of water. Transfer this mixture to a heavy walled glass jar, and to it add 2.9 gm. of silver nitrate dissolved in 400 cc. of water. The silver solution should be added in a slow stream, and the addition must be accompanied by constant and exceedingly vigorous stirring with a heavy glass rod. If properly made, the silver dissolves completely, giving a clear solution, which is at once available for use. If the solution is turbid, it should be set aside to settle for three or four days and the clear supernatant liquid removed by means of a siphon.

In the clear reagent a new sediment gradually forms, so that the solution deteriorates slowly and after a few months is not serviceable for quantitative work, though still good for qualitative tests.

Titration of Acetone and Preparation of Standard Acetone Solutions.—Standard solutions of iodine, sodium thiosulfate, and potassium permanganate are needed in this work, the latter being used only as a basis for making the other two accurate. 0.5 N permanganate solution may be used.

Iodine.—Weigh roughly 10 to 12 gm. of potassium iodide in a beaker and add 50 cc. of water. Weigh out 6.4 gm. iodine in a small beaker covered with a watch glass and add this to the potassium iodide solution. Stir until the iodine is dissolved and then transfer the resulting solution to a 500 cc. volumetric flask. Dilute to the mark with water and mix.

Sodium Thiosulfate.—Weigh out 12.425 gm. of the salt ($Na_2S_2O_3 \cdot 5H_2O$), dissolve in water, transfer to a 500 cc. volumetric flask, fill to the mark with water, and mix.

The two solutions thus prepared should be approximately tenth normal. Their relative values are determined by titration as follows:

Pipette 20 cc. of the iodine solution into a flask (capacity 500–600 cc.) and add about 100 cc. water. From a burette, run in the thiosulfate solution until the reddish-brown iodine color has faded to a faint straw yellow. Now add a few drops of starch solution (*see* p. 105) and continue the titration till the blue iodide of starch color disappears. The end-point of this titration is very sharp.

The value of the thiosulfate solution is now determined as follows:

Weigh roughly 2 gm. potassium iodide, transfer to a flask, and dissolve in about 150 cc. water. Add 5 cc. diluted hydrochloric acid (1 : 5) and 50 cc. 0.05 N potassium permanganate solution.

The permanganate sets free an equivalent quantity of iodine according to the following equation:

$$2KMnO_4 + 10KI + 16HCl = 12KCl + 2MnCl_2 + 8H_2O + 5I_2$$

The iodine thus liberated is then titrated with the sodium thiosulfate solution in the same manner as the original iodine solution.

From the relative values of the iodine, the thiosulfate, and

the permanganate solutions, the exact values of the first two (in terms of tenth normal solutions) are calculated.

Standard Stock Solution of Acetone.—Add about 1 cc. of *pure* acetone (from the bisulfite compound) to water in a one liter volumetric flask, dilute to the mark, and mix. The titration of acetone with iodine is based on the fact that in alkaline solutions the acetone is converted into iodoform. Several reactions are involved in this process:

Iodine is converted into hypo-iodite,

1. $I_2 + 2KOH = KOI + KI + H_2O$

Hypo-iodite is then slowly converted into (useless) iodate,

2. $3KOI = KIO_3 + 2KI$

The hypo-iodite converts acetone into iodoform and acetic acid,

3. $3KOI + CH_3COCH_3 = CH_3COCI_3 + 3KOH$

4. $CH_3COCI_3 + KOH = CH_3COOK + CHI_3$

On acidifying, after the iodoform has been formed, the surplus iodine present as hypo-iodite (and iodate) is set free, and can then be titrated with the standard sodium thiosulfate solution as described above.

Each molecule of acetone uses up three molecules of hypo-iodite, and as each molecule of the latter is formed at the expense of two atoms of iodine, six atoms of iodine correspond to one molecule of acetone. One cc. of 0.1 N iodine solution corresponds therefore to 0.968 mg. acetone. Because of the iodate formation a considerable excess must be added.

The titration of the acetone solution is carried out as follows:

Transfer 25 cc. of the stock acetone solution to a flask, add 150 to 200 cc. water, then 50 cc. of the standardized iodine solution, and 10 cc. strong sodium hydroxide (40 per cent). Let stand with occasional shaking for 5 minutes. Add 18 cc. concentrated hydrochloric acid, and titrate the liberated excess of iodine with the standard thiosulfate solution.

If the standard solutions are exactly tenth normal, subtract the volume of thiosulfate employed from the volume of iodine

solution taken, and multiply the remainder (in cc.) with 0.968 to obtain the acetone content (in mg.).

Calculate the acetone content of the stock solution (in mg. per cc.). Transfer to a distilling flask as much of it as contains exactly 50 mg. of acetone. Add water enough to make a volume of 500 to 600 cc. and distill with vigorous cooling of the condenser. The receiver should be a large flask (750 to 1000 cc.) containing about 250 cc. approximately normal sulfuric acid. Boil for 20 to 30 minutes, or until at least 150 cc. of distillate has gone over. Transfer this distillate to a volumetric (liter) flask and dilute to the mark with water. Ten cubic centimeters of the acetone solution so obtained contain 0.5 mg. acetone. This solution, as well as the original stock solution, should be kept in a well stoppered bottle. The sulfuric acid present in the dilute standard acetone solution is added to prevent polymerization.

Preparation of Standard Acetone Solutions from the Acetone Bisulfite Compound.—A standard acetone solution can be prepared without distillation from the "acetone sulfite" used in photography as follows:

Transfer 2.5 gm. of the powder to a volumetric (1000 cc.) flask by means of a little water (50 cc.), and fill up to the mark with dilute (1 in 5) hydrochloric acid. Transfer 25 cc. of the solution to a flask. Add 20 cc. tenth normal iodine, let stand for five minutes, and titrate the surplus iodine with tenth normal thiosulfate solution. This titration gives the SO_2 or the sodium bisulfite content.

To another 25 cc. of the acetone solution add 50 cc. tenth normal iodine, let stand five minutes, then add 10 cc. strong sodium hydroxide, followed after five minutes by 18 cc. concentrated hydrochloric acid. Titrate the liberated iodine with thiosulfate. From the 50 cc. of iodine taken subtract (a) the figure of the last thiosulfate titration and (b) the iodine corresponding to the SO_2. The remainder corresponds to the acetone. From the standardized stock solution prepare the more dilute standard solution (5 cc. or 10 cc. of which should contain exactly half a mg. of acetone).

If the "acetone sulfite" is not available, acetone sodium bisulfite is easily prepared by slowly adding (with stirring) two-thirds volume of ordinary acetone to one volume (100 or 200

cc.) of saturated sodium bisulfite solution (freshly prepared and filtered). The precipitate should be freed as completely as possible from the mother liquor by filtering on a Büchner funnel with suction. Then wash rapidly two or three times with alcohol. Let dry in the open air for two or three days. Sieve to make the preparation uniform, and preserve in a glass stoppered vessel.

Quantitative Determination of Acetone in Urine.—(*J. Biol. Chem.*, 18: 263.)—To about 1 cc. of 10 per cent sulfuric acid in a large test-tube add enough urine (0.5 to 5 cc.) to give about 0.5 mg. of free acetone (0.3 to 0.7 mg.). Connect the test-tube, as in ammonia determinations, with a second test-tube containing 10 cc. of fresh approximately 2 per cent sodium bisulfite solution. Warm the first test-tube to 35° to 40° C., as in the qualitative test for acetone, and aspirate the acetone into the bisulfite solution by means of a moderate air current (time about 10 minutes). Transfer the sulfite-acetone solution to a 100 cc. volumetric flask together with distilled water enough to make 50 to 60 cc. To each of two other 100 cc. flasks add 10 cc. of the standard acetone solution containing 0.5 mg. acetone, add 10 cc. of the 2 per cent bisulfite solution, and dilute with distilled water to 50 to 60 cc.

To each of the three flasks add 15 cc. (clear) Scott-Wilson reagent, and immediately (before turbidity formation) dilute with distilled water to the mark, mix, and let stand for 12 to 15 minutes. Read the turbid contents of one of the standard acetone suspensions against itself in the Duboscq colorimeter. The best source of light for these comparisons is diffuse daylight coming through an opening (about 25 cm. square) cut through the shade of a (north side) window. The colorimeter metal screen must also be used. The instrument must be adjusted until 20 mm. of the two suspensions are equal. Now replace the contents of one of the colorimeter cups with the contents of the second standard suspension, and the other with the unknown acetone mercury suspension obtained from the urine, and make the turbidity comparison in the same manner as colorimetric comparisons—setting the standard at 20 mm.

Twenty multiplied by 0.5 and divided by the reading of the unknown (in mm.) gives the acetone content (in mg.) of the volume of urine taken for the analysis.

Qualitative Test for Acetoacetic Acid (in traces).—To 5 cc. of urine in a test-tube add 1 to 2 cc. dilute acetic acid (10 per cent) and a small crystal of sodium nitroprusside. Shake a few times to dissolve the salt, then add an excess of concentrated ammonia (2 to 3 cc.), and mix. A violet color indicates acetoacetic acid.

Gerhardt's Ferric Chloride Test for Acetoacetic Acid.—This test is useful for showing the presence in urine of relatively large amounts of acetoacetic acid. It is made as follows: To 5 cc. of urine in a test-tube add ferric chloride solution (10 per cent), drop by drop. At first a white precipitate of ferric phosphate is obtained, then, as the addition of the reagent is continued, a dark red color is produced if acetoacetic acid is present (in more than traces).

A number of substances used as drugs, such as salicylic acid, phenacetin, etc., give a similar reaction. If confusion due to such drugs is to be suspected, boil the deep red solution for 2 to 3 minutes. If the color is due to acetoacetic acid, it should disappear during the boiling and not reappear on cooling. The disappearance is due to the destruction by boiling of the unstable acetoacetic acid.

Quantitative Determination of Acetoacetic Acid and Acetone.—Acetone urines contain from two or three to nine or ten times as much as acetoacetic acid as acetone. In strictly fresh urines the latter proportions prevail; but the older the urine the greater becomes the relative proportion of acetone, because of the spontaneous decomposition of the acetoacetic acid. Urines giving a strong ferric chloride reaction usually contain more than 0.5 mg. of acetoacetic acid per cubic centimeter, and must be diluted so that an appropriate fraction of 1 cc. (of the original urine) can be taken for a determination.

The amount of urine taken should yield approximately 0.5 mg. of acetone (from 0.3 to 0.7 mg.). Transfer this amount of urine to a large test-tube containing 1 cc. of 10 per cent sulfuric acid, and connect with a second test-tube containing 10 cc. of 2 per cent sodium bisulfite solution. Immerse the test-tube containing the urine in a beaker of boiling water, and the second test-tube in cold water, then pass through an *extremely* slow air current for ten minutes. Increase slightly the speed of the air

current and continue the aspiration for another five minutes. The acetoacetic acid plus acetone is thus transferred, in the form of acetone, to the bisulfite solution. Rinse the solution into a 100 cc. volumetric flask, and determine the acetone exactly as in the determination of the preformed acetone.

One mg. of acetone is equivalent to 1.8 mg. of acetoacetic acid. From the "total acetone" of the 24-hour quantity of urine is subtracted the total preformed acetone, and the remainder multiplied by 1.8 gives the acetoacetic acid.

Determination of β-Hydroxybutyric Acid in Urine.—The method described below was at first thought by its authors (Folin and Denis) to give strictly all the β-hydroxybutyric acid present in urine. But it now appears that the yield is only 85 to 95 per cent, just as in the original method of Shaffer.

The urine is diluted from 10 to 100 times, depending on how much of the substance is present. The ammonia content of the urine is the best index as to how much urine is required to yield the desired amount of β-hydroxybutyric acid (1.5 to 3.5 mg.). The ferric chloride test for acetoacetic acid is also helpful, but without considerable experience only a preliminary determination can give the desired information.

Measure diluted urine, equivalent to 1.5 to 3.5 mg. of β-hydroxybutyric acid, into a 500 cc. Kjeldahl flask, add a little dilute sulfuric acid (5 cc.), and water enough to make a volume of about 150 cc. Boil the mixture gently for ten minutes (to drive off the preformed acetone and the acetoacetic acid), then add to the solution (with a cylinder) 25 cc. of a solution containing 1 per cent potassium dichromate and 35 per cent sulfuric acid, and connect the flask, in the usual manner, with a condenser by means of a specially treated rubber stopper.

The rubber stopper should be boiled twice for an hour in 10 per cent sodium hydroxide solution (or better, heated in an autoclave in the same solution for half an hour at 130 to 140° C.), and then thoroughly washed. It is also necessary to wrap the stopper thoroughly in tin foil during the distillation, so as to exclude the volatile sulfur impurities which otherwise are given off and interfere with the subsequent turbidity formation.

Distill very slowly, for one and one-half hours, collecting the distillate (about 100 cc.) in another 500 cc. Kjeldahl flask, previously charged with about 100 cc. of water.

To the distillate add a small amount of sodium peroxide (2 gm.), and redistill by ordinary rapid boiling. Collect this final distillate in a 100 cc. volumetric flask (or cylinder). About 80 cc. should be obtained.

Dilute this distillate to the 100 cc. mark with distilled water and mix. Transfer from 25 to 50 cc. into a 100 cc. volumetric flask, and determine the acetone content by the turbidity method, as in the case of the two preceding (acetone) determinations. No bisulfite is used in this case to hold the acetone, and none should therefore be added to the standard. Each milligram of acetone obtained corresponds to 1.78 mg. of β-hydroxybutyric acid.

Shaffer's Short Method for the Determination of β-Hydroxybutyric Acid.—To 50 cc. of urine add 100 cc. of water, then 50 cc. of basic lead acetate solution (Goulard's Ext. U.S.P.), and stir. Add 50 cc. approximately normal NaOH, and stir again. Filter. A clear filtrate containing but traces of lead or glucose is usually obtained, even though the original urine contained considerable quantities of sugar. Traces of sugar do not interfere with the determination.

Introduce 50 cc. of the filtrate into a 500 cc. Kjeldahl flask previously marked at the level of 100 cc. with a "glass pencil." Add 25 cc. of water and 50 cc. of half concentrated sulfuric acid. The latter, if freshly prepared by mixing with water (1 : 1), must be cooled before it is used.

Connect the Kjeldahl flask with a dropping funnel and with a condenser. Distill off about 25 cc., collecting the distillate in another Kjeldahl flask.

The first distillate thus obtained contains the preformed acetone, as well as the acetone derived from the acetoacetic acid of the urine. By adding to it 5 cc. of strong alkali and redistilling for 10 minutes, this acetone is obtained in the second distillate, free from impurities, and can be titrated with iodine and thiosulfate.

After replacing the Kjeldahl flask used as a receiver with another one, the distillation of the urine filtrate is continued, while adding slowly (about 15 drops per 10 seconds) a 0.2 per cent potassium dichromate solution.

During this distillation the volume in the distilling flask should be kept at approximately 100 cc. (i.e., at the level indi-

cated by the pencil mark). This is readily accomplished by regulating the speed of the distillation so that it just about equals the speed with which the dichromate solution is added. The speed of the oxidation is much greater with increasing concentration of sulfuric acid. With too great concentration of the acid, however, when the volume approaches a level of about 70 cc., the hydroxybutyric acid is in part converted into crotonic acid, and thus escapes oxidation to acetone.

The dichromate solution is added only so fast as to maintain a *very slight* excess; the blue green color should largely predominate in the boiling mixture. Occasionally it may be necessary to interrupt the addition of dichromate for a few minutes, but the volume in the distilling flask should not be allowed to sink below 85 or 90 cc. The addition of dichromate should be continued until no more appears to be converted into the green chromium salt. From 50 cc. to 100 cc. dichromate solution (=0.1 gm. to 0.2 gm. $K_2Cr_2O_7$) is usually required for each distillation. The addition (and distillation) lasts 20 to 30 minutes.

The distillate obtained must be redistilled, after the addition of 5 cc. strong alkali and about 20 cc. of 30 per cent hydrogen peroxide. This final distillation need not last more than 10 minutes. The distillate thus obtained is titrated in the usual manner (p. 205) with iodine and thiosulfate. The yield of acetone obtained is about 90 per cent of the theoretical amount when working with solutions of pure β-hydroxybutyric acid. A correction of 10 per cent should therefore be added to the results obtained.

Slightly higher results (93 to 94 per cent) may be obtained by a very slow addition of the dichromate, and a considerable prolongation of the distillation period, but since the theoretical amounts of acetone cannot be obtained, the advantage so gained is doubtful.

Tentative Colorimetric Method for the Determination of Phenols in Urine.—No strictly reliable method for the determination of phenols in urine (or in blood) is known. Some value and significance can probably be attached to the results obtained by the simple procedure described here, but the values are probably always too high because tyrosine (free or combined) is included in the values obtained.

Transfer 4 gm. of Lloyd's reagent, 0.3 gm. of powdered

oxalic acid and about 40 cc. of urine to a small dry flask. Shake gently, but continuously, for 5 minutes, and filter. All the uric acid is removed by this treatment.

To determine the "free" (non-conjugated) phenols transfer 5 cc. of the "uric acid-free urine" (filtrate) to a 100 cc. volumetric flask, and add 5 cc. of the phenol reagent (see page 339) together with 20 cc. of saturated sodium carbonate solution. Dilute to volume, mix, let stand for about 20 minutes or longer, and filter.

Transfer 5 cc. of the standard phenol solution to another 100 cc. flask, add 5 cc. of phenol reagent plus 20 cc. of carbonate solution and, after about 20 minutes, make the color comparison in the usual manner.

To determine the total phenols (free and conjugated) transfer 3 cc. of the uric acid-free urine to a 100 cc. volumetric flask, add 10 drops of concentrated hydrochloric acid, and heat in boiling water for 10 to 15 minutes. Cool, add 5 cc. of phenol reagent and 25 cc. of saturated sodium carbonate solution. Dilute to volume, let stand for 20 minutes, and filter. Compare the color so obtained with that obtained from 5 cc. of the standard phenol solution plus 10 drops of HCl treated with 5 cc. of the phenol reagent plus 25 cc. of carbonate solution.

The standard is a solution of pure phenol in 0.01 N HCl, containing 0.5 mg. of the former substance in 5 cc. As phenol is an exceedingly hygroscopic substance, it is necessary to standardize the solution by means of the iodometric titration.

This titration is carried out as follows: Make a phenol solution in 0.1 N HCl, containing 1 mg. of crystallized phenol per cc. Transfer 25 cc. of the phenol solution to a 250 cc. flask, add 50 cc. 0.1 N sodium hydroxide, heat to 65° C., add 25 cc. 0.1 N iodine solution, stopper the flask, and let stand at room temperature thirty to forty minutes. Add 5 cc. of concentrated hydrochloric acid, and titrate excess of iodine with 0.1 N sodium thiosulfate solution. One cc. of 0.1 N iodine solution corresponds to 1.567 mg. of phenol. On the basis of the results, dilute the phenol solution so that 10 cc. contains 1 mg. of phenol.

Because of the red precipitate in the solution it is rather difficult to see the end-point of the titration. For those who have not had much experience it may be advisable to dilute the solution to a definite volume (after adding the hydrochloric acid), then to filter, and to titrate a portion of the filtrate as recommended by Sutton; with a little practice, however the titration can be made without this procedure.

Quantitative Determination of Hippuric Acid in Urine.—(*J. Biol. Chem.*, 11:257.)—In this method the hippuric acid is first hydrolyzed, and the resulting benzoic acid is extracted with chloroform, and the chloroform solution is titrated with standard sodium ethylate.

Transfer 100 cc. of urine to an evaporating dish, add 10 cc. 5 per cent sodium hydroxide solution, and evaporate to dryness on the water-bath. Rinse the residue into a 500 cc. Kjeldahl flask by means of 25 cc. of water and 25 cc. concentrated nitric acid. Add 0.2 gm. copper nitrate, a couple of pebbles to prevent bumping, and boil very gently over a micro burner for four and one-half hours. During this boiling, a miniature Hopkins' condenser (made from a large test-tube) is kept within the neck of the boiling flask to prevent loss of benzoic acid, which is volatile with steam.

After cooling, rinse the condenser with 25 cc. water, and transfer the contents of the flask to a separatory funnel (capacity 500 cc.). Rinse the flask with 25 cc. water, thus making the total volume in the separatory funnel 100 cc.

Add to this solution 55 gm. of ammonium sulfate, shake until dissolved, and extract with neutral (freshly washed) chloroform four times, using 50, 35, 25, and 25 cc. of chloroform respectively. Collect the chloroform extracts in another separatory funnel, and wash this by shaking with 100 cc. saturated solution of pure sodium chloride, to each liter of which has been added 0.5 cc. concentrated hydrochloric acid.

Draw off the chloroform which contains the benzoic acid into a dry flask, and titrate with 0.1 N sodium ethylate solution and 4 to 5 drops of phenolphthalein as indicator. The first distinct coloration diffusing through the whole liquid is taken as the end-point without regard to subsequent fading.

The sodium ethylate solution is made by dissolving from 1.8 gm. to 2.3 gm. metallic sodium in absolute alcohol and diluting to a liter with absolute alcohol. It is standardized against chloroform solutions of benzoic acid.

One cubic centimeter of 0.1 N ethylate corresponds to 12.2 mg. benzoic acid or 17.9 mg. hippuric acid.

Turbidity Method for the Determination of Albumin in Urine.—(*J. Biol. Chem.*, 18:273.)—To about 75 cc. of water in

each of two 100 cc. volumetric flasks add 5 cc. of a 25 per cent solution of sulfosalicylic acid. To one flask add 5 cc. of a standard protein solution, prepared as described below, and containing 10 mg. of albumin. To the other add the albuminous urine 1 cc. at a time (by means of an Ostwald pipette) until the turbidity obtained seems to be reasonably near that of the standard. Fill the two flasks up to the mark with water, cautiously inverting a few times to secure mixing. The standard must invariably first be read against itself to secure the adjustment of the colorimeter (and of the eye). Then replace the contents of one of the Duboscq colorimeter cups by the suspension of the unknown, and make the turbidity comparison in the usual manner.

Set the standard containing 10 mg. of protein at 20 mm. The unknown must not read less than 10 nor more than 30 mm. Dividing 200 by the product of the reading of the unknown and the number of cubic centimeters of urine taken, gives the albumin in milligrams per cubic centimeter of urine.

It is very important not to shake the albuminous suspensions in the volumetric flasks because of the tendency of the precipitate to agglutinate. The preliminary mixing must therefore be accomplished by means of a few gentle inversions.

The standard protein solution is prepared from fresh blood serum free from hemoglobin. For the preparation of this serum either slaughter house or normal human blood may be used. The so-called blood serum sold for the preparation of bacteriological culture media should be avoided, as it is usually several days old and is frequently partially decomposed. The dried preparations of "blood albumin" listed by chemical dealers are also not satisfactory for the preparation of standard solutions. To prepare the standard, dilute 35 cc. of serum with a 15 per cent solution of chemically pure sodium chloride to about 1000 cc. Mix and filter. By means of nitrogen determinations ascertain the protein content of the filtrate (protein $=$ N $\times$ 6.25) and on the basis of the figure obtained, dilute the solution with 15 per cent sodium chloride solution so that it contains 2 mg. of protein per cubic centimeter. Sodium chloride in the concentration mentioned is fairly effective as a preservative. Nevertheless, it is best to saturate the standard albumin solution with chloroform (20 cc.).

The above method is not applicable to urines which are very deeply colored with blood or bile pigments. The method is, of course, applicable to other albuminous fluids than urine, as, for example, exudates, transudates, and the cerebrospinal fluid.

Gravimetric Method for the Determination of Albumin in Urine.—The method is as follows: Pipette 10 cc. of urine into an ordinary conical centrifuge tube, which has been previously weighed; add 1 cc. of 5 per cent acetic acid, and let stand for fifteen minutes in a beaker of boiling water. At the end of this time remove the tube from the water bath, and centrifuge for a few minutes. Pour off the supernatant liquid, stir up the precipitate in the tube with about 10 cc. of boiling 0.5 per cent acetic acid, and again centrifuge. Remove the supernatant liquid from the precipitate in the tube, and wash once more, this time with 50 per cent alcohol. After centrifuging and pouring off the supernatant alcohol, place the tube for two hours in an air bath at 100 to 110° C., then cool in a desiccator, and weigh.

Determination of Total Fixed Base in Urine.—(Fiske, *J. Biol. Chem.*, 51: 55.)—Measure into a large lipped pyrex test-tube (200×25 mm.) a sample of urine representing about 0.1 hour. (The sample should not contain more than 5 mg. of inorganic phosphorus, so less than the amount prescribed must be used if the phosphorus content is more than 50 mg. per hour, or 1.2 gm. per day.) Add 1 cc. of approximately 4 N sulfuric acid, 0.5 cc. of concentrated nitric acid, and a quartz pebble, and boil down until white fumes appear. If the residue does not soon become colorless after this stage has been reached, cool slightly, add a few drops more of nitric acid, and continue the heating. When the remaining drop of sulfuric acid has become clear and colorless, let cool for a few minutes, and rinse into a test-tube (accurately marked at 25 cc.) with four 2 cc. portions of water. Add a drop of a saturated alcoholic solution of methyl red. Neutralize with powdered ammonium carbonate until the color of the indicator just begins to change, and restore the pink color by adding 4 N sulfuric acid, 1 drop at a time. Heat to boiling, and again restore the pink color if necessary. Add a 10.5 per cent solution of ferric chloride crystals (in 0.1 N HCl) in the proportion of 0.1 cc. for each mg. of inorganic phosphorus present, shake, and run in 1 cc. of a 5 per cent solution of ammonium acetate. Add sufficient water to make the total volume 10 or 11 cc., heat again to boiling, and dilute to the 25 cc. mark with cold water. Close the mouth of the tube with a clean, dry rubber stopper, invert 2 or 3 times, and filter immediately through a

dry 9 cm. ashless paper into a dry test-tube or some other small receptacle. The filter should be kept nearly filled as long as possible, and only about 20 cc. of filtrate should be collected. Stopper the tube containing the filtrate, and cool.

Transfer 5 cc. of the filtrate to a small platinum dish, add 1 cc. of 4 N sulfuric acid, and evaporate on a water bath until nearly dry. Place the dish on a metal triangle, and heat, cautiously at first, over a micro burner, gradually raising the flame until fumes have ceased to come off. Let cool, sprinkle over the residue a little powdered ammonium carbonate, and ignite again, finally raising the flame to its maximum and moving the triangle about until each part of the dish has been momentarily subjected to a dull red heat. When the dish has cooled, add 2 cc. of water. Agitate until the residue is dissolved, using a rubber-tipped rod to assist in dissolving it if necessary. Transfer the contents of the dish to a large lipped pyrex test-tube, and rinse four times with 2 cc. of water. Determine the sulfate content of the solution by the benzidine method (p. 165).

If 0.02 N NaOH is used in titrating the benzidine sulfate precipitate, the titration figure (after subtracting a temperature correction of 1 per cent) gives the number of cubic centimeters of 0.1 N fixed base in the sample of urine used.

Determination of Calcium in Urine.—(Fiske and Logan, *J. Biol. Chem.*, 93: 211.)—Into a large lipped pyrex test-tube (200 ×25 mm.) measure a sample of urine containing between 0.25 and 0.75 mg. of calcium (usually 3 to 5 cc. of human urine). Add 1 cc. of 10 N sulfuric acid and boil down (with a quartz pebble to prevent bumping) until white fumes appear. While the boiling is continued, run concentrated nitric acid,[1] a drop or two at a time, down the wall of the test-tube until the residue is colorless. Cool, and add 10 cc. of water and 2.5 cc. of 5 N ammonium hydroxide.[1] Neutralize the excess ammonia with N nitric acid[1] until the precipitate of phosphate has just disappeared. Add 10 cc. of 2.5 per cent oxalic acid,[1] and slowly neutralize the mixture to pH 5 with 5 N ammonium hydroxide, finally adjusting the reaction with a few drops of the oxalic acid solution if necessary. Shake the tube gently for 3 minutes after precipitation starts, and let stand over night.

[1] Calcium-free (see p. 231).

Filter with suction through a filtration tube 8 mm. in diameter (p. 243), fitted with a mat of filter paper pulp so tight that the rate of filtration does not exceed 3 cc. per minute with the suction pump on full. Wash the precipitate with four 2 cc. portions of filtered 3 per cent ammonium oxalate solution.[2] Introduce into the filtration tube about 1 cc. of water and, with the aid of a sharpened nichrome wire, poke the precipitate and mat through the hole in the lower end of the tube into a platinum dish or crucible. Rinse the test-tube and the filtration tube with 2 cc. of N nitric acid [2] and two 2 cc. portions of water.

Evaporate the contents of the platinum dish or crucible to dryness on the steam bath. Add 0.5 cc. of 2.5 per cent oxalic acid solution,[2] and evaporate again to dryness. Support the dish on a metal triangle and heat gradually with a micro burner flame until the paper chars and finally begins to glow. Remove the flame until the glowing stops, and then apply at intervals until the paper is completely carbonized. Finish the ignition by heating with the full flame of the micro burner for a few seconds.

As soon as the dish is cool enough to handle, add a drop of methyl red (saturated alcoholic solution) and 1 cc. of 0.02 N hydrochloric acid. Rotate the dish or stir with a small glass rod until solution is complete. If 1 cc. does not suffice, add a second cc. of standard acid. Titrate the excess acid with 0.02 N sodium hydroxide (carbonate- and silicate-free, p. 247) from a micro burette to the full yellow color of the indicator, adding only 0.005 cc. at a time as the end-point is approached. 1 cc. of 0.02 N acid is equivalent to 0.4008 mg. of calcium.

Commercial ammonium hydroxide is rarely calcium-free. The 5 N solution may be prepared by adding concentrated ammonium hydroxide from a dropping funnel to stick sodium hydroxide contained in a suction flask, collecting the gas in water in a cooled receiver. The solution, diluted to the desired concentration, is kept in a paraffined bottle.

To test the reagent for calcium, evaporate 50 cc. to dryness in a platinum dish, and add 1 drop of concentrated nitric acid and 3 cc. of water. Pour into a 15 cc. conical centrifuge tube, neutralize to methyl red with the same ammonium hydroxide solution, and add 2 cc. of filtered 3 per cent ammonium oxalate solution. Let stand over night, and centrifuge. If no sediment can be detected on careful examination of the tip of the centrifuge tube, the reagent is satisfactory.

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[2] Base-free (see p. 231).

Test 50 cc. of the concentrated nitric acid in the same manner. Then evaporate 10 cc. of the 10 N sulfuric acid to dryness in a platinum dish, ignite, and apply the ammonium oxalate test to the residue. If calcium is found in either of these reagents, it must be purified by distillation before it can be used.

The oxalic acid is tested by the same procedure after igniting 5 gm. of the crystals in a platinum dish.

Some of the reagents (ammonium oxalate, oxalic acid, nitric acid) must also be practically free from fixed base of any kind.

Test the ammonium oxalate and the oxalic acid as follows:

Ignite 1 gm. of the solid in a platinum dish. Cool, add a drop of methyl red and 1 cc. of 0.02 N hydrochloric acid, and titrate with 0.02 N sodium hydroxide. At least 0.9 cc. of the standard alkali should be required.

To test the nitric acid, evaporated 10 cc. to dryness in a platinum dish. Add 0.5 cc. of 2.5 per cent oxalic acid (base-free), evaporate again to dryness, and ignite. Cool, add methyl red and 1 cc. of 0.02 N hydrochloric acid and titrate as before.

The oxalic acid and the ammonium oxalate, if not sufficiently pure, must be recrystallized from water.

Determination of Magnesium in Urine. — (Fiske and Logan.)—Boil down the filtrate and washings from the calcium oxalate precipitate (p. 229) in a large lipped pyrex test-tube (200×25 mm.) to a small volume, or better, evaporate to dryness in a current of air with apparatus especially designed for the purpose (*J. Biol. Chem.*, 86: 761).[3] Oxalic acid is to be added in a later step, but the residue at this stage contains enough oxalate to interfere with the precipitation of magnesium ammonium phosphate in most instances. It is accordingly destroyed by heating with concentrated nitric acid, adding a few drops at the start, and later a drop at a time until practically no foam is produced when the nitric acid comes into contact with the residue.

For the purpose of destroying the oxalic acid, the test-tube may be heated with a micro burner, but since overheating is likely to cause the

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[3] The sample should contain between 0.25 and 0.7 mg. of magnesium. In the case of urine having an unusually high ratio of magnesium to calcium, it may be necessary to use an aliquot of the filtrate and washings (previously diluted to a convenient volume) rather than the entire amount. If less than half is used, enough additional 10 N sulfuric acid must be added, before evaporation, to make the total quantity of sulfate present between 0.5 and 1 cc. of 10 N.

separation of insoluble material (largely metaphosphate) a better arrangement is a molten sulfate bath kept at 220–230° C. The bath consists of a 600 cc. beaker containing 100 gm. of ammonium sulfate, 10 cc. of water, and 10 cc. of concentrated sulfuric acid, and is heated with a Bunsen burner. The mixture softens between 180 and 200° C., and does not give off fumes below 300°. The beaker is not likely to break on cooling if the mixture is stirred as it solidifies, and if about 10 cc. of water are added each time before reheating.

Colorimetric Method.—Allow the residue to cool, and add 2 cc. of water, 2 cc. of 2.5 per cent oxalic acid, 0.2 cc. of 25 per cent citric acid,[4] 1 cc. of a solution of 4.5 per cent monoammonium phosphate,[5] and a drop or two of thymol blue (0.04 per cent aqueous solution). Add concentrated ammonium hydroxide until the indicator turns yellow, cool under the tap if the solution has become warm, and continue the addition of ammonium hydroxide to the maximum blue color of the indicator. Scratch the wall of the test-tube below the surface of the liquid with a glass rod of small diameter until crystals begin to form (from a few seconds to a minute or more). Remove the rod after rinsing with 2 drops of dilute sulfuric acid (about 0.1 N) and about 0.5 cc. of water. Shake gently for 5 minutes, and let stand over night.

Filter with suction, using the 8 mm. filtration tube described on p. 243. In this case a mat of paper pulp 1 mm. thick is adequate, and it need not be tightly packed. Wash with four 2 cc. portions of approximately N ammonium hydroxide, and finally rinse off the tip of the filtration tube with water.

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[4] Some citric acid must be added to prevent contamination of the precipitate with iron. Citrate also, if present in sufficient quantity, insures the formation of a precipitate containing phosphate and magnesium in the correct proportion. But in addition it increases the solubility of magnesium ammonium phosphate, or at least delays precipitation. The formation of a precipitate containing phosphorus and magnesium in a ratio exceeding 1 : 1 can be prevented by oxalate as well, and with less interference with the separation of the triple phosphate ($MgNH_4PO_4 \cdot 6H_2O$). In the presence of iron, a mixture of oxalate and citrate is better than either one alone. Correct results cannot be obtained without adding some polybasic acid.

[5] The ammonium phosphate should be recrystallized: Dissolve 50 gm. of the salt in 50 cc. of boiling water, and filter. Let the filtrate cool, with occasional shaking. Filter with suction on a small Büchner funnel, and wash the crystals with about 40 cc. of cold water.

In analyzing samples already containing a large excess of phosphate, less of this reagent should be used. 1 cc. of 4.5 per cent monoammonium phosphate contains 12 mg. of phosphorus, and the amount present during the precipitation should never be a great deal more than that.

To dissolve the precipitate, place the pyrex test-tube inside a dry 1000 cc. suction flask, and insert the stopper holding the filtration tube into the neck of the flask. Dissolve the magnesium ammonium phosphate by running 2 cc. of 10 N sulfuric acid through the filtration tube, using only very gentle suction (to avoid spattering), and rinse with four 2 cc. portions of water. (In this procedure the paper pulp is not disturbed, and the same mat may be used repeatedly.) Remove the rubber stopper from the neck of the suction flask, and rinse the tip of the filtration tube with 1 or 2 cc. of water to collect any of the solution that may have crept up the outside wall.

Transfer the phosphate solution in the test-tube to a 50 cc. volumetric flask, rinsing three times with 5 cc. of water, and add 1 cc. of a solution of 12.5 per cent ammonium molybdate in 5 N sulfuric acid (Molybdate IIIa, p. 341). In another similar flask prepare a standard containing 5 cc. of monopotassium phosphate solution (0.1755 gm. KH_2PO_4 per liter),[6] to which are added 25 cc. of water and 5 cc. of 2.5 per cent ammonium molybdate in 5 N sulfuric acid (Molybdate I, p. 341). To the contents of each flask now add 2 cc. of 0.25 per cent aminonaphtholsulfonic acid reagent. Dilute to the mark, mix, and read after 3 or 4 minutes, with the standard set at 20 mm.

The amount of magnesium in the sample analyzed (in mg.) is 3.14 divided by the reading. Whenever the color appears to be much more than twice that of the standard, the solution may profitably be transferred to a 100 cc. volumetric flask, adding 5 cc. of 2.5 per cent ammonium molybdate in 5 N sulfuric acid (besides the molybdate already present) and an additional 2 cc. of aminonaphtholsulfonic acid reagent before diluting to the mark; the magnesium content is in this case obtained by dividing 6.27 by the colorimeter reading.

If the filtrate and washings from the calcium oxalate precipitate contain less than 0.25 mg. of magnesium, a larger sample of urine must be used. An alternative method, which is somewhat more time-consuming but has a wider range (0.15 to 1.2 mg. of magnesium) is described below.

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[6] The standard, as well as all the reagents used in developing the blue color, are identical with those described under the determination of phosphorus (pp. 175, 341).

Alkalimetric Titration Method.—When the residue left after decomposing the oxalic acid has cooled, dilute to 15 cc., add 0.5 cc. of 10 N sulfuric acid, 2 cc. of 25 per cent citric acid solution, and a drop or two of thymol blue (0.04 per cent aqueous solution). Heat the tube to about 90° C. in a water bath and add ammonium hydroxide to the maximum blue color of the indicator.

Remove the tube from the bath and add, drop by drop with shaking, 0.5 cc. of a 5 per cent alcoholic solution of 8-hydroxyquinoline. Add a few drops of ammonium hydroxide if necessary to maintain the deep color of the indicator. To promote precipitation, scratch the glass as the solution cools with a thin glass rod. Remove and rinse the rod before or just as precipitation starts. Let stand 10 minutes and shake gently for 3 minutes.

After 1 hour, and before 3 hours, filter with suction through an 8 mm. filtration tube (p. 243) provided with a thin mat of paper pulp. Because the precipitate tends to float and creep, fill the filtration tube not more than three quarters full and permit it to empty before adding more of the suspension. Wash with three 2 cc. portions of a solution made by adding 2 cc. of the 5 per cent alcoholic solution of 8-hydroxyquinoline to 100 cc. of approximately 0.1 N ammonium hydroxide. Introduce into the filtration tube about 1 cc. of water and, with the aid of a sharpened nichrome wire, poke the precipitate and mat through the hole in the lower end of the tube into a platinum dish or crucible. Rinse the test-tube and the filtration tube with 2 cc. of N nitric acid [7] and two 2 cc. portions of water.

Evaporate the contents of the platinum dish or crucible to dryness on the steam bath. Add 0.5 cc. of 2.5 per cent oxalic acid,[7] and evaporate again to dryness. Support the dish on a metal triangle and heat gradually with a micro burner flame until the paper chars and finally begins to glow. Remove the flame until the glowing stops, and then apply at intervals until the paper is completely carbonized. Finish the ignition by heating with the full flame of the micro burner for a few seconds.

As soon as the dish is cool enough to handle, add a drop of methyl red (saturated alcoholic solution). Then add an excess of 0.02 N hydrochloric acid (1 cc. at a time unless the amount of

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[7] Base-free (see p. 231).

magnesium is already known approximately). Rotate the dish or stir with a small glass rod until solution is complete. Titrate the excess acid with 0.02 N sodium hydroxide (carbonate- and silicate-free, p. 247) from a micro burette to the full yellow color of the indicator. 1 cc. of 0.02 N acid is equivalent to 0.2432 mg. of magnesium.

Phosphate in amounts found in urine (or in bone) does not interfere in this procedure. A small amount of iron likewise does no harm even though it precipitates.

Determination of Potassium in Urine.—(Fiske and Litarczek.)—The following reagents will be needed:

1. Sodium Cobaltinitrite.—Dissolve (A) 50 gm. of sodium nitrite in 50 cc. of warm water, and (B) 17 gm. of cobaltous nitrate in 17 cc. of 50 per cent acetic acid. Mix A and B, and aerate for 30 minutes. Let the solution stand at about 0° C. for 2 days, decant, and filter with suction through hardened paper. Centrifuge a portion of the filtrate; if any yellow sediment can be detected in the tip of the centrifuge tube, centrifuge the whole solution. To the clear solution, which is meanwhile stirred continuously (preferably with a mechanical stirrer), add 0.85 volume of alcohol in a fine stream from a burette. Let the precipitate settle for a few hours, and filter with suction, washing thoroughly with absolute alcohol, and finally with ether.

The reagent used for the precipitation of potassium is prepared just before use by dissolving the air-dried sodium cobaltinitrite in the proportion of about 0.3 gm. of salt to 1 cc. of water.

2. Sodium Chloride-Acetic Acid Solution.—To 200 cc. of 30 per cent sodium chloride add 200 cc. of concentrated hydrochloric acid. Cool, and filter with suction through hardened paper. Wash 3 times with 25 cc. of 1 : 1 HCl, then with five 25 cc. portions of alcohol. Dry the salt on the steam bath. Prepare a 12 per cent solution of this purified sodium chloride in 10 per cent acetic acid.

3. Tartaric Acid.—A 5 per cent solution in 95 per cent alcohol, freshly prepared every day or two.

4. Aqua Regia.—Mix 3 parts of concentrated hydrochloric acid and 1 part of concentrated nitric acid.

5. 80 Per Cent Alcohol.

6. 95 Per Cent Alcohol.

7. Absolute Alcohol.

8. 0.02 N Sodium Hydroxide.—Prepared as described on p. 247.

Transfer to a 100 cc. beaker a sample of urine containing between 0.8 and 4 mg. of potassium (usually 1 cc.). Add 1 cc. of aqua regia and evaporate on the steam bath, continuing the heating for 10 minutes after the residue is apparently dry. Dissolve the residue with 1 cc. of water, add 1 cc. of sodium chloride-acetic acid solution and (drop by drop, while rotating the beaker) 1 cc. of fresh sodium cobaltinitrite solution. Cover the beaker with a watch glass. After 15 minutes filter with suction through an 8 mm. filtration tube (p. 243) provided with a mat of short-fibered asbestos.[8] Wash with three 2 cc. portions of 80 per cent alcohol, and continue the suction until the alcohol has been evaporated.

Remove the filtration tube from its stopper, and place the former in a dry beaker (150 cc. capacity, tall form). Heat on a wire gauze with a Bunsen burner for 7 minutes (the temperature in the interior of the beaker should reach about 370° C.), and let cool. Then heat in the same way (for 5 minutes) the beaker in which the potassium cobaltinitrite was precipitated. (If any trace of yellow precipitate adhering to the lip of the beaker has not also turned black, it should be decomposed by gentle heating over—not in—the flame of a micro burner.)

Return the filtration tube to its stopper, and insert the latter into the neck of a 1000 cc. suction flask into which has been set a pyrex test tube (200×25 mm.) drawn out at the upper end to form a neck about 3 cm. long and 12 mm. in internal diameter, and provided with a lip. Transfer 1 cc. of water to the 100 cc. beaker, and heat just to boiling on a hot plate. Pour the hot solution into the filtration tube, and draw it into the pyrex test-tube with very gentle suction. Follow with alcohol (2 cc. at a time), heated just to boiling in the same manner and then passed through the filtration tube, until the total volume of the contents of the test-tube has reached 11 cc. (A mark made on the tube with a glass pencil is accurate enough.)

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[8] The same mat may be used for several analyses without removing the cobalt oxide residue.

Cool the contents of the test-tube to room temperature, and add 5 cc. of the alcoholic solution of tartaric acid. Shake gently for 5 minutes. Filter the acid potassium tartrate through a filtration tube of the same type used in the preceding step, but in this case using a mat of paper pulp. Wash with four 2 cc. portions of absolute alcohol, then rinse the lip of the test-tube with 1 cc., and finally wash off the tip of the filtration tube with a small amount of alcohol.

With a sharpened nichrome wire poke the precipitate and mat through the hole in the bottom of the filtration tube into the same pyrex test-tube, first introducing 1 cc. of water and partially breaking up the mat. Rinse with 9 cc. of water in small portions. (Water blown from a wash bottle must not be used.)

Insert a rubber stopper into the neck of the test-tube, and invert a few times to dissolve the tartrate. Now titrate to the turning point of phenol red (1 or 2 drops of a 0.5 per cent aqueous solution) with 0.02 N sodium hydroxide, adding only 0.01 cc. at a time as the end-point is approached. Again insert the rubber stopper, invert the test-tube once, and finish the titration to the first definite red color. Subtract the blank (determined by titrating 10 cc. of water in the same way), which should be only about 0.01 cc. 1 cc. of 0.02 N sodium hydroxide is equivalent to 0.782 mg. of potassium.

Special Filtration Tube.—(Fiske, *J. Biol. Chem.*, 46: 285) —Many crystalline precipitates, when centrifuged, tend to float on the surface of the liquid, resulting in loss of material when the supernatant fluid is poured off. If, on the other hand, the small precipitates dealt with in microanalytical work are filtered by gravity through paper held in the ordinary conical filter funnel, the filter is usually so large in proportion to the size of the precipitate that a wholly unreasonable amount of washing is required to remove the mother liquor adhering to the paper.

Many suitable filters have been devised for use with suction in microgravimetric analysis, where the precipitate is weighed together with the filter, but in volumetric analysis on a small scale it is often necessary to remove both the precipitate and the mat from the filter *quantitatively*. The risk of mechanical loss in this operation can be avoided, and the amount of water re-

quired for rinsing materially reduced, if the contents are removed through the *bottom* of the filter.

Especially when the amount of liquid used for washing the precipitate must be as small as possible, the filtration tube should likewise be of small dimensions. A tube suitable for many purposes can be made from a piece of glass tubing of 8 mm. bore and 120 mm. long (capacity about 6 cc.), flanged at the upper end and constricted at the other end by heating in a Bunsen burner flame until the diameter of the hole has shrunk to about 2 mm. The filtration tube is inserted through the hole in a rubber stopper fitting the neck of the suction flask, in which a large test-tube has been placed to catch the filtrate.

Depending upon the nature of the precipitate, the mat is made of either paper pulp or short-fibred asbestos. Ashless paper must be used, and ordinarily a loose-textured variety (*e.g.* Schleicher and Schüll No. 589, black ribbon) is most suitable. The pulp is readily prepared by vigorously shaking one 15 cm. disk with 200 cc. of water for 2 or 3 minutes in a stoppered bottle. The pulp is transferred to the bottom of the filtration tube by pipetting with a glass tube of 6 or 7 mm. outside diameter. The same glass tube may then be used to pack down the edges of the mat.

For the retention of very fine precipitates, instead of using a very thick mat of paper pulp, a thin mat covered with an 8 mm. disk of close-textured filter paper (cut out with a cork bore) is sometimes to be preferred. The paper disk is inserted with the aid of a solid glass rod (cut off squarely and not fire polished) just large enough to slip into the filtration tube.

When the filtration and washing have been finished, the precipitate and mat are partly broken up, after adding a little water, by manipulation with a stiff nichrome wire (which has been sharpened at one end with a file), and then poked through the 2 mm. hole into a suitable receptacle with the same instrument.

Filtration Tube for Sulfate and Total Base Determinations. —(Fiske, *J. Biol. Chem.*, 47: 59.)—For precipitates that are inclined to clog the filter, a larger tube may be required. The one shown in the cut (Fig. 7) is 75 mm. long and has an internal diameter of 15 mm. The upper end is flanged as usual, while the other end is drawn out in the flame to make an elongated tip.

Since more paper pulp must be used with this larger tube, the hole at the lower end is also somewhat larger (3 mm.).

Preparation of 0.02 N Sodium Hydroxide (Carbonate- and Silicate-Free).—(Fiske and Logan, *J. Biol. Chem.*, 93: 211.)—Sharp end-points in most alkalimetric titrations of small amounts of material are possible only when the standard alkali is free from all but traces of either carbonate or silicate. The proper preparation of this important reagent requires: (1) the use, as starting material, of a clear saturated solution of sodium hydroxide, in which sodium carbonate is practically insoluble, (2) avoidance of contact with glass while the alkali is being dissolved in water and until the solution has cooled down, (3) protection

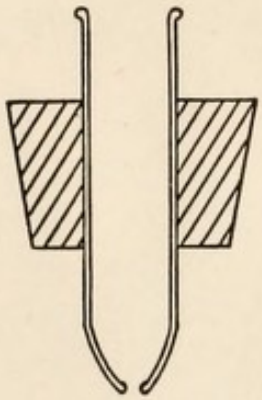


FIG. 7.

against subsequent contamination with carbonate or silicate from the moment of dilution.

Heat a liter bottle of hard glass under the hot-water tap, and pour into it a liberal amount of melted paraffin (of melting point not less than 55° C.). When the paraffin is on the verge of beginning to solidify, pour it out slowly, rotating and tipping the bottle so that a layer thick enough to prevent seeing through the glass is deposited over the entire inner surface. After the paraffin lining has become solid, remelt the paraffin that has been poured out, and transfer enough of it to the bottom of the bottle to form a layer 1 cm. thick. This is best done by pouring through a warmed long-stemmed funnel, otherwise some of the lining on the neck and side-walls may be destroyed.

After the bottle has become thoroughly cool, nearly fill it with distilled water. Insert a 2-hole rubber stopper bearing (*a*) a

soda-lime tube, and (*b*) a paraffined glass tube long enough to reach nearly to the bottom of the bottle, and drawn out at the upper end (which should project several cm. beyond the stopper) to a bore of about 1 mm. Seal in the stopper with paraffin, and attach a short piece of small rubber tubing to the projecting end of the glass tube.

Prepare a saturated solution of sodium hydroxide by dissolving C. P. alkali in water in a large nickel crucible or a platinum dish. (The sodium hydroxide now obtainable in pellet form is easier to handle than the sticks.) Cool the solution in a dish of water, and remove the carbonate by suction filtration, using either a Büchner funnel with hardened paper or, better, a Jena filter with sintered glass disk (discarding the first portion of the filtrate, which may contain silicate extracted from the disk). Collect the remainder of the filtrate in a test-tube set inside the suction flask.

Transfer enough of the filtered alkali solution to make a liter of approximately 0.02 N solution to the paraffined bottle, by running it through the rubber tubing from a graduated pipette. Immediately introduce a few cc. of water from a pipette in the same way. Close the open end of the rubber tubing with a piece of thin glass rod, and mix thoroughly by rotating the bottle. To insure complete mixing, it is advisable to let the solution stand over night. Before using, draw out a few cc. through the rubber tubing with a pipette, and discard.

The standard alkali is withdrawn as needed by connecting the tip of a micro burette (p. 97) with the rubber tubing, and applying suction at the upper end of the burette through a rubber connection.

The titration of standard hydrochloric acid with 0.02 N alkali prepared in this manner should give the same result (within 0.1 or 0.2 per cent) whether the indicator used is methyl red or phenolphthalein.

Method for the Determination of Reducing Sugar Materials in Normal Urine.—(*J. Biol. Chem.*, 1922, 51 : 209.)—The colorimetric method for the estimation of sugar in blood is equally applicable to the determination of sugars in urine, provided that other disturbing substances present in urine are first removed. This removal is adequately accomplished by shaking

the urine with Lloyd's "alkaloid reagent," which is made by J. U. Lloyd, Cincinnati.

The process is as follows: To 5 cc. of urine add 5 cc. tenth normal oxalic acid and 10 cc. of water. Add 1.5 gm. of Lloyd's reagent and shake gently for 2 minutes. Filter. Two cubic centimeters of the filtrate is the usual amount used for the sugar determination. The above mentioned dilutions are for concentrated urines. With more dilute urines, one takes 10 or 15 cc. and reduces the amount of water taken.

The colorimetric determination of the sugar in the filtrate is made in exactly the same manner as in the case of blood filtrates. See p. 301.

The standard sugar solutions to be used are the same as for the blood; namely, such as contain 0.1 and 0.2 mg. of glucose per cc.

Calculation.—For the colorimetric reading the standard is usually set at 20 mm. If 2 cc. of filtrate is used, 20 divided by the reading times the value of the standard employed gives the sugar, in milligrams, present in 0.5 of a cubic centimeter of urine.

Colorimetric Determination of Sugar in Diabetic Urine.—The macro titration method for use with undiluted diabetic urine (see page 95) is very satisfactory, particularly with reference to the sharpness of the end-point, and the absence of reoxidation, which is so disturbing when sugar is titrated in beakers or porcelain dishes. The drawback to that method is that it requires special reagents and equipment not used for any other purpose. Attention is therefore called here to the fact that the sugar in diabetic urines or other "sugar urines" can readily be determined colorimetrically by the same method as is used for normal urine and for blood filtrates. For the most accurate results, especially with urines containing little sugar, less than 2 per cent, the preliminary extraction with Lloyd's reagent, as described for normal urine, should not be omitted. But when the preliminary qualitative test has indicated the presence of much sugar that treatment may be omitted and the urine simply diluted.

A dilution of 1 to 1000 is suitable for urines containing from 3.5 per cent to 10 per cent of sugar. For weaker urines take 2 cc. (or 10 cc. of the filtrate from the extraction with Lloyd's reagent). Half fill a volumetric liter flask with tap water. With

a Folin-Ostwald pipette introduce 1 cc. of the strong diabetic urine, or 2 cc. of weak urine (the latter preferably in the form of filtrate from Lloyd's reagent). Dilute to volume with tap water, mix thoroughly.

Transfer 2 cc. and 1 cc. plus 1 cc. of water to Folin-Wu blood sugar tubes and finish the determination in the same way as for blood sugar (page 301).

Colorimetric Method for the Determination of the Amino Acid Nitrogen in Urine.—For a description of the various reagents involved in this determination, see the description of the method as given for the determination of the amino acid nitrogen in blood.

Due to the shade of color produced in an amino acid nitrogen determination in urine, the standard used for the comparison should contain only glutamic acid. The standards are prepared by diluting the stock glutamic acid solution to solutions of required strengths with 0.07 N HCl containing 0.2 per cent sodium benzoate. The presence of interfering substances in urine which limit the range of true proportionality in this determination makes it necessary to prepare several standards for each determination and to use for the comparison the standard in which the amount of color is nearest to that of the unknown. The standards that are convenient to use contain 0.03, 0.04, and 0.05 mg. of amino nitrogen as glutamic acid.

For the colorimetric determination of the amino acid nitrogen in urine it is necessary first to remove the ammonia.

The removal of the ammonia is easily accomplished by means of the aeration method described for the determination of ammonia in blood (page 331).

The procedure is as follows:

Pipette 10 cc. of urine into a 300 cc. Erlenmeyer flask, add 2 to 3 drops of phenolphthalein and 1 cc. of 5 per cent sodium hydroxide solution. Blow air over the urine for about 20 minutes. Then neutralize the solution with either dilute H_2SO_4 or HCl until the pink color just disappears. Transfer quantitatively the contents of the Erlenmeyer flask to a 100 cc. volumetric flask, dilute to volume with distilled water, and mix.

The amount of this diluted urine required for each determination may be quite variable. Usually 2 cc. or 3 cc. are satis-

factory. To test-tubes graduated at 25 cc., add 2 cc. and 3 cc., respectively, of the diluted urine. To these add 1 cc. of 0.07 N HCl, to balance the acidity of the standard, 2 cc. of the 1.5 per cent borax solution, 7 cc. and 6 cc. of water, respectively, 2 cc. of a freshly dissolved 0.5 per cent solution of the amino acid reagent, and mix well.

To other test-tubes graduated at 25 cc., add 1 cc. each of the 0.03, 0.04, and 0.05 mg. standards. To these add 2 cc. of 1.5 per cent borax, 9 cc. of water, 2 cc. of the amino acid reagent and mix thoroughly.

Let the tubes stand in a dark place for 18 to 24 hours. It is often advisable to take out the test-tubes and inspect them after they have stood for 15 to 30 minutes. If the tube containing the urine appears much darker than the darkest standard, or lighter than the lightest standard, it will be necessary to start another sample, using more or less of the ammonia-free diluted urine, as the case may call for.

After the 18 to 24 hour period of standing, add to each test-tube 2 cc. of the acid formaldehyde solution and 2 cc. of the 0.1 M sodium thiosulfate solution. Dilute to a volume of 25 cc. and mix. Allow to stand for 4 to 5 minutes to permit the bleaching of the excess amino acid reagent. The color comparisons are made in the usual manner by the use of a colorimeter.

For the calculation it is, of course, essential to know which standard is used and the actual volume of undiluted urine taken for the determination.

The volume of diluted urine used in this determination should contain between 0.03 and 0.05 mg. of amino nitrogen. Although the amino acid nitrogen may be determined in solutions of greater strength, it is desirable to use as dilute a urine as practical, due to the fact that urea inhibits somewhat the color production. This effect is minimized by dilution. Too high a dilution should also be avoided.

The amino acid nitrogen in urine is subject to wide variations, but it may often reach values one half as great as those of the ammonia. The so-called formol titration for the determination of ammonia in urine gives therefore necessarily very unreliable figures.

BLOOD

The first step in the analysis of blood is usually the preparation of an extract or filtrate which contains the product or products to be determined, and which at the same time contains as little as possible of other interfering materials. This preliminary step involves the quantitative removal of all the various protein materials in the blood cells and the plasma. No single protein precipitant is serviceable even for the isolation of all the water soluble products. But the most extensively used procedure for this purpose is the tungstic acid precipitation introduced by Folin and Wu in 1919 as part of their "System of Blood Analysis." The filtrate from this precipitation has been used for the determination of nonprotein nitrogen, urea, uric acid, creatinine, creatine, amino acids, chlorides, and sugar. This filtrate is still used more extensively than any other for the determinations mentioned above. In 1930, however, this precipitation method was altered in a technically simple, but theoretically important way (*J. Biol. Chem.*, 1930, 86: 173).

In the original method the blood is first laked by diluting it with 7 volumes of water; in the new method the laking is prevented by diluting the blood with a slightly hypertonic solution. The change was introduced originally as an improvement in the method for the determination of uric acid, because it was found that the red blood cells, when laked, set free materials which not only gave a color with the uric acid reagent, but which also greatly reduced the color obtainable from uric acid, so that one could not know just what was being determined. It was found, further, that the partly unknown materials set free from the broken down blood cells had been responsible for large errors in the determinations of the blood sugar, the creatinine, and particularly the non-protein nitrogen. Doubtless there is still room for differences of opinion as to whether the indiffusible, fixed materials of the red blood cells should or should not be included

in some of the analyses, but the view taken here is that the inclusion of suddenly disintegrated cell materials does not belong in the determination of the levels at which definite well known materials are being transported to and from tissues. The fixed constituents of the white cells are excluded even in the original method.

Collection of the Blood.—Blood intended for analysis should not be hemolyzed to any extent and must not be partly clotted. The prevention of hemolysis involves only the exclusion of wet syringes and wet containers for the collection of the blood.

The prevention of clotting without introducing substances which interfere with the analysis is a little more complicated. Citrated blood is almost unusable for most purposes because of the large amount of citrate required to prevent clotting. Potassium oxalate is the most commonly used anticlotting reagent, and it is perfectly good, if too much is not used. 2 mg. of potassium oxalate for each cc. of blood is ample to prevent clotting, if the collected blood is promptly and adequately mixed with it. 4 mg. per cc. is apt to give turbidity in the uric acid determinations, and if more than that amount is taken, the protein precipitation will be incomplete.

The most convenient effective procedure for supplying clinicians with suitable amounts of oxalate is to supply it in the form of small strips of cloth impregnated with the requisite amount of the salt.

Dissolve 50 gm. of potassium oxalate in 250 cc. of water in a large porcelain dish. Cut about 100 gm. of bird's-eye cotton cloth (diaper cloth or old toweling) into strips about 10 cm. by 40 to 50 cm. Soak these strips in the solution by drawing them through it once, as for the making of litmus paper or urease paper. Let each strip drain for about a minute, then hang it up to dry over two parallel strings about 15 to 20 cm. apart.

The weight of the cloth before and after drying will show closely enough the per cent of oxalate in it. A very small piece of the cloth (50 mg.) will be adequate for 15 to 20 cc. of blood.

The preparation of similar strips impregnated with lithium oxalate, in some respects better than potassium oxalate, was described in earlier editions of this Manual.

Blood should never be *shaken* with an anticlotting reagent. 8 to 10 gentle inversions of the test-tube or flask are adequate, and produce no foam.

Preparation of Protein-free Filtrate from Laked Blood.—Reagents required for the precipitation of the proteins:

1. ⅔ N sulfuric acid.
2. A 10 per cent solution of sodium tungstate.

This solution should be slightly, but permanently alkaline to phenolphthalein. At the present time (1933) Eimer and Amend's best grade of sodium tungstate is serviceable. Merck's blue label sodium tungstate, which we formerly used, is now less good.

Transfer 5 cc. of oxalated non-clotted blood to 35 cc. of water in a small flask. Add 5 cc. of the tungstate solution and finally add, rather slowly, and with shaking, 5 cc. of ⅔ N sulfuric acid. Close the mouth of the flask and shake hard. If the conditions are right, scarcely a single air bubble will form as a result of the shaking. Let stand for 5 to 10 minutes and filter. The filtrate must be water-clear, though a very slight trace of a secondary precipitate may form later. This precipitate can be ignored.

If too much oxalate is present, foaming on shaking and incomplete precipitation will be encountered. Some such mixtures may be saved by the addition of a little more acid, but some determinations, including that of the sugar, may be affected by the resulting greater acidity of the filtrate.

Preparation of Protein-free Filtrate from Unlaked Blood.—Reagents required for the protein precipitation:

1. 1/3 N sulfuric acid.
2. A solution containing 6 gm. of sodium tungstate and 15 gm. of anhydrous sodium sulfate per liter.

The sodium sulfate must be neutral and the sodium tungstate slightly but permanently alkaline to phenolphthalein. Merck's blue label sodium sulfate and Eimer and Amend's best grade of sodium tungstate fulfil the requirements.

Transfer 40 cc. (8 volumes) of the tungstate-sulfate solution to a small flask; with a 5 cc. pipette, or "blood pipette," add 5 cc. (1 volume) of blood. Mix without any rough shaking and let stand, with occasional gentle shaking, for 5 minutes, or as much longer as may be convenient. At the end of the diffusion period add with a pipette, with gentle shaking, 5 cc. (1 volume) of 1/3 N sulfuric acid. Continue the gentle shaking for another minute, then centrifuge or filter without delay.

The filtration in this case is much more rapid and can be made to yield more filtrate than is obtained from the laked blood filtrations, but it is also subject to specific difficulties. The intact corpuscles in the unlaked blood mixture quickly clog up the pores of the filter paper, and the filtration stops at the end of about 15 minutes, yielding less than 20 cc. of filtrate. A prolonged filtration period is further excluded by the fact that in 30 to 35 minutes the cells begin to disintegrate and the albuminous material set free at this stage will pass through the paper. These difficulties are not insurmountable. By using funnels coated on the inside with high melting paraffin (m.p. 50° C.), both the hemolysis and the clogging of the filter pores are delayed, so that one gets about 30 cc. of filtrate in 35 minutes. By using so-called folded filters (Green's No. 588, diam. 15 cm.) with or without paraffined funnels one can get from 35 to over 40 cc. of filtrate in from 20 to 35 minutes. In routine work it is not safe to continue the filtering for more than 25 or 30 minutes. In these filtrations only 2 or 3 cc. should be added at first and, if turbid, the first few drops of filtrate should be discarded.

Although less tungstate and only one half as much acid are used for the preparation of unlaked blood filtrates as for the preparation of laked blood filtrates, the former are somewhat more acid than the latter. The titrateable acidity of 10 cc. of (hot) unlaked blood filtrate with phenolphthalein as indicator is equivalent to from 0.6 to 0.7 cc. of 0.1 N alkali. The titratable acidity of plasma filtrates is just about the same.

Preparation of Protein-free Plasma Filtrates.—Plasma filtrate may be prepared by exactly the same procedure as is used for the unlaked blood except that the waiting period introduced before adding the 1/3 N acid is omitted.

Plasma filtrate can be prepared also by first diluting it with 7 volumes of water, then adding 1 volume of 5 per cent sodium tungstate and adding, with shaking, 1 volume of 1/3 N sulfuric acid.

The only marked difference between these two filtrates is in their sodium sulfate content, the first containing more than the second. This difference is of little or no consequence in connection with any of the analyses.

Plasma, because of its relatively low total protein content,

can be made to yield more concentrated filtrates than are readily obtainable from whole blood. A filtrate representing a dilution of 1 in 5 can be made as follows: To 5 cc. of plasma and 10 cc. of water add 5 cc. of 5 per cent sodium tungstate solution and 5 cc. of 1/3 N sulfuric acid, shake and filter.

Determination of Non-protein Nitrogen.—For the digestion of 5 cc. of blood filtrate it is not necessary to use more than one-half cubic centimeter of the phosphoric-sulfuric acid mixture described on pp. 65–67. Dilute 50 cc. of the acid mixture with 50 cc. of water, and keep well protected to prevent the absorption of ammonia. Use 1 cc. for each digestion.

The digestion is most conveniently made in ignition test-tubes (pyrex, 200 mm.×25 mm.) which have been graduated at 35 cc. and at 50 cc.

Transfer 5 cc. of the blood filtrate to such a test-tube. The test-tube should either be dry or rinsed with alcohol to reduce the danger of bumping. Add 1 cc. of the diluted acid mixture and a quartz pebble. Boil vigorously over a micro burner until the characteristic dense fumes begin to fill the tube. This will happen in from 3 to 7 minutes, depending on the size of the flame. When the test-tube is nearly full of fumes, reduce the flame sharply so that the speed of the boiling is reduced almost to the vanishing point. Cover the mouth of the test-tube with a watch glass. Continue the gentle heating for 2 minutes, counting from the time the test-tube became filled with fumes. If the oxidations are not visibly finished at the end of two minutes, the heating must be continued until the solution is nearly colorless. Usually the solution becomes colorless at the end of 20 to 40 seconds. At the end of 2 minutes remove the flame and allow the digestion mixture to cool for 70 to 90 seconds. Then add 15 to 25 cc. of water. Add 1 or 2 drops of gum ghatti solution. Cool further, approximately to room temperature, and then fill to the 35 cc. mark with water. Add 15 cc. of Nessler's solution (p. 337). Insert a clean rubber stopper and mix. If the solution is turbid, centrifuge a portion before making the color comparison with the standard.

The standard most commonly required is 0.1 mg. N. Transfer 5 cc. of the standard ammonium sulfate (containing 0.02 mg. N per cc., made by diluting 2 cc. of stock solution to 100

cc.) to a test-tube, graduated at 35 cc. and 50 cc. Add 1 cc. of the phosphoric-sulfuric acid mixture to balance the acid in the unknown, and 2 to 4 drops of gum ghatti solution. Fill to the 35 cc. mark with water. Add 15 cc. of Nessler's solution and mix. The unknown and standard should be Nesslerized simultaneously.

Calculation.—If the standard is set at 20 mm. for the color comparison, 20 divided by the reading and multiplied by 0.2 gives the non-protein nitrogen in 1 cc. of blood, because 0.5 cc. is the amount of blood represented in 5 cc. of the blood filtrate.

The non-protein nitrogen per 100 cc. of blood is, therefore, 20 divided by the reading and multiplied by 0.2×100, or 20.

If a standard containing 0.2 mg. N is used, the calculation becomes 20 divided by $R \times 40$.

Many seem to have trouble in obtaining perfectly clear solutions when Nesslerizing the digestion mixtures obtained with blood filtrates. The cause is lack of suitable alkalinity in the Nessler solution. The following data will help to overcome the difficulty:

Twenty cubic centimeters of normal hydrochloric acid may be titrated with the Nessler solution, and if the solution is substantially correct, a good end-point will be obtained at 11 to 11.5 cc., with phenolphthalein as indicator. If an end-point is obtained much below 11 cc., as at 9.5 cc., the Nessler solution is too alkaline and turbidity is likely to occur.

Turbidity, due to excess of alkalinity, may likewise be produced because the sulfuric-phosphoric acid mixture is too weak. If 5 cc. of the dilute acid (1 : 1) are further diluted 10 times (to 50 cc.), 10 cc. of the solution so obtained, when titrated with the Nessler solution and phenolphthalein as indicator, should give a fairly good end-point at 9 cc. to 9.3 cc.

Several (European) investigators have published modifications of the method described above, the common object of which has been to avoid turbidity in the Nesslerization, without recognizing that the cause of their turbidities has been an unsuitable relationship between the acidity of their digestion mixtures and the alkalinity of their Nessler solutions. By the addition of a little gum ghatti solution as now recommended most of the turbidity difficulties should disappear. But it is still more or less

important that the addition of the Nessler reagent to the acid digestion-mixture should yield a solution of the right range of alkalinity.

The colored turbidity due to incorrect Nesslerization should not be confused with the white sediment (silica) which is obtained by excessive digestion and which can be removed by centrifuging. This sediment is often due to the use of unsuitable flames during the digestion. A small micro Bunsen burner (Eimer and Amend catalogue No. 19270) is very nearly essential for this digestion. Overheating should be particularly superfluous when making nonprotein nitrogen determinations on filtrates from unlaked blood, because with such filtrates there is almost no charring and the digest becomes clear and colorless almost as soon as the white fumes begin to fill the test-tube.

In normal human *unlaked* blood the non-protein nitrogen is approximately 10 mg. lower than in *laked* blood, the average being about 20 mg. instead of 30 mg. per cent. This large difference is mostly due to the fact that nearly the whole of the so-called undetermined nitrogen of laked blood is excluded from unlaked blood filtrates. The upper strictly normal level of non-protein nitrogen in unlaked blood should be below 30 mg., probably even below 25 mg., in persons whose 24-hour urinary nitrogen does not exceed 12 gm.

Gum Ghatti Solution.—Gum ghatti is not only much more effective than gum arabic as a protective colloid, but unlike the latter it is practically free from ammonia. The required solution is prepared as follows:

Fill a 500 cc. cylinder with distilled water and suspend at the top, just below the surface, in a wire basket of galvanized iron, 10 gm. of the gum. Leave it to dissolve over night, but not for 24 hours. Then remove the wire basket with the remaining undissolved material. A little dirt may escape into the solution when the wire screen is removed, but this material soon settles, and the clear solution can be used without further purification. After the solution has been transferred to a bottle, add 0.4 to 0.5 gm. of benzoic acid dissolved in 5 cc. of alcohol and shake at once. This will keep out moulds. The gum ghatti can be obtained from Howe and French, in Boston, or from Eimer and Amend, in New York.

Micro Method for the Determination of Non-Protein Nitrogen.—The method described here is based on the use of 0.2 cc. of blood. It is doubtless possible with slight modifications to apply the method to 0.1 cc. of blood, but it is not sound practice to use only 0.1 cc. if the larger amount can be obtained; hence it seems better to base the description on 0.2 cc. Those who wish to use the smaller amount will need to be doubly careful about the purity of the chemicals.

The following reagents are needed for the non-protein nitrogen determination.

1. Sulfate-Tungstate Solution.—Transfer to a 500 cc. volumetric flask 10 gm. of C.P. anhydrous sodium sulfate and 15 cc. of 10 per cent sodium tungstate solution. Half fill the flask with distilled water and shake until the sulfate has dissolved. Dilute to volume and mix.

2. Sulfuric Acid.—Dilute 12 cc. of 2/3 N sulfuric acid to 100 cc. and mix.

3. Mixture of Sulfuric Acid, Phosphoric Acid, and Copper Sulfate.—Transfer first about 50 cc. of water to a 250 cc. volumetric flask, then introduce in the order named, 15 cc. of 85 per cent phosphoric acid, 10 cc. of concentrated sulfuric acid, and 5 cc. of 5 per cent copper sulfate solution. Cool the mixture and dilute to volume. This acid mixture should be kept in a glass-stoppered flask so as to protect it from contamination. The sulfuric acid is the most likely to contain some nitrogen; we use the Baker and Adamson brand.

4. Standard Ammonium Sulfate Solution.—From the regular standard ammonium sulfate solution used for the ordinary non-protein nitrogen method, dilute 1 mg. of ammonia nitrogen to a volume of 100 cc.

5. Regular Nessler Reagent.

Determination.—Transfer 4 cc. of the sulfate-tungstate solution to a clean, dry 15 cc. centrifuge tube. With a micro blood pipette, graduated in 0.1 cc. and 0.2 cc., introduce 0.2 cc. of blood, not omitting the rinsing with the solution in the tube. Stir with the pipette and let stand for 15 minutes (longer standing does no harm). At the end of about 15 minutes add 1 cc. of the dilute sulfuric acid solution. Stir carefully, but rather thoroughly, and centrifuge at a fairly good speed for 5 minutes. Decant the colorless clear supernatant solution and transfer 4 cc. of it to a

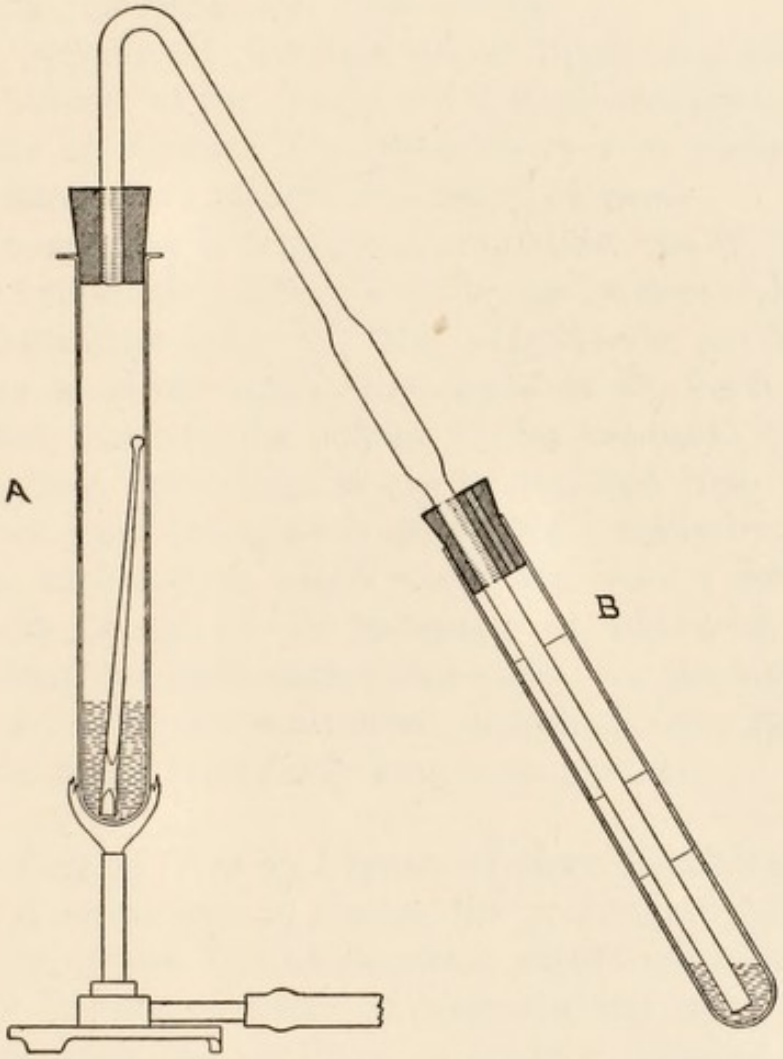
pyrex test-tube, graduated (all around) at 25 cc. Add 1 cc. of the sulfuric acid-phosphoric acid mixture, also an antibumping tube of pyrex glass. Boil off the water and finish the digestion as in the regular Folin-Wu digestion for non-protein nitrogen. Let cool for about 40 seconds and add 3 to 4 drops of water. Then add about 5 cc. more of water and again apply the flame and boil the mixture for about 1 minute to clean out the antibumping tube. Remove the antibumping tube, rinsing with a few cc. of water. Cool the mixture, add 2 drops of gum ghatti solution, and dilute to a volume of 18 to 20 cc. Transfer to another graduated test-tube 4 cc. of the standard ammonium sulfate solution and 1 cc. of the sulfuric acid-phosphoric acid mixture. Add 2 drops of gum ghatti solution. Dilute to about 18 cc. Finally, Nesslerize the solutions at about the same time with 4 cc. of the Nessler reagent. Mix and make the color comparison.

Calculation.—The standard, 0.04 mg. of N, corresponds to 25 mg. per cent of non-protein nitrogen, since the actual analysis is made on the extract corresponding to 0.16 cc. of blood. $\frac{0.04 \times 100}{0.16} = 25$. $\frac{20}{x}$ times 25 gives, therefore, the non-protein nitrogen in mg. per cent when the standard is set at 20 mm. and x is the reading of the unknown. This calculation is not strictly accurate because no allowance has been made for the volume of the added blood.

If the Nesslerized unknown is seen by inspection to be much stronger than the standard, Nesslerize immediately another standard containing 0.08 mg. of nitrogen plus 1 cc. of the acid mixture, and then wait 15 minutes before making the color comparison.

One step in the process described above calls for some discussion. It is practically impossible to boil off the water for the Kjeldahl digestion unless really effective means are used to prevent the occurrence of explosive bumping. The antibumping tube will prevent the explosions, but it is also important properly to regulate the flame from the micro burner, particularly when the mixture is just beginning to boil. Some adequate arrangement for securing perfect control over the gas flame is therefore important. The filtrates from unlaked blood normally contain less organic matter to be destroyed than do filtrates from laked

blood. The digestion takes, therefore, very little time after the water has been driven off. Unless much sugar is present the mixture becomes colorless almost as soon as the white fumes begin to appear in the tube, and 15 seconds later the flame may be removed. The antibumping tubes used in this determination should be longer than the ones used in the urea determination because they must be removed before one dilutes to volume.



Note anti-bumping tube inside tube A

FIG. 8.

Determination of Urea.—It is unfortunate that accurate urea determinations can not be made by direct Nesslerization of hydrolyzed blood filtrates. The reason why this can not be done is not so much due to the insidious turbidities, for these can be largely eliminated by the use of gum ghatti, but rather because of the presence of relatively large amounts of nitrogenous mate-

rials which have a profound effect both on the quality and on the quantity of color which the urea-ammonia gives with Nessler's reagent. Since the preliminary isolation of the ammonia is necessary, and since beginners find this isolation somewhat difficult, it seems best to describe the method in considerable detail. The problem is essentially how to transfer by distillation from one test-tube to another the small amounts of ammonia (the urea-ammonia from 0.5 cc. of blood). The inherent difficulties are foaming, bumping, and back-suction.

1. To prevent the foaming, use 2 drops of a solution containing 1 volume of the crudest mineral oil obtainable and about 10 volumes of toluene. The heavy oil used in oil-burning furnaces or heavy black machine oil should be used.

2. The bumping is best prevented by the use of the recently described antibumping tube. This tube can be seen inside Tube A in the illustration (Fig. 8). The effectiveness of this tube is due to the small air bubble imprisoned in the lower open part of the tube, touching the bottom of the test-tube. It should be of pyrex glass, preferably. It can be obtained from Eimer and Amend, but from soft glass it can be made easily by any one.

3. The back-suction which completely ruins a determination is primarily caused by the bumping. As additional protection against disastrous back-suction, one should use the kind of delivery tube shown in the illustration; suitable delivery tubes of this sort can be made from fairly long 5 cc. pipettes.

Hydrolysis of Urea by Urease.—Before describing the determination it seems best to discuss the preliminary hydrolysis of the urea by urease. Too much urease should not be used, partly because it introduces traces of ammonia and partly because it greatly increases the tendency to produce foaming during the distillation. 1 cc. of 2.5 per cent jack bean meal extract is enough for 5 cc. of blood filtrate. This extract is made as follows: Transfer 0.5 gm. of jack bean meal to a clean 50 cc. flask; add 20 cc. of 30 volume per cent alcohol (30 cc. alcohol diluted to 100 cc.). Shake for 10 minutes and filter or centrifuge. This extract must be made fresh (on the same day) because it develops ammonia if kept more than a few hours.

Instead of using freshly prepared urease extract, one can use permanently stable *urease paper*, prepared as follows:

Transfer to a clean 200 cc. flask 30 gm. of jack bean meal and 100 cc. of 30 volume per cent alcohol. Add also 1 cc. of the acid acetate buffer mixture described on page 151. Stopper tightly and shake vigorously, for at least 5 minutes, and then shake less hard for 10 minutes. Filter or centrifuge in centrifuge tubes covered with tin foil. The centrifuging will take 30 minutes, if the whole mixture can be centrifuged at once; the filtration will take about 2 hours. It does not pay to try to secure the maximum amount of extract by either method.

Transfer the extract to a perfectly clean porcelain dish and take it up at once on strips of rather heavy filter paper, such as S. and S. No. 597. The strips should be about 5 cm. wide. These strips are drawn through the urease solution, drained for a few moments, and air-dried while hanging over two parallel threads about 15 cm. apart, in a place protected from air currents. As soon as the strips are dry cut them into pieces about 1 cm. by 2.5 cm., and preserve in wide mouth bottles.

The Determination.—Transfer 5 cc. of the blood filtrate to a pyrex or Jena test-tube, having a capacity of about 30 cc. Add 2 drops of the acid acetate buffer mixture (containing 15 gm. of sodium acetate and 1 cc. of 99 per cent acetic acid in 100 cc.). Then add 1 cc. of the urease solution or a piece of urease paper. Insert a cork and digest at room temperature for 25 minutes or at 45° for 10 minutes. If the urease paper is used, it is absolutely essential that the test-tube be shaken during the digestion period. Cool the tube (if warm), remove the stopper and add (a) an antibumping tube, (b) 2 drops of antifoaming mixture and, finally, (c) 2 cc. of 4 per cent borax solution. Connect at once with the delivery tube and a graduated test-tube receiver containing 2 cc. of 0.05 N acid. Fasten the boiling tube in a clamp, and make sure that the delivery tube reaches the acid in the receiver B. It is convenient, but not essential, that the receiver be held in place by means of a two hole rubber stopper as shown in the illustration.

Start the distillation by the help of a small responsive Bunsen micro burner. As soon as the contents are nearly boiling, cut the flame down sharply, so that the first minute of boiling is very gentle. Then boil briskly for about 3 minutes, and finally, boil for a moment with the delivery tube just above the liquid in the

receiver. The total boiling period from the first beginning of boiling should be only 4½ to 5 minutes.

Transfer 0.1 mg. of ammonium sulfate nitrogen to another test-tube like the receiver, both graduated at 25 cc., add 2 drops of gum ghatti solution to each, dilute to a volume of about 20 cc., and add 2.5 cc. of Nessler reagent. Dilute to volume, mix, and make the color comparison.

The standard corresponds to 20 mg. per cent of urea nitrogen. Normal blood filtrates will therefore be weaker than the standard. If more than 40 mg. of urea nitrogen is found, the determination should be repeated with less than 5 cc. of the filtrate plus water enough to make a volume of 5 cc.

Urea Determination by Means of the Autoclave.—When a large number of urea determinations are to be made or when creatine determinations are also made, it is sometimes convenient to decompose the urea of the blood filtrate by heating under pressure. To 5 cc. of the blood filtrate in a large test-tube add 1 cc. of normal hydrochloric acid, cover with tin foil, and heat to 150° C. for 10 minutes. Distill off the ammonia exactly as in the preceding process, except that 2 cc. of 10 per cent sodium carbonate must be substituted for the borax, because of the added hydrochloric acid.

Aeration Process in Urea Determination.—The removal of the ammonia formed from the blood urea by urease, or by heating under pressure, can, of course, be driven into the receiver by an air current plus an alkali, instead of by the distillation process described above. The aeration process gives perfectly reliable results.

To the decomposed blood filtrate in a large test-tube add a little paraffin oil and 1 or 2 cc. of 10 per cent sodium hydroxide. Connect with a receiver and finish the aeration process as described for the colorimetric determination of ammonia in urine.

Preformed Creatinine.—It has not been proved that the creatinine reaction obtained with alkaline picrates and blood filtrates is due only to creatinine, but the determination is, nevertheless, important, because of its extensive use in renal function tests after the administration of creatinine.

The values obtained from laked blood filtrates are undoubt-

edly too high, but it is by no means clear that the same statement holds true for filtrates obtained from unlaked blood. The character of the reaction as obtained with such filtrates under the new conditions selected here for the determination of the creatinine is certainly more in harmony with the view that the only chromogenic substance determined in the filtrates is creatinine.

The creatinine in blood is remarkably constant in the neighborhood of 1 mg. per 100 cc. and much higher values are found only in cases of renal insufficiency.

The Determination.—Reagents:

1. Standard Stock Solution of Creatinine. This is the regular solution used in urine analysis. It contains 1.61 mg. of creatinine zinc chloride or 1 mg. of creatinine per cc. The solvent is 0.1 normal hydrochloric acid.

2. Standard Creatinine Solution. 1 cc. of the regular standard used in urine analysis, containing 1 mg. of creatinine is diluted with water to 100 cc.

3. Sodium Picrate Solution. 1 per cent.

4. Sodium Hydroxide Solution. 2 per cent.

Transfer to two clean and dry test-tubes 2 cc. and 4 cc. of the standard creatinine solution and dilute to 20 cc. Transfer 10 cc. of blood filtrate to another similar test-tube. To each of the standards add 2 cc. of the picrate solution and add 1 cc. to the blood filtrate. Then add 2 cc. of the alkali to each standard and 1 cc. to the blood filtrate. Mix by a couple of inversions and let stand for not less than 30 minutes. When the unknown is obviously much darker than the stronger standard the waiting period should be extended to about 1 hour. Toward the end of the waiting period, if the unknown should be very much darker than the stronger standard, dilute it to the same volume as the standard by the addition of 10 cc. of water, 1 cc. of picrate solution, and 1 cc. of the alkali, and mix again before making the color comparison.

Calculation: $\frac{20}{x}$ multiplied by 1 or 2 (or 4, if the unknown has been diluted) gives the creatinine in mg. per 100 cc. of blood.

When working with plasma or serum, it is often more satisfactory, for creatinine determinations, to secure filtrates repre-

senting a dilution of 1 in 5, as indicated on page 263. With such more concentrated filtrates one must employ twice as much creatinine in the standard (4 cc. and 8 cc. of the standard creatinine solution) and the calculation must be altered to correspond.

In connection with experiments requiring several successive creatinine determinations, as in renal function tests, it may be necessary to work with less than 10 cc. of filtrate. It is not very practical to work with smaller total volumes of liquid than 10 to 12 cc. in the ordinary type of colorimeter, but almost equally precise work can be accomplished with only 3 or 4 cc. of liquid by using a suitable type of colorimeter. The modifications in the creatinine method which are called for in connection with such micro determinations are so obvious as to require no special description.

Determination of Creatinine plus Creatine.—This determination is of doubtful validity, because it is not certain that the required hydrolysis does not yield other chromogenic substances besides creatinine. The method is still useful, however, at least in connection with experiments involving the administration of creatine.

Transfer 5 cc. of unlaked blood filtrate to a test-tube graduated at 25 cc. Add 2 cc. of 0.1 N hydrochloric acid. Cover the mouth of the test-tube with tin foil and heat in an autoclave at 130° C. for 20 minutes (or in connection with simultaneous urea determinations to 155° C. for 10 minutes). Cool.

Transfer 0.04 mg. and 0.08 mg. of creatinine to graduated test-tubes, add 2 cc. of 0.1 N hydrochloric acid to each. Dilute the contents of the test-tubes to about 18 cc. Then add 2 cc. of picrate solution and 2 cc. of the 2 per cent alkali; dilute to volume, mix, and finish the determination as for preformed creatinine.

Preparation of Pure Sodium Picrate and Picric Acid.—It has been recognized for a long time that only purified picric acid should be used for the colorimetric determination of such small amounts of creatinine as are involved in blood analysis. The impurities which must be removed turn deep red when the picric acid solutions are rendered alkaline. The yellow color of even the purest picric acid shifts toward red upon addition of an alkali and the degree of the shift is more or less proportionate to the degree of alkalinity, but this unavoidable source of error is insignificant in comparison with the errors which may come in when crude picric acid is used. Since picric acid can best be

purified in the form of sodium picrate and since sodium picrate is fully as convenient and serviceable for the color reaction as the less soluble free acid, the latter is really superfluous for colorimetric creatinine determinations. The preparation of both is described here.

Sodium Picrate.—Transfer 500 gm. of moist picric acid to a Florence flask of 1500 cc. capacity. Add 500 cc. of acetone. Shake, with a little warming under hot tap water, until all the crystals have dissolved. Add 20 gm. of active charcoal (norit). Shake, and filter into another flask. During this filtration keep the funnel closed with a watchglass to prevent evaporation.

Dissolve 250 gm. of anhydrous sodium carbonate and 100 gm. of sodium chloride in 2500 cc. of warm water in a 4 liter beaker. While stirring with an agate-ware spoon, add the acetone solution gradually to the alkaline salt solution. When the reaction (CO_2 evolution) is finished, let stand, preferably in cold water, for about 30 minutes, and filter on a large Büchner funnel (diam. 20 cm.). Wash with about 2 liters of sodium chloride solution (7 per cent) and suck as dry as possible.

If the original picric acid is of good quality, the sodium picrate on the Büchner funnel will be pure, but it is a little safer to recrystallize it once as follows:

Return the precipitate to the 4 liter beaker and add 2 liters of boiling water and 20 gm. of sodium carbonate. To the resulting hot solution add gradually, with stirring, 150 gm. of sodium chloride, cool, filter, and wash as before with 7 per cent sodium chloride solution. Then wash once or twice with a more dilute sodium chloride solution (2 per cent) and finally wash once with methyl alcohol to remove most of the remaining chloride and water. Dry, either at room temperatures or over a radiator.

Test for the Purity of Sodium Picrate.—Make 100 cc. of a 3 per cent solution. Transfer 5 cc. and 10 cc. to test-tubes graduated at 25 cc. Dilute each to about 22 cc., add 2 cc. of 5 per cent sodium hydroxide, dilute to volume, mix, and let stand for 10 minutes. Then add 4 gm. of powdered potassium chloride, mix by inversion for about 1 minute, filter on a 9 cm. quantitative filter paper, and compare the two filtrates in the colorimeter. If the picrate is pure, the two filtrates will have the same color.

Picric Acid.—The process for the preparation of pure picric acid is exactly the same as described above, up to the final washing with methyl alcohol,—except that hardened filter paper should be used on the Büchner funnel.

One simply converts the purified sodium picrate in the Büchner funnel into picric acid by treating it with dilute hydrochloric acid.

Prepare at least 2 liters of such acid (1 vol. of conc. acid to 4 vols. of water). Disconnect the filtering flask from the suction pump. Pour the acid over the picrate. Stir up the precipitate with a porcelain or glass spoon, so as to make sure that the acid has acted on it all. Use plenty of the acid. Unchanged picrate can be distinguished by its darker color. When no more picrate is visible, connect again with the suction pump, and filter to dryness. Then wash 5 or 6 times with cold distilled water and suck as dry as possible. Temperatures up to 90° C. can safely be used for the drying of picric acid.

Uric Acid.—STANDARD URIC ACID SOLUTION.—The solution described here will keep for at least 5 years. Weigh out on a watchglass exactly 1 gm. of uric acid and transfer it to a not too small, dry funnel, inserted into a volumetric liter flask. Tap the funnel, so as to transfer nearly the whole of the uric acid to the flask. Transfer 0.6 gm. of lithium carbonate to a 250 cc. Florence flask, add 150 cc. of water; shake until solution is obtained (5 minutes). Some insoluble material remains and it is usually best to filter. Heat the solution or filtrate to 60° C. Also warm the liter flask under running warm water. Pour the warm lithium carbonate solution into the liter flask, incidentally washing into the flask the traces of uric acid which adhere to the watchglass and funnel. Shake so as to dissolve the uric acid promptly, a little additional warming under hot tap water is permissible. The lithium carbonate solution is not always perfectly clear, even when filtered, and one should not mistake this little turbidity for undissolved uric acid and keep warming and shaking too long. In 5 minutes all of the uric acid should be dissolved. Shake the flask under running cold water without undue delay. Add 20 cc. of 40 per cent formalin, and half fill the flask with distilled water. Add a few drops of methyl orange solution and finally add, from a pipette, rather slowly and with

shaking, 25 cc. of normal sulfuric acid. The solution should turn pink, while 2 or 3 cc. of acid are still left in the pipette, thus showing that the total acidity from adding 25 cc. of the acid is not great. Dilute to volume, mix thoroughly, and transfer to a clean, tightly stoppered bottle. This stock solution containing 1 mg. of uric acid per cc. should be kept away from light.

To prepare the working standard dilute 1 cc. of the stock solution, with water only, to 250 cc. It behaves exactly like a lithium carbonate solution of uric acid. This diluted solution should keep perfectly for at least two weeks.

Uric Acid Reagent, Free from Phenol Reagent.—The first step in the preparation should be to dissolve a few crystals of the tungstate and test the reaction with phenolphthalein solution to make sure that it is permanently alkaline to phenolphthalein. If it is not alkaline it is apt to be of poor quality in other ways, especially with respect to its molybdenum content, and at all events, it should first be rendered alkaline by boiling with a slight excess of sodium hydroxide.

Transfer 100 gm. of sodium tungstate (of the requisite alkaline reaction) and 150 cc. of water to a 500 cc. Florence flask. Dilute 20 cc. of phosphoric acid with 50 cc. of water and pour this gradually, and with shaking, into the tungstate-water mixture. Shake until the tungstate has dissolved and cool under running water. Pass H_2S into the solution for 10 minutes.

Transfer the solution to a 500 cc. separatory funnel and add (gradually at first), with gentle shaking, a total of 150 cc. alcohol. Shake vigorously for 7 to 8 minutes. Let the mixture settle, and then withdraw the more or less yellow bottom layer, the weight of which should be 160 to 170 gm.

Discard the highly colored upper layer and rinse the separatory funnel. Return the phosphotungstate solution, together with 100 cc. of rinsing water, to the separatory funnel. Add 75 cc. of alcohol and shake thoroughly as before. Withdraw the bottom layer, which should now be substantially colorless, into a weighed 500 cc. Florence flask and dilute the contents to a weight of about 250 gm. Boil vigorously for 5 minutes to remove the H_2S. Dilute again to a weight of 250 gm., and add 15 cc. of phosphoric acid (85 per cent). Boil with a reflux condenser for one hour. Remove the condenser, add a little liquid bromine or strong

bromine water, and boil another 5 minutes to remove the surplus bromine. Cool and dilute to a volume of 500 cc.

New Simplified Method for the Preparation of Uric Acid Reagent.—*Folin.*—The method described below is based on a recent (unpublished) discovery of how to prepare sodium tungstate completely free from molybdate. Mallinckrodt Chemical Company, St. Louis, are making such tungstate for the market and samples received from them have been satisfactory. It seems best, however, to give a brief description of the preparation of such tungstate.

Dissolve 1 kg. of sodium tungstate in 2 liters of water; add slowly and with stirring hydrochloric acid (dilution 1 to 1) until the solution is nearly neutral to litmus paper. Transfer the solution to a large flask or bottle. Pass a moderate H_2S current into the solution for 15 to 20 minutes. Stopper the container and let stand over night. By this treatment the molybdate is transformed into colored sulfomolybdates which are soluble in alcohol.

Transfer the solution to large beakers and add slowly and with stirring about 2/3 volume of alcohol. Let stand till the next day; decant and filter on a large Büchner funnel and wash with 50 per cent alcohol until the filtrate is colorless.

Transfer the precipitate to a 4 liter beaker, add 1.5 liter of water and about 2 cc. of bromine, and stir. Then heat over a burner and continue the stirring until the surplus bromine has disappeared. Continue the heating and add saturated clear sodium hydroxide solution until the mixture becomes *permanently* alkaline to phenolphthalein paper. Cool and filter, if necessary. Then precipitate as before with alcohol and dry.

From sodium tungstate entirely free from molybdate the uric acid reagent may be prepared as follows:

Transfer 100 gm. of the sodium tungstate to a 500 cc. Florence flask. Dissolve 32 to 33 cc. of 85 per cent phosphoric acid in 150 cc. of water. Pour this acid solution on to the tungstate and shake. Add a few pebbles and boil gently with a suitable condenser (a 10 cm. funnel and a 200 cc. flask filled with cold water) for 50 to 60 minutes. Decolorize with a little bromine water, boil off the surplus bromine, cool and dilute to a volume of 500 cc.

The uric acid reagent thus prepared can contain no phenol

reagent, but it may nevertheless give a slight color in the test described below with the cyanide solution.

The color so obtained is due to a trace of the hypersensitive uric acid reagent "A" described by Wu (*J. Biol. Chem.*, 1920, 43: 198) and its formation seems to be due to the use of a little too much phosphoric acid. If the test indicates more than an insignificant trace of the "A" reagent, add 5 gm. of sodium tungstate (but no more) to the solution and boil again for 10 to 15 minutes; then decolorize with bromine (if necessary), cool and dilute to volume as before.

Without testing for the blank color, we sometimes add that extra tungstate at the end of the first boiling period and boil another 10 minutes before decolorizing.

Test.—It is imperative that the uric acid reagent be completely free from phenol reagent, for otherwise it will yield disastrous blanks with the urea-cyanide solution. To 5 cc. of water in a test-tube add 4 cc. of the uric acid reagent and 10 cc. of the urea-cyanide solution. Mix and let stand for 15 minutes. The mixture will gradually turn yellow, but no blue color should form, if Merck's Reagent cyanide has been used for the preparation of the urea-cyanide reagent. If a blue color is obtained the most probable cause is the use of too much phosphoric acid in the preparation of the reagent.

Urea-cyanide Solution. — Transfer 75 gm. of Merck's Reagent sodium cyanide to a 2 liter beaker, add 700 cc. of water and stir until the cyanide is completely dissolved. Add 300 gm. of urea and stir. Then add 4 to 5 gm. of calcium oxide and stir for about 10 minutes. Filter at once if necessary for immediate use, but preferably not until the next day. Add to the filtrate about 2 gm. of powdered lithium oxalate, shake occasionally for 10 to 15 minutes and filter.

The *lithium oxalate* is prepared as follows:

Transfer 50 gm. of lithium carbonate and 85 gm. of oxalic acid to a 3 liter beaker. Pour on the mixture about 1 liter of hot water (70° C.). Stir cautiously to avoid loss by foaming until the evolution of CO_2 ceases. Add 1 liter of alcohol and filter on a Büchner funnel.

Revised Macro Method for the Determination of Uric Acid in Blood.—We now make the regular (macro) determination of uric acid in blood in the following manner:

Transfer 5 cc. of unlaked blood filtrate to a test-tube graduated at 25 cc., and transfer to another similar test-tube 5 cc. of the standard uric acid solution containing 5/250 milligram of uric acid. With a cylinder add 10 cc. of the urea-cyanide solution. Mix by whirling the test-tubes at an angle of about 60° C. Add 4 cc. of concentrated uric acid reagent. Let stand for about 20 minutes. Dilute to volume, mix, and make the color comparison.

$\frac{20}{x} \times 4$ gives the uric acid content of the blood in milligrams per cent when the standard is set at 20 mm. x represents the colorimetric reading of the blood filtrate.

The uric acid reagent should be added at the same time to the standard and the unknown, and the tubes should be in a vertical position when the reagent is added so that it does not flow down one side.

The maximum obtainable color is not quite reached during a 20 minute waiting period, and if it is more convenient, one can just as well wait for 40 minutes or longer before finishing the determination, but the colorimetric readings obtained at the end of 20 minutes are reliable.

Micro Method for the Determination of Uric Acid.—The modified micro method corresponding to the macro method for the determination of uric acid in blood is as follows:

The blood filtrate for the determination of the uric acid by the micro method is obtained by adding 0.2 cc. of blood to 4 cc. of tungstate-sulfate mixture in a centrifuge tube and, 15 minutes later, adding 1 cc. of sulfuric acid and centrifuging.

The sulfate-tungstate solution contains 20 gm. of anhydrous sodium sulfate and 3 gm. of sodium tungstate per liter. The sulfuric acid solution is obtained by diluting 12 cc. of 2/3 N sulfuric acid to 100 cc.

Transfer 4 cc. of the extract to a test-tube graduated at 25 cc. To two other similar test-tubes add 4 cc. and 2 cc. (plus 2 cc. of water) of a standard uric acid solution containing 1 mg. of uric acid in 500 cc.

To each of the three tubes add 10 cc. of the same urea-

cyanide solution as is used in the macro method, and mix. Then add 4 cc. of the concentrated uric acid reagent and let stand for 20 to 30 minutes. Dilute to volume, mix, and make the color comparison.

$\frac{20}{x} \times 5.2$ (or 2.6) gives the uric acid in mg. per 100 cc. of blood.

It will be noted that in this calculation the filtrate is regarded as representing a dilution of 1 in 26 instead of 1 in 25 as given in the original method.

The Preparation of Crystallized Uric Acid.—Because of its insolubility, the uric acid obtained from dealers or manufacturers is usually nearly pure. For the preparation of standard uric acid solutions and also for injection experiments with human subjects it is, nevertheless, desirable to have this interesting metabolism product in the highest possible state of purity. Beautiful crystalline preparations can be obtained on a large scale as follows:

Transfer about 7 liters of distilled water to a 12 liter pyrex flask and heat this water to a temperature of 55 to 60° C.

Heat about a liter of water in one flask, and transfer to another flask, capacity 1500 cc., 11 gm. of lithium carbonate. Pour 750 cc. cold distilled water on to the lithium carbonate, and heat to about 90° C. Most of the lithium carbonate dissolves. To this hot mixture add, through an improvised paper funnel, 25 gm. of uric acid. Rinse down any uric acid that may stick to the neck of the flask with the previously heated hot water, and shake. The uric acid dissolves at once, though a part may come down again as lithium urate. Pour the mixture at once through a good quality filter (diam. 25 cm.) on a funnel into the warm water in the large flask and wash with hot water. Mix thoroughly, and to the rotating urate solution add 25 cc. of 99 per cent acetic acid. Large, shiny rectangular uric acid crystals begin to come down at once. After 15 minutes standing, shake and add another 20 cc. of acetic acid. After another 15 minute interval, shake up once more and to the rotating mixture add 40 cc. more of acetic acid. Let stand for about 30 minutes. Remove the supernatant liquid by decantation, and filter the remainder through a hardened filter paper on a Büchner funnel. Wash with distilled

water until the filtrate is no longer acid to litmus paper. Yield: 23 gm.

It is important to decant and filter before the mixture is cold, because the additional uric acid which comes down, on cooling, is apt to be dark in color and less pure. A somewhat smaller yield, but larger and more uniform crystals are obtained by omitting the third addition of (40 cc.) acetic acid.

Blood Sugar.—Practically all competent investigators in the field of blood sugar determinations are agreed that the modern methods in use up to about 1930 yield values which are at least 10 to 15 per cent too high for normal human blood. As a result of the intense recent investigations at least three different methods have come into vogue, all of which give results which are almost identical with the fermentable sugar and which are therefore accepted as giving the true glucose content of blood. The essential feature of the colorimetric method described here is the preparation of filtrates from unlaked blood. Any dependable sugar method will give substantially the same values when applied to such blood filtrates.

Improved Folin-Wu Method.—(*J. Biol. Chem.*, 1920, 41, 367; 1929, 82, 83.)—Reagents:

1. Standard Sugar Solutions.—The best preservative for a number of different substances which are likely to be decomposed by moulds is a nearly saturated benzoic acid solution. Dissolve 2.5 gm. of benzoic acid in 1 liter of boiling water and cool. Transfer to a bottle; the solution will keep indefinitely.

Dissolve 1 gm. of pure anhydrous glucose in 50 cc. of the benzoic acid solution. (The most reliable glucose for such standard solutions is that obtainable from the Bureau of Standards, Washington, D. C.) Transfer the solution to a 500 cc. volumetric flask and dilute to volume with the saturated benzoic acid solution. Transfer to a bottle, label and preserve. The solution will keep indefinitely.

Transfer 5 cc. of this stock solution, containing 10 mg. of glucose, to a 100 cc. volumetric flask and dilute to volume with water. This solution will keep for at least a week without any additional preservative, but if it is to be kept for a long time it is best to add a few drops of toluene.

For work with diabetic blood it is necessary to have on hand

another working standard which is twice as strong, 10 cc. diluted to 100 cc., and which accordingly contains 0.2 mg. per cc. or 0.4 mg. in the 2 cc. actually used for each analysis.

2. A 5 Per Cent Solution of Crystallized Copper Sulfate.—Dissolve 25 gm. of the salt in a little water, transfer to a 500 cc. volumetric flask, add 5 or 6 drops of concentrated sulfuric acid, dilute to volume and preserve in a clean bottle.

3. Alkaline Tartrate Solution.—Transfer 35 gm. of anhydrous sodium carbonate to a volumetric liter flask, add 175 to 200 cc. of water and shake for a few moments to bring the carbonate into solution. Then add 13 gm. of sodium tartrate (Merck's highest purity) and 11 gm. of sodium bicarbonate. Add water to a volume of about 800 cc. and shake until a clear solution is obtained. Dilute to volume and mix.

4. Alkaline Copper Tartrate Solution.—This solution should be prepared fresh each day, because it may deteriorate by auto-reduction if kept over night. The reagent is simply a mixture of 9 volumes of the alkaline tartrate solution with 1 volume of the copper sulfate solution.

5. Acid Molybdate Reagent.—The active ingredient of this reagent is probably some phosphomolybdate of unknown constitution. The reagent was originally devised as a part of the Folin-Wu method, and it is still probably the most dependable reagent for the colorimetric estimation of cuprous oxide.

For temporary use (not over 1 week) the reagent can be quickly prepared as follows:

Dissolve 40 gm. of sodium molybdate in 100 cc. of distilled water in a 500 cc. beaker. To the turbid solution add, with stirring, 55 cc. of 85 per cent phosphoric acid, 40 cc. of cool sulfuric acid (25 per cent: 1 volume of H_2SO_4 to 3 volumes H_2O), and finally 20 cc. of 99 per cent acetic acid. The resulting mixture is at once ready for use.

For the preparation of a permanent reagent which will not turn blue on standing it is convenient to keep on hand a brominated 30 per cent solution of sodium molybdate.

By means of a funnel and a glass rod transfer 300 gm. of sodium molybdate to a volumetric liter flask. Add about 800 cc. of water and shake until solution is complete except for the turbidity. Dilute to volume, mix and transfer this stock solution

to a bottle. Add 0.2 to 0.3 cc. of bromine. Shake and set aside until wanted.

Transfer 500 cc. of the clear supernatant solution to a 1500 cc. Florence flask. Add, with stirring, 225 cc. of 85 per cent phosphoric acid. Some bromine is liberated and imparts a yellow color to the solution. Next add 150 cc. of cool 25 volume per

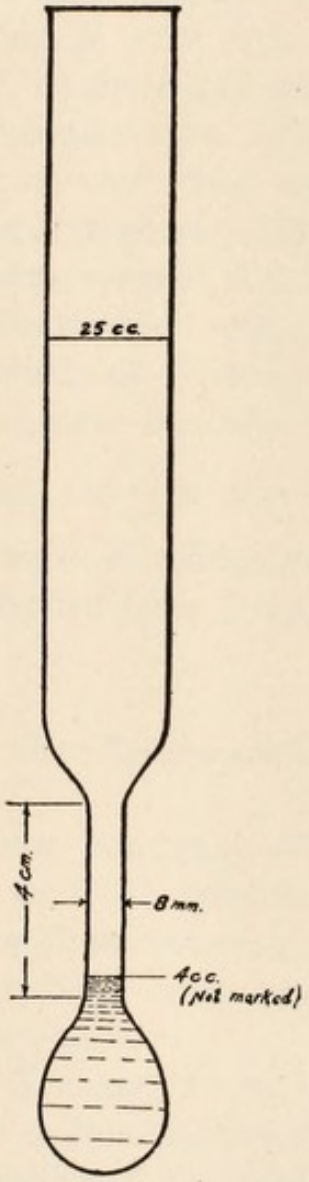


FIG. 9.

cent sulfuric acid, and let stand over night. Remove the remaining bromine by means of an air current. Then add 75 cc. of 99 per cent acetic acid, mix, and dilute to a volume of 1 liter. If kept protected from organic matter, this reagent will remain colorless for years.

The special "blood sugar tube" shown in the sketch is indispensable, because in open tubes with large liquid surfaces there

always occurs re-oxidation of cuprous oxide. If noticeable re-oxidation does not occur in a method like that of Shaffer and Somogyi, which does not permit the use of constricted tubes, it can be so only because they work with such large volumes that the surface becomes *relatively* insignificant.

The blood sugar determination is made as follows:

Transfer 2 cc. of blood filtrate to a Folin-Wu sugar tube, or 1 cc. plus 1 cc. of water, if very high blood sugar values are expected. Transfer 2 cc. of the sugar standard (0.1 mg. of glucose per cc.) to another similar tube. Add 2 cc. of freshly mixed copper tartrate reagent to each tube and heat in a beaker of rapidly boiling water for 8 minutes. Cool in running water. Add 4 cc. of the acid molybdate reagent and, after waiting for about 1 minute, dilute to volume with water, or preferably with a solution containing 1 volume of the molybdate reagent to 4 volumes of water. Mix, and make the color comparison.

Calculation: $\frac{20}{x}$ times 100 (or 200 if the stronger standard is used) gives the sugar in milligrams per 100 cc. of blood. The result must be multiplied by 2 if only 1 cc. of blood filtrate is used.

Micro Method for the Determination of Blood Sugar.—Reagents and apparatus:

1. Sulfate-Tungstate Solution.—Transfer to a 500 cc. volumetric flask 10 gm. of C. P. anhydrous sodium sulfate and 15 cc. of 10 per cent sodium tungstate solution. Half fill the flask with distilled water and shake until the sulfate has dissolved. Dilute to volume and mix.

2. Sulfuric Acid.—Transfer 12 cc. of 2/3 N sulfuric acid and 2 gm. of anhydrous sodium sulfate to a 100 cc. volumetric flask. Shake till the sulfate has dissolved, dilute to volume and mix.

3. Potassium Ferricyanide Solution.—Dissolve 1 gm. of C. P. potassium ferricyanide in distilled water and dilute to a volume of 250 cc. The major part of this solution should be kept in a brown bottle and in a dark closet. The part in daily use should also be kept in a brown bottle.

4. Sodium Cyanide-Carbonate Solution.—Transfer 8 gm. of anhydrous sodium carbonate to a 500 cc. volumetric flask.

Add 40 to 50 cc. of water and shake, to promote rapid solution. With a cylinder, add 150 cc. of freshly prepared sodium cyanide solution, 1 per cent; dilute to volume and mix. It is easiest and best to prepare more sodium cyanide solution than is needed, and to throw away the unused portion.

5. Ferric Iron-Gum Ghatti Solution.—Fill a liter cylinder with water. Suspend on a wire screen of copper or galvanized iron (just below the surface of the liquid) 20 gm. of gum ghatti, and leave over night. Remove the screen, and strain the liquid through a double layer of a clean laboratory towel.

Dissolve in a 250 cc. beaker, by the help of heat, 5 gm. of anhydrous ferric sulfate and 75 cc. of 85 per cent phosphoric acid in 100 cc. of water. Cool this solution and add it, with stirring, to the strained gum ghatti solution. Finally, add to the mixture, a few cc. at a time, about 15 cc. of 1 per cent potassium permanganate solution. The purpose of the permanganate is to oxidize certain reducing impurities present in the gum ghatti. The slight turbidity of the solution will disappear completely if the solution is kept in a warm place (37° C.) for a few days. This reagent keeps indefinitely.

6. Standard Stock Solution of Glucose.—Dissolve 1 gm. of benzoic acid in about 300 cc. of hot distilled water. Weigh out exactly 980 milligrams of C. P. anhydrous glucose on a watch glass. Rinse the sugar through a funnel into a volumetric liter flask, by the help of the warm benzoic acid solution, add about 400 cc. of distilled water, then cool to room temperature, and finally, make up to volume and mix. Transfer to a clean, dry, glass stoppered bottle.

This stock solution preserved with 0.1 per cent of benzoic acid will keep indefinitely. It is made to contain 0.98 mg. per cc. instead of 1 mg. only in order to make the calculations easier.

From this stock solution one obtains the dilute working standard by diluting 1 cc. to 100 cc. By incorporating a little benzoic acid (no more than 25 mg.) into this solution, it will keep for a long time.

Besides the reagents described above, the following equipment is called for:

1. A Colorimeter.—The biological colorimeter of Bausch and Lomb, with rack and pinion for each colorimeter cup, is satisfactory.

2. A Chalet Lamp.—Have a "daylite" light screen in one window and a yellow light filter in the other.

The yellow light filter, mentioned above, which we have used, is made by impregnating filter paper with an acid picrate solution (see p. 315), but a more permanent light filter of frosted yellow glass is obtained from the Spencer Lens Company, at Buffalo, N. Y.

3. Micro Blood Pipettes.—These pipettes are made to contain (not to deliver) 0.1 cc. and 0.2 cc. They can be obtained from Eimer and Amend, New York City. A liberal supply of these pipettes is desirable, for they should always be available in dry and absolutely clean condition. If they are perfectly clean, they fill quickly and easily by capillary attraction, i.e., without any suction. To clean the pipettes soak them in a saturated solution of sodium dichromate in concentrated sulfuric acid ("cleaning fluid") before rinsing.

4. Lancets.—The best and most durable spring lancet for pricking a finger or an ear is the U. S. Army lancet (Arthur Thomas Catalogue, number 3644).

5. Test-tubes.—Graduated at 25 cc.

6. Centrifuge and 15 cc. Centrifuge Tubes.

7. Volumetric Pipettes. — Capacity 4 cc., or so-called "Folin blood pipettes," capacity 5 cc., graduated to the tip in 1 cc. divisions.

The Determination.—Transfer to a clean dry centrifuge tube 4 cc. of the sulfate-tungstate solution. Prick a finger with the lancet, and collect exactly 0.1 cc. of blood, by means of the micro blood pipette. Transfer at once (before clotting has had time to occur) to the solution in the centrifuge tube. Rinse the pipette two or three times (by suction) and stir a little with the pipette. Let stand for about 15 minutes, or longer if convenient. Then add 1 cc. of the acid sulphate solution and stir with the micro pipette still in the centrifuge tube, or with a fine glass rod. Centrifuge for 5 minutes.

Transfer 2 cc. of the water-clear supernatant solution in the centrifuge tube and also 2 cc. of water to a 25 cc. test-tube graduated at 25 cc. And to another similar test-tube add 4 cc. of the standard dilute glucose solution. Add to each tube 1 cc. (or 2 cc.) of the 0.4 per cent potassium ferricyanide solution

and then add to each 1 cc. of the cyanide-carbonate solution. Heat both test-tubes together in a beaker of boiling water for 8 minutes, cool, and to each tube add 5 cc. of the ferric iron-gum ghatti solution.

Dilute the contents of the tube to 25 cc. and mix. Make the color comparison in the usual manner, always first reading the standard against itself, and never omitting to make use of the yellow light filter. When the standard is set at 20 mm., $\frac{20}{x}$ times 100 gives the sugar value in mg. per cent.

It will be noted from the above brief description how simple the determination is when one once has acquired the necessary reagents and equipment.

One detail in the determination has been left to the experience of the worker. He has been given the choice of using 1 or 2 cc. of the potassium ferricyanide solution. If 1 cc. is used, the results are a little more accurate, particularly if the blood happens to be low in sugar. On the other hand, if only 1 cc. is used, the determination is dependable only up to a little over 300 mg. per cent, and if more than this amount is found, the determination should be repeated with 1 cc. of the blood extract plus 3 cc. of water. When 2 cc. of potassium ferricyanide are used, one single determination will cover the range from far below 100 to over 500 mg. per cent, but the highest values so obtained will tend to be a shade too low, and the lowest values a trifle too high. The reason for this is that the blank (auto-reduction of the ferricyanide) is appreciably stronger when 2 cc. are used.

Those who are easily puzzled by calculations may find it a little difficult to understand why the dilute standard glucose solution is made to contain 0.0098 mg. of glucose per cc. instead of 0.01 mg. The reason for this procedure is that 0.1 cc. of blood is diluted to a volume of 5.1 cc. instead of 5 cc. Since 5.1 is 2 per cent greater than 5, the standard glucose has been made 2 per cent weaker than 0.01. The calculation can then be made as if the standard contained 0.01 mg. per cc., and as if 0.1 cc. of blood had been diluted to 5 cc.

The blood sugar values obtained by this micro method are very close to the true glucose values of the blood.

Preparation of Acid Picrate Light Filter.—Dissolve 5 gm. of picric acid in 100 cc. of methyl alcohol and add 5 cc. of 10 per cent sodium hydroxide solution. Place a pack of eight to ten filter papers on a level and smooth mat of newspapers. Pour the acid picrate solution onto the filters until the papers are saturated and an excess of solution filters through at the bottom and flows out a distance of at least 2 cm. on the newspaper mat. When all the liquid has evaporated and the filter papers are perfectly dry, pour over the pack an excess of a 3 per cent solution of paraffin in benzine (gasoline) and again leave the papers to dry. All of the filter papers will be evenly stained, canary yellow to golden yellow, and the stain will not rub off, because of the paraffin. A heavy filter with good absorbing qualities, such as Schleicher and Schüll No. 604, is best. Large or small filters stain equally well.

Amino Acid Nitrogen in Blood.—Reagents:

1. STANDARD AMINO ACID SOLUTION.—Two stock solutions are prepared; one contains 0.1 mg. of amino nitrogen per cc. as glycine dissolved in 0.07 N HCl plus 0.2 per cent sodium benzoate, and the other contains 0.1 mg. of amino nitrogen per cc. as glutamic acid in 0.07 N HCl plus 0.2 per cent sodium benzoate. The sodium benzoate is used as a preservative. Standards used in the analysis of blood filtrates are prepared from these by mixing equal volumes of the two stock solutions and diluting with 0.07 N HCl containing 0.2 per cent sodium benzoate to a concentration of 0.03 mg. and 0.05 mg. of amino nitrogen per cc. A 0.03 mg. standard is prepared by adding 15 cc. each of the glycine stock solution and the glutamic acid stock solution to a 100 cc. volumetric flask. The contents are made up to a volume of 100 cc. with 0.07 N HCl containing 0.2 per cent sodium benzoate. The 0.03 mg. standard is used for the analysis of filtrates prepared from normal human unlaked blood. The amino acid concentration in most animal bloods is appreciably greater than that of human blood, therefore the strength of the standard used for such comparisons should be increased accordingly. It is not permissible to use 2 cc. of a weaker standard to prepare a standard of double strength, due to the fact that the standard is so prepared that 1 cc. contains about the equivalent of acid found in 10 cc. of tungstic acid blood filtrate. If a stronger standard is required it is necessary to prepare it from the stock solutions.

The use of both glycine and glutamic acid in the standard is made necessary because the shades of color produced by the different amino acids vary somewhat. The standard as recom-

mended produces a color that matches very nearly that produced in the tungstic acid blood filtrates.

2. Borax Solution.—As a source of alkali a 1.5 per cent solution of borax ($Na_2B_4O_7 \cdot 10H_2O$) is used. A definite amount of borax is added to each determination. If the amino acid nitrogen is to be determined on a solution of unknown acidity or basicity, the solution should first be neutralized with sodium hydroxide or hydrochloric acid using phenolphthalein as indicator and then the required amount of borax solution added, usually 2 cc. Too much borax decreases the amount of color developed.

3. Fresh 0.5 Per Cent Solution of the Sodium Salt of β-Naphthoquinonesulfonic Acid.—For the preparation of this quinone in pure form see page 323. Enough of this compound for several thousand amino acid determinations can be made in the course of two mornings. In solution β-naphthoquinonesulfonic acid is gradually decomposed, and the solution becomes visibly darker in the course of a few hours, particularly if it is not kept in the dark. For this reason only freshly prepared solutions should be used. For practical work it is convenient to charge a series of clean specimen tubes with 100 or with 500 mg. of the dry quinone in roughly powdered form. The samples need not be weighed any more accurately than can be done on a small torsion balance, because a variation either way of 5 per cent makes no difference. Transfer 100 mg. of the quinone to a small flask, add 20 cc. of water, and shake. Complete solution is obtained almost at once.

4. Bleaching Reagents.—Two solutions are needed for the bleaching of the excess β-naphthoquinonesulfonic acid reagent: (1) a 0.1 M solution of sodium thiosulfate which need not be standardized, and (2) an acid formaldehyde solution prepared by mixing 3 volumes of 1.5 N HCl and 1 volume of glacial acetic acid with 4 volumes of 0.15 M formaldehyde. The 0.15 M formaldehyde solution may be prepared accurately enough by diluting 11.3 cc. of ordinary 40 per cent formaldehyde to 1000 cc.

Sodium thiosulfate in acid solution has the property of destroying the color of β-naphthoquinonesulfonic acid. If acetic acid is used in making this solution acid, the bleaching of the quinone is far from complete; but if a strong acid like hydrochloric acid is used, the color is bleached completely. However,

in the presence of a strong acid, sodium thiosulfate immediately decomposes with the liberation of sulfur. The addition of an amount of formaldehyde equivalent to the thiosulfate present will delay this decomposition for many hours. In the solutions as recommended the thiosulfate is present in slight excess, *i.e.* 2 cc. of 0.1 M thiosulfate and 2 cc. of 0.075 M formaldehyde. Under these conditions the bleaching is more prompt and complete, and the solution remains perfectly clear for several hours.

The addition of acetic acid to the acid formaldehyde is made necessary because an amino acid determination made on a solution containing tryptophane will become cloudy in the absence of acetic acid. Tryptophane is the only amino acid we have studied that behaves in this manner. Determinations on unlaked blood filtrates develop a very slight cloud if acetic acid is not included in the acid formaldehyde solution.

5. Sulfate-Tungstate Solution for Addition to Standard.—This solution contains 15.0 gm. of Na_2SO_4 (anhydrous) and 1.5 gm. of $Na_2WO_4 \cdot 2H_2O$ per 1125 cc. of solution.

The unlaked blood filtrate contains about 1.5 per cent Na_2SO_4 and about 0.15 per cent $Na_2WO_4 \cdot 2H_2O$ which remains from the solutions used in the preparation of the filtrate. The presence of these salts in the above concentrations has very little or no effect on the amount of color developed in the determination, but sodium tungstate in particular and sodium sulfate to a much smaller degree alter the shade of color produced, making exact color comparisons difficult. The presence of sodium tungstate produces a disturbing greenish shade. To balance this effect and to bring the volume of the standard up to that of the unknown, add 9 cc. of the above solution to the standard.

If sodium sulfate is present in concentrations of 3 per cent or more, or sodium tungstate in concentrations of 0.6 per cent or more, an error of 2 per cent or more, depending on the concentrations, will be introduced.

The Determination.—The amino acid nitrogen content of a blood filtrate may be determined on either 5 cc. or 10 cc. of filtrate.

Transfer 10 cc. of the filtrate into a test-tube graduated at the 25 cc. mark. To this add 2 cc. of 1.5 per cent borax solution

and 2 cc. of a freshly prepared 0.5 per cent β-naphthoquinonesulfonic acid solution and mix thoroughly. The standard is prepared by introducing 1 cc. of the standard solution of desired strength into a test-tube similar to that used for the filtrate. To the standard that is to be used in the comparison of an unlaked blood filtrate or plasma filtrate add 9 cc. of the sulfate-tungstate solution prepared for this purpose. If the filtrate to be analyzed contains no tungstic acid and very little sodium sulfate (laked blood filtrate), add 9 cc. of water. Then add to the standard 2 cc. of the 0.5 per cent β-naphthoquinonesulfonic acid solution and mix well. Both standard and unknown are then set in a dark closet for 18 to 24 hours. After this period of standing add 2 cc. of the acid formaldehyde solution and 2 cc. of the 0.1 M sodium thoisulfate solution. Dilute the contents of each tube to a volume of 25 cc. with distilled water and then mix thoroughly. After standing for 4 to 5 minutes to allow for the complete bleaching of the excess quinone reagent, the unknown is compared with the standard by the use of a colorimeter.

If 5 cc. of the blood filtrate are used for the determination, transfer the sample (5 cc.) to a test-tube graduated at the 15 cc. mark, add 1 cc. of the borax solution and 1 cc. of the freshly prepared 0.5 per cent β-naphthoquinonesulfonic acid solution. The standard is prepared as described above. After the 18 to 24 hour period of standing add to the unknown 1 cc. each of the acid formaldehyde and the sodium thiosulfate solutions, dilute to 15 cc., and mix. To the standard add 2 cc. portions of the acid formaldehyde and sodium thiosulfate solutions, dilute to 30 cc., and mix. Color comparisons are made as directed above.

The calculation in either case is made by the use of the following formula

$$\frac{20}{x}\times 0.03\times 100=\text{mg. per cent or } \frac{20}{x}\times 3=\text{mg. per cent}$$

when the colorimeter setting for the standard is 20. x is the reading of the unknown and 0.03 mg. is the concentration of the amino nitrogen in the standard.

When the amino acid nitrogen of fluids other than blood filtrates is to be determined, the sample taken should contain between 0.03 and 0.15 mg. of amino nitrogen and should be neutralized with sodium hydroxide or hydrochloric acid, depending on whether the sample is acidic or basic, phenolphthalein

being used as indicator. In order to balance the acidity of the unknown with the standard add 1 cc. of 0.07 N HCl to the sample used after it has been neutralized. The determination is then carried out in the same manner as described for blood filtrates.

AMINO ACID NITROGEN (MG. PER CENT) IN BLOOD OF NORMAL YOUNG MEN

After a night's fast	Whole Blood	Plasma	Corpuscles
Minimum	2.3	4.0	0.34
Maximum	3.74	5.65	2.19
Average	3.0	4.84	1.04

Detailed Description of Preparation of the Amino Acid Reagent.—The process described below for the preparation of strictly pure sodium β-naphthoquinonesulfonate is the outcome of a great many trial experiments. No effort has been spared to make the preparation simple as well as reliable. From the list of needed chemicals and from the different steps in the process enumerated, the preparation may seem a rather formidable undertaking, but in actual practice it will be found that the amount of work involved is not very large. The description has purposely been made so explicit that a person with limited chemical experience cannot go astray, except by not following the directions. I would warn against introducing variations or modifications of any kind, for I have tried a great number of plausible short cuts and variations, only to find that they had no merit.

The following chemicals are needed for the preparation of 75 to 90 gm. of the pure reagent:

Cold 10 per cent sulfuric acid, 1000 cc.
Concentrated nitric acid, 100 cc.
" hydrochloric acid, 500 cc.
10 per cent sodium hydroxide solution, 300 cc.
10 " " " chloride solution, 2000 cc.
Bromine, 1 cc.
Sodium nitrite, 50 gm.
" nitrate, 100 gm.
" sulfite, 50 gm.
" bisulfite, 100 gm.
Borax, 400 gm.
Resublimed β-naphthol, 100 gm.
Alcohol, about 2000 cc.
Ether, about 200 cc.
Ice, 1000 gm.

1. Transfer 100 gm. of β-naphthol to a liter beaker; add 300 cc. of 10 per cent sodium hydroxide solution and stir with a glass rod until complete solution is obtained (usually requires 10 to 15 minutes).

2. Transfer 50 to 55 gm. of sodium nitrite to a 4 liter beaker; add 600 cc. of water and shake until solution is obtained (3 to 5 minutes).

3. Pour the alkaline β-naphthol solution into the 4 liter beaker holding the nitrite solution, and rinse with about 100 cc. of water.

4. Add 800 gm. of crushed ice to the naphthol-nitrite mixture.

5. Fill a 200 cc. cylinder with cold dilute (10 per cent) sulfuric acid, and pour it slowly down one side of the beaker, while stirring vigorously and continuously with a heavy glass rod. Continue the stirring for 1 to 2 minutes after all the acid in the cylinder has been added. Then fill the cylinder again and add in the same way. Repeat this addition of dilute sulfuric acid, 200 cc. at a time, until 800 cc. have been added. The additions should be continued until the mixture in the beaker gives a distinct and *permanent* acid reaction with Congo red paper (time 15 to 20 minutes).

A yellow precipitate begins to form with the first addition of acid and increases in quantity until the whole mixture becomes a semisolid paste. The precipitate will have a slight greenish tint; if it is distinctly green, the conditions are not right and a less good yield is obtained. Let stand for 1 hour after the last of the acid has been added. It is important not to omit this detail because without such a period of standing much unchanged β-naphthol remains in the mixture and is encountered when trying to dissolve the precipitate in sulfites. Longer standing does no harm, but is superfluous.

6. Filter through a 20 cm. Büchner funnel with *moderate* suction, and wash with about 1500 cc. of cold water.

7. Transfer the precipitate (nitroso-β-naphthol), by blowing, to a large evaporating dish, and sprinkle over it 100 gm. of sodium bisulfite and 50 gm. of sodium sulfite; stir with a spoon (glass or enameled ware). An extremely soluble bisulfite addition product is formed, and the mixture becomes liquid. Filter immediately on a Büchner funnel (diameter 12 to 15 cm.)

through a double layer of good (quantitative) filter paper from the small amount of black residue. After filtering wash with a small amount of water.

8. Transfer the filtrate and washings at once (to avoid excessive darkening) to a 5 liter flask, or wide mouth (colored) bottle, containing 2000 cc. of water and 500 cc. of concentrated hydrochloric acid. Cover with a funnel and one or two watch-glasses, and let stand in a *dark* closet for about 36 hours (24 hours is not quite enough). The whole flask becomes filled with a network of white needles, which carry down a little dark and pink matter as impurities. The greater the exposure to light the more of the dark decomposition products will be formed.

Filter on a 20 cm. Büchner funnel with moderate suction, and wash with about 2 liters of cold water.

9. Blow the precipitate (1-amino-2-naphthol-4-sulfonic acid) to a large filter paper, and from there transfer it to a large beaker (3 to 4 liter capacity). Sprinkle over the precipitate 100 gm. of sodium nitrate. Dilute 100 cc. of concentrated nitric acid with 350 cc. of water, and pour the whole of this lukewarm dilute acid into the beaker. Reaction begins immediately and nitric oxide fumes begin to come off. Leave without stirring for 10 minutes while the greater part of the reaction takes place. Then stir thoroughly for a few minutes and let stand for another 20 to 30 minutes.

If no visible reaction takes place on adding the nitric acid, the cause is probably to be found in the presence of sodium carbonate in the sodium nitrates used. Even samples of sodium nitrate which are labeled "The Standard of Purity" may contain considerable amounts of carbonates. If no reaction takes place, add a little (1 to 5 cc.) of concentrated nitric acid.

At the end of about half an hour filter on a Büchner funnel (diameter 15 cm.) and wash with about 1000 cc. of 10 per cent sodium chloride solution.

The light brown product on the funnel is the desired sodium salt of 1, 2, 4-naphthoquinonesulfonic acid. But it is not pure. It contains dark-colored decomposition products and also traces of ammonia. Considerable ammonia is formed (from the amino group) during the oxidation with the nitric acid, but because of the large amount of sodium nitrate present, only traces of the ammonia are carried down as the ammonium salt of the quinone.

By recrystallization under the conditions described in the next section (Section 10) the product is freed from all disturbing impurities.

10. Transfer the moist precipitate to a large porcelain dish. Add 200 gm. of powdered borax and 450 cc. of water. Mix with a pestle until all but a few flakes of the quinone have dissolved. Filter through a good quality (quantitative) filter paper on a 10 cm. Büchner funnel from the surplus borax and a little undissolved black residue. Because of the latter the filtration is apt to be rather slow, and it is better not to apply too strong a suction. Wash with 100 to 150 cc. of water.

While the filtration is proceeding, transfer 850 cc. of 95 per cent alcohol and 150 cc. of concentrated hydrochloric acid to a Florence flask. Cover the mouth of the flask with a beaker and cool under running water.

Transfer the quinone-borax filtrate to a 4 liter beaker. Add a few drops of liquid bromine to the cooled acid-alcohol. Shake until the bromine is dissolved, and then pour the resulting straw-yellow solution into the quinone-borax mixture and stir quickly and vigorously for a few moments so as to secure complete mixing. Let stand for 5 minutes. The quinone has all come down at the end of this time.

Filter on a Büchner funnel (diameter 15 cc.) and wash with 700 to 800 cc. of 10 per cent solution of sodium chloride.

This one recrystallization is adequate as far as the color of the product is concerned, but it still contains some ammonia. The recrystallization must, therefore, be repeated once more. This final recrystallization is conducted in the same way, except with the difference that the washing of the quinone on the Büchner funnel with sodium chloride is omitted, and for it is substituted the washing, first with 300 to 400 cc. of alcohol, and finally, with about 200 cc. of ether.

Seventy-five to 90 gm. of perfectly pure quinone is thus obtained.

The purification process described above is the outcome of a large number of experiments directed toward obtaining this quinone free from colored decomposition products. The compound cannot be recrystallized from water alone, because each time it is dissolved there is some decomposition, and when precipitation is made (usually by the addition of sodium chloride)

the decomposition products adhere to the compound, and it thus becomes darker than it was before.

The following tests for the purity of the sodium salt of 1, 2, 4-naphthoquinonesulfonic acid will be found useful.

Color.—Prepare a fresh 1 per cent solution of the quinone in water, and compare its color with that of a 0.5 N solution of potassium dichromate, with the latter set at 20 mm. in the colorimeter. The quinone solution will read 26 to 27 mm.

Colored Decomposition Products.—Transfer 2 cc. of the fresh 0.5 per cent quinone solution to a test-tube, add 2 cc. of 1.5 per cent borax solution and mix; allow to stand in a dark closet for 18 to 24 hours. Then add 2 cc. of the acid formaldehyde solution and 2 cc. of the 0.1 M sodium thiosulfate solution, dilute to a volume of 25 cc. and mix. After about five minutes the solution will bleach so completely that it is only by looking through the length of the tube that a faint yellow shade is visible.

Ammonia in Blood.—(*J. Biol. Chem.*, 1932, 97: 141.)—The ammonia in arterial or systemic blood is subject to relatively large fluctuations, but the amounts found in strictly fresh blood (0.06 to 0.14 mg. per cent) are too small to serve as the supply from which the urinary ammonia is drawn. The spontaneous ammoniacal decomposition in drawn blood represents a relatively rapid process so that correct ammonia values can not be obtained unless the blood is analyzed within the first two or at the most three hours. It has been claimed that the spontaneous ammoniacal decomposition does not occur (Nash and Benedict) or that it can be prevented by adjustment of the pH of the blood (Parnas), but these claims are erroneous, and merely indicate that the underlying analytical work was not quite so reliable as the authors believed it to be. In the light of these facts it is no wonder that no correlation has been established between the ammonia in blood and any metabolic process or clinical condition.

For the determination of ammonia in blood it is essential to use only reagents which have been rendered free from every trace of ammonia. The potassium oxalate, for example, which is

used to prevent the clotting of the blood, must be freed from the large traces found in all so-called C.P. brands. Reagents:

1. Potassium Oxalate Free from Ammonia.—Transfer 100 gm. of neutral potassium oxalate and 500 cc. of distilled water to a 1500 cc. Florence flask, add 20 cc. of 10 per cent solution of potassium hydroxide, and pass an air current through it, at 4 to 6 liters per minute, for 24 to 36 hours; that is, until 5 cc. of the solution fails to give a trace of color with Nessler's reagent. Transfer the solution to a 2 liter beaker, add with stirring 1000 to 1200 cc. of alcohol, cool for a couple of hours, and filter with suction on a Büchner funnel. Wash three or four times with alcohol and two or three times with ether. Dry in a protected place. Yield, 85 to 90 gm.

The potassium oxalate so prepared has one especial merit besides that of being completely free from ammonia. It is very light and fluffy instead of compact and heavy like ordinary powdered oxalate and therefore goes into solution more quickly. This is helpful in the prevention of coagulation of the blood, at least when the minimum safe quantity is used (20 mg. for each 10 cc. of blood). It is neutral in reaction as the added KOH is washed away with the alcohol.

2. Carbonate-Oxalate Solution.—This is the best alkali to use for the removal of ammonia from blood by aeration. A potassium carbonate-oxalate mixture which gives absolutely no color with Nessler's reagent, even after several hours, can be made by the simple process described below:

Dissolve 10 gm. of anhydrous potassium carbonate and 15 gm. of potassium oxalate in about 100 cc. of water in a 300 cc. Erlenmeyer flask. Add to this solution 0.1 to 0.2 cc. of bromine and shake until all of the bromine globules have dissolved. If any precipitate is formed, as may happen with some samples of bromine, filter on a quantitative filter paper into another 300 cc. flask. Dilute the straw-yellow filtrate (or solution) to about 150 cc., and boil down to a volume of 80 or 90 cc. Cool and dilute to 100 cc.

By this treatment the ammonia in both the oxalate and the carbonate, as well as the unknown disturbing impurity of the carbonate, is completely destroyed and the surplus hypobromite is destroyed during the boiling.

Test.—Transfer 2 drops of 2 per cent gum ghatti solution

and about 10 cc. of the carbonate-oxalate solution to a clean test-tube. Add 2 cc. of Nessler's reagent. No trace of color should develop in the course of an hour.

Isolation of the Blood Ammonia by Aeration.—Aeration with air free from ammonia represents the theoretically soundest method by which the almost infinitesimal quantities of ammonia in fresh blood can be isolated without including significant quantities of ammonia due to decompositions. For strictly dependable retentions, in the receiver, of the traces of ammonia carried away by the air, one must use the special ammonia absorption tube shown on page 129, and must keep the speed of the air current somewhere between 5 and 7 liters per minute. If the directions given below are followed, a suitable speed of air current will be obtained without the use of a gas meter.

The Determination.—Transfer 10 cc. of blood to a clean, dry Erlenmeyer flask (capacity 300 cc.). Add 2 cc. of potassium oxalate-carbonate solution (10 per cent potassium carbonate (K_2CO_3) plus 15 per cent potassium oxalate). Insert a clean 2-hole rubber stopper carrying two glass tubes. The tube reaching to within about 1 cm. of the bottom of the flask is drawn out to a point having an internal diameter of about 1.5 mm. It connects with a large bottle containing 5 volume per cent of sulfuric acid, where the air is washed free from ammonia by passing through an ammonia absorption tube. The other glass tube connects with the ammonia absorption tube in the receiver. The receiver, a test-tube 260 mm. by 25 mm., is graduated at 25 cc. and contains 1 cc. of 0.1 N acid, together with water enough to reach the upper openings in the absorption tube. Start the air current and regulate it to a speed of about 6 liters per minute (5.5 to 6.5 liters). The bottom of the Erlenmeyer flask is exposed by a circle having a diameter of 1 to 1.2 cm. Keep the air current running at this speed for 40 to 45 minutes.

To each of two test-tubes, graduated at 25 cc., add 0.01 mg. and 0.007 mg. respectively of ammonia nitrogen, and to each add 1 cc. of 0.1 N acid, together with water enough to give a volume of about 22 cc.

Rinse the ammonia absorption tube with about 9 cc. of water, contained in a 10 cc. volumetric pipette, on the outside and on

the inside through the opening in the top and through the large openings in the outside jacket of the tube. Add 2 drops of gum ghatti solution to each of the three tubes and then add 2 cc. of Folin-Wu Nessler's reagent to each of the three tubes. Make up to volume, mix, let stand for about 15 minutes (or longer if desired), and make the color comparison in the usual manner if the 0.01 mg. standard can be used. For values less than 0.07 mg. per cent straight test-tube comparisons are fully as reliable as those made by the help of the colorimeter, but they will require additional standards.

Nessler's Reagent.—This reagent is essentially a solution of the double iodide of mercury and potassium (HgI_2, 2KI) containing sodium or potassium hydroxide. A stock solution of the double iodide is best prepared as follows:

Transfer 150 gm. of potassium iodide and 110 gm. of iodine to a 500 cc. Florence flask; add 100 cc. of water and an excess of metallic mercury, 140 gm. to 150 gm. Shake the flask continuously and vigorously for 7 to 15 minutes, or until the dissolved iodine has nearly all disappeared. The solution becomes hot. When the red iodine solution has begun to become visibly pale, though still red, cool in running water, and continue the shaking until the reddish color of the iodine has been replaced by the greenish color of the double iodide. This whole operation usually does not take more than 15 minutes. Now separate the solution from the surplus mercury by decantation and washing with liberal quantities of distilled water. Dilute the solution and washings to a volume of two liters. If the cooling was begun in time, the resulting reagent is clear enough for immediate dilution with 10 per cent alkali and water, and the finished solution can at once be used for Nesslerizations.

The cost of the chemicals called for in this rather interesting process of making Nessler's solution is less than when starting with mercuric iodide, and the disagreeable impurities present in many samples of mercuric iodide are avoided. From the stock solution of mercuric potassium iodide, made as described above, prepare the final Nessler solution as follows:

From completely saturated sodium hydroxide solution containing about 63 gm. of NaOH per 100 cc. decant the clear supernatant liquid and dilute to a concentration of 10 per cent.

(It is worth while to determine by titration that a 10 per cent solution has been obtained, with an error of not over 5 per cent.) Introduce into a large bottle 3500 cc. of 10 per cent sodium hydroxide solution, add 750 cc. of the double iodide solution, and 750 cc. of distilled water, giving 5 liters of Nessler's solution.

In the absence of modifying circumstances, such as the presence of much acid or alkali, this reagent should be added in the proportion of 10 cc. per 100 cc. of the volume to which the Nesslerized solution is to be diluted. As a general rule the volumetric flask (or volumetric test-tube) should be at least two-thirds full before adding the Nessler reagent. If attention is not given to this detail, turbid mixtures are obtained, and turbid solutions must never be used for color comparisons.

Preparation of Phenol Reagent.—Transfer to a flask (capacity about 1500 cc.):

750 cc. of water,
100 gm. of sodium tungstate,
20 gm. of phosphomolybdic acid,
50 cc. of phosphoric acid (85 per cent H_3PO_4),
100 cc. of concentrated hydrochloric acid.

Insert a funnel in the flask and partly close the opening of the funnel with a watchglass. Boil the mixture gently for two hours. A deep straw-yellow solution should be obtained. It should not turn appreciably blue when a sample, 5 cc., is rendered alkaline with sodium carbonate. Dilute to a liter.

Determination of Chloride.—(Whitehorn, *J. Biol. Chem.*, 45:449, 1920–21.)—This simple method is doubtless open to theoretical errors, but practically it seems to give accurate and dependable results.

Reagents required:

1. Silver Nitrate Solution.—Dissolve 4.791 gm. of silver nitrate in distilled water and dilute to 1 liter. 1 cc.=1 mg. of Cl.

2. Potassium Thiocyanate Solution.—Dissolve about 3 gm. of the salt in a liter of water. Determine its exact value as described under "Procedure," and on the basis of the titration, dilute so that 5 cc. are equivalent to 5 cc. of the silver solution.

3. Powdered Ferric Ammonium Sulfate.
4. Concentrated Nitric Acid.

Procedure.—Pipette 10 cc. of the protein-free filtrate into a porcelain dish. Add with a pipette 5 cc. of the standard silver nitrate solution and stir thoroughly. Add about 5 cc. of concentrated nitric acid, mix, and let stand for 5 minutes, to permit the flocking out of the silver chloride. Then add, with a spatula, an abundant amount of ferric ammonium sulfate (about 0.3 gm.), and titrate the excess of silver nitrate with the standard thiocyanate solution until the definite salmon-red (*not yellow*) color of the ferric thiocyanate persists in spite of stirring for at least 15 seconds.

Calculation.—5.00 − titer (in cc.) = mg. of Cl per cc. of blood (or plasma).

Since each cubic centimeter of thiocyanate solution used is equivalent to 1 cc. of silver nitrate solution, the difference between the volume of silver nitrate solution taken and the excess determined by the titration, that is, 5 − titer, represents the volume which reacted with chloride in the ratio of 1 cc. to 1 mg. of Cl. And the 10 cc. of filtrate taken represents 1 cc. of blood (or plasma).

To convert Cl figures into NaCl figures divide by 0.606.

Chloride determinations are usually made on plasma rather than on whole blood. To precipitate the proteins from plasma (or serum) see p. 263.

Determination of Phosphorus.—(Fiske and Subbarow, *J. Biol. Chem.*, 1925, 66: 375.)—The special reagents needed are as follows:

1. Molybdate I.—2.5 per cent ammonium molybdate in 5 N sulfuric acid. Dissolve 25 gm. of the salt in 500 cc. of 10 N sulfuric acid, and make up to 1 liter with water.

2. Molybdate II.—2.5 per cent ammonium molybdate in 3 N sulfuric acid. Dissolve 25 gm. of the salt in 500 cc. of 10 N sulfuric acid, and make up to 1 liter with water.

3. Molybdate IIIa.—12.5 per cent ammonium molybdate in 5 N sulfuric acid (replacing the former Molybdate III, made up without sulfuric acid, which sometimes develops a blank on

standing). Dissolve 62.5 gm. of the salt in about 400 cc. of 5 N sulfuric acid, and make up to 500 cc. with sulfuric acid of the same strength.

4. 0.25 PER CENT AMINONAPHTHOLSULFONIC ACID REAGENT. —See p. 175.

5. 10 PER CENT TRICHLOROACETIC ACID.—This solution should be phosphate-free. Dilute 16 cc. with 7.5 cc. of water, and add 0.5 cc. of Molybdate IIIa and 1 cc. of the aminonaphthosulfonic acid reagent. Pour the solution into a test-tube large enough to hold it all, and compare with a mixture containing 21.5 cc. of water, 2.5 cc. of Molybdate I, and 1 cc. of aminonaphtholsulfonic acid (made up at the same time), looking through the solutions from the top. If any blue color at all develops in the course of a few minutes, it is better to redistill the trichloroacetic acid, preferably under diminished pressure.

6. STANDARD PHOSPHATE (5 CC.=0.2 MG. P).—Dissolve 0.1755 gm. of pure monopotassium phosphate in water. Transfer quantitatively to a liter volumetric flask, add 10 cc. of 10 N sulfuric acid, dilute to the mark, and mix.

Transfer to an Erlenmeyer flask 4 volumes of 10 per cent trichloroacetic acid. While the flask is being gently rotated, run in from a pipette 1 volume of blood, plasma, or serum. Close the mouth of the flask with a clean, dry rubber stopper, and shake vigorously a few times. Filter through ashless paper.

Inorganic Phosphate.—Measure 5 cc. of the trichloroacetic acid filtrate into a test-tube graduated at 10 cc. (or a 10 cc. volumetric flask). Add 1 cc. of Molybdate II and 0.4 cc. of the aminonaphtholsulfonic acid reagent. At the same time prepare a standard by mixing in a 50 cc. volumetric flask, in order, 5 cc. of monopotassium phosphate solution (containing 0.2 mg. of phosphorus), 25 cc. of water, 5 cc. of Molybdate I, and 2 cc. of aminonaphtholsulfonic acid reagent. Dilute both standard and unknown to the mark, and mix. Read in the colorimeter after 3 or 4 minutes, with the standard set at 20 mm.

If the room temperature is low, or if there is any chance that an excess of oxalate or citrate (*i.e.*, more than 2 or 3 mg. per cc.) was added to the blood to prevent coagulation, the reading should be checked a few minutes later.

80 divided by the reading gives the number of mg. of inor-

ganic phosphorus per 100 cc. From this result, to correct for the volume occupied by the protein precipitate, 2 per cent should be subtracted in the case of serum or plasma, and 4 per cent with whole blood.

Total Acid-Soluble Phosphorus.—Boil down 5 cc. of trichloroacetic acid filtrate in a large lipped pyrex test-tube with 2 cc. of 10 N sulfuric acid if the material being analyzed is whole blood, or 0.4 cc. of the same reagent if it is serum or plasma. A quartz pebble may be added to prevent bumping. When white fumes appear, continue heating with a very low flame, which should never reach above the level of the liquid in the test-tube, and add concentrated nitric acid down the wall of the tube, one drop at a time, until the residue is colorless. Boil for a few seconds more, and cool the tube.

For whole blood, rinse the contents into a 50 cc. volumetric flask with 30 cc. of water, add 1 cc. of Molybdate IIIa and 2 cc. of the aminonaphtholsulfonic acid reagent. Dilute to the mark, and mix. Read against a standard prepared as described under the determination of inorganic phosphate. 400 divided by the reading will (after subtracting the usual 2 per cent correction) give the result in mg. per 100 cc. of blood.

For serum or plasma, rinse the ashed residue with three 2 cc. portions of water into a graduated test-tube or volumetric flask marked at 10 cc. Add 0.2 cc. of Molybdate IIIa and 0.4 cc. of aminonaphtholsulfonic acid reagent, and read against the customary standard. The calculation is identical with that for inorganic phosphate.

Determination of Total Fixed Base.—(Fiske, *J. Biol. Chem.*, 51 : 55.)—Precipitate the protein with 10 per cent trichloroacetic acid, using 4 volumes in the case of serum or plasma, and 9 volumes for whole blood. Measure the trichloroacetic acid solution into a dry Erlenmeyer flask, and run in the blood, etc., from a pipette, meanwhile rotating the flask. Close the flask with a rubber stopper, shake thoroughly, and filter through ashless paper. (If the same filtrate is not to be used for the determination of inorganic phosphate, less trichloroacetic acid is required. The concentration in the filtrate should in any event be not less than 5 per cent.)

Transfer 5 cc. of the serum or plasma filtrate, or 10 cc. in

the case of whole blood, to a 200×25 mm. lipped pyrex test-tube, and boil down with 1 cc. of 4 N sulfuric acid, 2 or 3 drops of concentrated nitric acid, and a quartz pebble until white fumes appear and the sulfuric acid residue is colorless.

Cool, and rinse with four 2 cc. portions of water into a test-tube marked at 25 cc. After adding a drop of methyl red (saturated alcoholic solution), neutralize the solution with powdered ammonium carbonate. Heat to boiling, and adjust the reaction with 4 N sulfuric acid (and a few grains of ammonium carbonate if necessary), leaving the solution finally just alkaline (yellow) to methyl red. Add 0.1 cc. of a 10.5 per cent solution of ferric chloride crystals in 0.1 N hydrochloric acid, followed by 1 cc. of 5 per cent ammonium acetate and enough water to make the total volume 10 or 11 cc. Again heat to boiling, and dilute to the mark with cold water.

Close the tube with a clean, dry rubber stopper, invert 2 or 3 times, and filter promptly through a dry 9 cm. ashless paper into a dry test-tube, keeping the filter nearly filled. Collect only about 20 cc. of filtrate, and discard the rest. Stopper the test-tube at once. (The change in volume caused by the cooling of the filtrate is corrected for in the final calculation.)

Transfer 5 cc. of the cooled filtrate to a small platinum dish, add 1 cc. of 4 N sulfuric acid, and evaporate on the steam bath until nearly dry. Place the dish on a metal triangle and heat, cautiously at first, over a micro burner, gradually raising the flame until no more fumes come off. Let cool, sprinkle over the residue a little powdered ammonium carbonate, and ignite again, finally raising the flame to its maximum and moving the triangle about until each part of the dish has been momentarily subjected to a dull red heat. Let cool, and add 2 cc. of water. Rotate the dish until the residue has all dissolved, using a rubber-tipped rod to assist in dissolving it if necessary.

Transfer the contents of the dish to a large lipped pyrex test-tube, and rinse four times with 2 cc. of water. Add 2 cc. of benzidine reagent, and proceed with the determination of inorganic sulfate as given on p. 165, titrating with 0.02 N sodium hydroxide (p. 247).

Subtract 3 per cent from the titration figure (1 per cent for the temperature correction, and 2 per cent to allow for the volume of the protein precipitate). The result (if the alkali is

exactly 0.02 N) when multiplied by 100, gives the number of milliequivalents of fixed base per liter of blood, serum, or plasma.

For the determination of fixed base in plasma or whole blood, most of the customary anticoagulants are ruled out because they are either sodium or potassium salts. A concentrated solution of ammonium citrate is satisfactory, and can be prepared in the following manner:

Dissolve 15 gm. of citric acid in 10 cc. of water. Gradually add concentrated ammonium hydroxide (stirring well) in amount equivalent to about 16 cc. of a 10 N solution. Evaporate on the steam bath to about 15 cc. Cool, and continue adding ammonia until a small drop of the citrate solution added to 5 cc. of water gives an intermediate brownish color with phenol red (*i.e.*, neither red nor yellow). This will take about 5 cc. of 10 N ammonium hydroxide. Dilute to 25 cc. Mix thoroughly, and keep well stoppered. 0.02 cc. of this solution will be enough for 5 cc. of blood.

Instead of removing the protein with trichloroacetic acid, the material may be ashed directly with sulfuric acid (with the aid of nitric acid or some other base-free oxidizing agent), but this takes more time, especially with whole blood. In this case the correction for the volume of the protein precipitate is naturally omitted.

Determination of Calcium.—(Fiske and Logan.)—The determination of calcium (with an accuracy of 1 per cent) in the solution of the dry ashed residue of 2 cc. of plasma or serum, or that resulting from wet ashing the trichloroacetic acid filtrate of plasma, serum, or whole blood, differs from the determination in urine (p. 227) only in that the precipitation of the calcium oxalate is carried out at a smaller volume (10 cc.) and with proportionately less of each reagent.

Trichloroacetic Acid Method.—Add 1 volume of serum, plasma, or whole blood to 4 volumes of 10 per cent calcium-free trichloroacetic acid[1] in an Erlenmeyer flask. Insert a rubber stopper, shake vigorously a few times, and filter through ashless paper. Evaporate 10 cc. of the filtrate to dryness in a 200×25 mm. lipped Pyrex test-tube, adding a quartz pebble to prevent bumping. Ash the residue with 0.5 cc. of 10 N sulfuric acid[2] (with the aid of a few drops of nitric acid[2] added after the

[1] Ignite 10 gm. of the solid reagent in a platinum dish, and apply the ammonium oxalate test given on p. 229.

[2] Calcium-free (see pp. 229–231).

water has boiled off). Add 4 cc. of water and 4 cc. of 2.5 per cent oxalic acid,[2] adjust the reaction to pH 5.0 with 5 N ammonium hydroxide,[2] and complete the determination as described for urine (pp. 227–229). To correct for the volume occupied by the protein precipitate, subtract 2 per cent from the result in the case of serum or plasma (4 per cent with whole blood).

Dry Ashing Method.—Evaporate 2 cc. of plasma or serum to dryness in a platinum crucible on the steam bath, and heat in an oven at 110° C. for several hours. Cautiously ignite the residue to a white ash. Add 1 cc. of water, dissolve the ash on the steam bath with 1 cc. of 10 N hydrochloric acid[3] (the crucible being covered with a watchglass), and evaporate to dryness. Transfer the residue to a 200×25 mm. lipped pyrex test-tube with the aid of two 2 cc. portions of water, 0.5 cc. of 10 N hydrochloric acid[3] and 4 cc. of 2.5 per cent oxalic acid[2] in succession. Neutralize to pH 5.0 with 5 N ammonium hydroxide,[2] and complete the determination as described for urine (pp. 227–229).

The determination of calcium by direct precipitation may be less accurate, but the results are close enough (2 or 3 per cent) for some purposes. Since there is reason to believe that direct precipitation is incomplete in certain pathological conditions, the results should occasionally be controlled by means of one of the methods given above.

Direct Precipitation Method.—Pipette 1 cc. of filtered 3 per cent ammonium oxalate and exactly 2 cc. of plasma or serum into a 15 cc. centrifuge tube. Mix and let stand over night. Centrifuge for at least 4 minutes at 2000 R. P. M., and pour off the supernatant liquid. Add approximately 12 cc. of 3 per cent ammonium oxalate[4] from a wash bottle in such a way as to stir up the precipitate. Centrifuge, and pour off the washings. Dissolve the precipitate with the aid of 3 or 4 drops of concentrated nitric acid,[4] add 2 cc. of water, and transfer the solution quantitatively to a platinum dish or crucible, rinsing with 4 cc. of water (1 cc. at a time). Complete the determination from this point in the usual manner.

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[3] Base-free. Evaporate 10 cc. to dryness in a platinum dish, and test for fixed base according to the directions given for nitric acid on p. 231.

[4] Base-free (see p. 231).

Determination of Potassium.—(Fiske and Litarczek.)—Evaporate to dryness on the steam bath, in a 100 cc. beaker, from 10 to 20 cc. (depending upon the amount of material available and the degree of accuracy required) of the trichloroacetic acid filtrate (p. 243) prepared from serum or plasma (1 : 5), or 5 cc. of whole blood filtrate (1 : 10). Add 1 cc. of aqua regia, and again evaporate, heating for 10 minutes after the contents of the beaker are apparently dry. Dissolve the residue in 1 cc. of water, add 1 cc. of sodium chloride-acetic acid solution, and precipitate with sodium cobaltinitrite exactly as described for urine (p. 241). The remainder of the procedure is likewise identical with that given for the determination of potassium in urine, with the following exceptions. In the extraction of the ignited cobalt precipitate, use only 0.5 cc. of hot water (instead of 1 cc.), and continue the rinsing with hot alcohol only until the total volume of filtrate has reached 5.5 cc. (instead of 11). Again, in rinsing the filtration tube after removing the acid potassium tartrate precipitate (with its mat) use only 4 cc. of water (instead of 9 cc.), making the volume of solution to be titrated only 5 cc. The blank, determined in this case by titrating 5 cc. of water, will likewise be smaller.

To calculate the potassium content of the serum or plasma (in mg. per 100 cc.), multiply the number of cc. of 0.02 N alkali required (after subtracting a 2 per cent correction for the volume of the protein precipitate) by 391, and divide by the volume of trichloroacetic acid filtrate used. In the case of whole blood, proceed in the same manner, and multiply the result by 2.

Total Fatty Acids and Cholesterol in Blood Plasma.—(W. R. Bloor, *J. Biol. Chem.*, 1926, 77 : 53.)—The most frequently desired values in the field of the lipids are those of the total fatty acids and the cholesterol, and the consensus of opinion is that these should be determined on the plasma, rather than on whole blood. In connection with the methods described below it should be pointed out that the older determinations based on nephelometry have been largely abandoned and have been replaced by an ingenious process of complete oxidation of the isolated fatty material.

Isolation.—One cc. of blood plasma is run slowly with con-

tinuous gentle shaking into a 25 cc. volumetric flask containing 15 to 20 cc. of ether-alcohol mixture (3 volumes of alcohol to 1 of ether). The mixture is then warmed on a water bath with shaking until the ether boils freely. Cool to room temperature, dilute to volume with ether-alcohol mixture, mix, and filter into a clean test-tube.

Transfer 20 cc. of the filtrate to a 125 cc. Erlenmeyer flask, add 2 drops of 40 per cent potassium hydroxide, cover with a watchglass (to minimize oxidation) and heat until no trace of alcohol odor is noticeable. Add to the residue 1 cc. of dilute sulfuric acid (1 volume of concentrated acid to 3 volumes of water), warm for a few seconds, and extract with petroleum ether as follows:

Add 15 cc. of petroleum ether and stir by gentle rotation until all of the visible particles are dissolved. Pour the petroleum ether into a clean 25 cc. volumetric flask. (Because of the different surface properties of the two liquids it is possible to make a good separation by decantation.) Warm the residue in the Erlenmeyer flask on a water bath and shake so as to rinse down any material which may have collected on the sides of the flask. Continue the extraction with successive small portions of petroleum ether until the combined extracts in the 25 cc. volumetric flask reach nearly to the mark. Cool this flask with contents to room temperature, dilute to volume with petroleum ether and mix. The solution contains the cholesterol and the total fatty acids obtainable from 0.8 cc. of plasma.

Transfer 15 cc. of the petroleum ether extract into a 125 cc. glass stoppered Erlenmeyer flask, and rinse the remaining 10 cc. of the extract, with 10 cc. more of petroleum ether into a 50 cc. Erlenmeyer flask. The second 10 cc. portion of the extract is to be used for the separate determination of cholesterol.

Remove, by evaporation on a water bath, the petroleum ether from both flasks. It is quite essential that every trace of petroleum should be removed from the larger flask, and in this case the last remaining fumes should be blown out with a gentle air current.

Oxidation of the Fatty Acids and Cholesterol.—To the residue in the 125 cc. flask add 5 cc. of Nicloux's silver reagent and 3 cc. of normal potassium dichromate solution, mix, and insert

the glass stopper. Transfer to another similar glass stoppered flask 5 cc. of the silver reagent and 3 cc. of the normal potassium dichromate solution, insert the glass stopper and mix. Heat the two stoppered flasks in an oven for 20 minutes at a temperature of 124 to 126° C. or heat for 60 to 90 minutes on an actively steaming water bath, at 88 to 90° C.

Cool the flasks and add to each, first, 75 cc. of ice cold distilled water, then, 10 cc. of 10 per cent potassium iodide solution. Titrate the liberated iodine in each flask with 0.1 N thiosulfate in the usual way, with starch as indicator. The titration figure of the unknown subtracted from the titration figure of the control gives the oxidation value of the total lipid present.

In carefully controlled experiments it has been found that under the given conditions 0.1 mg. of fatty acids reduces 3.6 cc. of the dichromate, while 1 mg. of cholesterol is equivalent to 3.93 cc. of the dichromate.

From the titration figure representing the sum of the fatty acids and the cholesterol, one subtracts the dichromate equivalent of the cholesterol. This equivalent is calculated from the cholesterol value obtained in the colorimetric determination described below. The calculations involved are best illustrated by a typical example.

Calculation.—By examination of the different volumes used in the preliminary preparation and in the oxidation procedures, it will be found that the oxidation is applied to the extract from 0.48 cc. of plasma while the separate cholesterol determination is made on an extract representing 0.32 cc.

Blank titration = 36.58 cc. of thiosulfate.

Titration of sample = 27.00 cc. of thiosulfate.

The titration difference therefore is 9.58 cc. of thiosulfate, equal to 9.58 cc. of 0.1 N dichromate. The cholesterol content found in 0.32 cc. of plasma is 0.63 mg. which corresponds to 0.95 mg. in the 0.48 cc. of plasma used for the oxidation. 0.95 mg. cholesterol is equivalent to 0.95×3.93 or 3.73 cc. of 0.1 N dichromate.

9.58 cc. $-$ 3.73 cc. or 5.85 cc. of 0.1 N dichromate therefore has been used up in oxidizing the fatty acid. $\frac{5.85}{3.60}$ or 1.63 mg. of fatty acid were therefore present in 0.48 cc. of plasma, since

3.60 cc. of dichromate = 1 mg. of fatty acid. 1 cc. of plasma therefore contains $\frac{1.63}{0.48}$ or 3.39 of fatty acid or 339 mg. per 100 cc.

Cholesterol Determination.—The residue left in the 50 cc. Erlenmeyer flask after the evaporation of the petroleum ether is dissolved in successive very small portions of chloroform, all of which are poured into a 10 cc. glass stoppered graduated cylinder. The total volume of chloroform employed should be exactly 5 cc. as measured in the cylinder. Transfer to another similar cylinder 5 cc. of a cholesterol-in-chloroform solution containing 0.5 mg. of cholesterol. To the chloroform in each cylinder add 1 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid. Insert the glass stoppers, shake, and set the cylinders in water adjusted to 20° C. and leave them there for 15 minutes.

Then transfer the contents to colorimeter cups and make the comparison, with the standard set at 10 mm. It is preferable to use a red glass filter over the eye piece of the colorimeter.

Calculation.—If the unknown reads 8 mm. against the standard set at 10 mm., $\frac{10}{8} \times 0.5$ or 0.63 mg. of cholesterol is present in the 0.32 cc. of plasma used for the determination. This yields $\frac{0.63}{0.32} \times 100$, or 197 mg. of cholesterol per 100 cc. of plasma.

The required reagents are described below:

0.1 N Sodium Thiosulfate.

Normal Potassium Dichromate.

10 Per Cent Potassium Iodide Solution.

1 Per Cent Starch Solution.

Sulfuric Acid-Silver Chromate Reagent.—This reagent is prepared according to Nicloux (*Bull. soc. chim. biol.*, (1927) 9:758 as follows: To 5 gm. of silver nitrate dissolved in 25 cc. of water in a 100 cc. centrifuge tube, add 5 gm. of potassium dichromate previously dissolved in about 50 cc. of water Centrifuge and wash the sediment twice with water to get rid of the nitric acid. Dissolve the moist precipitate in 50 cc. of pure concentrated sulfuric acid.

Note.—The preparation of the acid silver solution described above may prove laborious and inconvenient in some laborato-

ries. Preliminary experiments have indicated that a solution of 5 gm. of silver sulfate in 500 cc. of concentrated sulfuric acid is just as serviceable and in some respects more suitable.

PETROLEUM ETHER.—Petroleum ether must be fractionated so as to get rid of that portion which does not boil below 60° C. Distill with a Clarke column (*Ind. and Eng. Chem.*, 15: 349 (1923), and reject the portion which distills over 60° C. Wash the low boiling distillate with concentrated sulfuric acid in a separatory funnel and distill once more, this time using an ordinary condenser. Fairly good results can be obtained with unpurified petroleum ether provided that sufficient care be taken to remove the last traces of solvent from the fatty substances before oxidation, but this is a rather difficult thing to do and it is therefore well worth while to use only properly rectified petroleum ether.

ALCOHOL-ETHER MIXTURE.—3 parts of redistilled 95 per cent alcohol to 1 part of redistilled ether.

CHOLESTEROL STANDARD.—The final working standard contains 0.5 mg. of cholesterol in 5 cc. A convenient way to make it is to prepare a 0.1 per cent solution of cholesterol in chloroform (1 cc.=1 mg.) and from this prepare the diluted solution for use.

$$\frac{20}{x} =$$

This table is helpful in connection with colorimetric comparisons.

12.0 = 1.67	15.0 = 1.33	18.0 = 1.11	21.0 = .95	24.0 = .83
12.1 = 1.65	15.1 = 1.32	18.1 = 1.10	21.1 = .95	24.1 = .83
12.2 = 1.64	15.2 = 1.32	18.2 = 1.10	21.2 = .94	24.2 = .83
12.3 = 1.63	15.3 = 1.31	18.3 = 1.09	21.3 = .94	24.3 = .82
12.4 = 1.61	15.4 = 1.30	18.4 = 1.09	21.4 = .93	24.4 = .82
12.5 = 1.60	15.5 = 1.29	18.5 = 1.08	21.5 = .93	24.5 = .82
12.6 = 1.59	15.6 = 1.28	18.6 = 1.08	21.6 = .93	24.6 = .81
12.7 = 1.57	15.7 = 1.27	18.7 = 1.07	21.7 = .92	24.7 = .81
12.8 = 1.56	15.8 = 1.27	18.8 = 1.06	21.8 = .92	24.8 = .81
12.9 = 1.55	15.9 = 1.26	18.9 = 1.06	21.9 = .91	24.9 = .80
13.0 = 1.54	16.0 = 1.25	19.0 = 1.05	22.0 = .91	25.0 = .80
13.1 = 1.53	16.1 = 1.24	19.1 = 1.05	22.1 = .90	25.1 = .80
13.2 = 1.52	16.2 = 1.23	19.2 = 1.04	22.2 = .90	25.2 = .79
13.3 = 1.50	16.3 = 1.23	19.3 = 1.04	22.3 = .90	25.3 = .79
13.4 = 1.49	16.4 = 1.22	19.4 = 1.03	22.4 = .89	25.4 = .79
13.5 = 1.48	16.5 = 1.21	19.5 = 1.03	22.5 = .89	25.5 = .78
13.6 = 1.47	16.6 = 1.20	19.6 = 1.02	22.6 = .88	25.6 = .78
13.7 = 1.46	16.7 = 1.20	19.7 = 1.02	22.7 = .88	25.7 = .78
13.8 = 1.45	16.8 = 1.19	19.8 = 1.01	22.8 = .88	25.8 = .78
13.9 = 1.44	16.9 = 1.18	19.9 = 1.01	22.9 = .87	25.9 = .77
14.0 = 1.43	17.0 = 1.18	20.0 = 1.00	23.0 = .87	26.0 = .77
14.1 = 1.42	17.1 = 1.17	20.1 = 1.00	23.1 = .87	26.1 = .77
14.2 = 1.41	17.2 = 1.16	20.2 = .99	23.2 = .86	26.2 = .76
14.3 = 1.40	17.3 = 1.16	20.3 = .99	23.3 = .86	26.3 = .76
14.4 = 1.39	17.4 = 1.15	20.4 = .98	23.4 = .85	26.4 = .76
14.5 = 1.38	17.5 = 1.14	20.5 = .98	23.5 = .85	26.5 = .75
14.6 = 1.37	17.6 = 1.14	20.6 = .97	23.6 = .85	26.6 = .75
14.7 = 1.36	17.7 = 1.13	20.7 = .97	23.7 = .84	26.7 = .75
14.8 = 1.35	17.8 = 1.12	20.8 = .96	23.8 = .84	26.8 = .75
14.9 = 1.34	17.9 = 1.12	20.9 = .96	23.9 = .84	26.9 = .74
				27.0 = .74

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