THE QUANTITATIVE HISTOCHEMISTRY OF BRAIN

I. CHEMICAL METHODS*

By OLIVER H. LOWRY, NIRA R. ROBERTS, KATHERINE Y. LEINER, MEI-LING WU, AND A. LEWIS FARR

(From the Department of Pharmacology, Washington University School of Medicine, St. Louis, Missouri)

(Received for publication, July 13, 1953)

Elucidation of the biochemistry of the brain is hindered by its extreme histological complexity. However, if it were possible to perform appropriate chemical analyses and enzymatic measurements on the individual histological components of the brain, one could then reassemble a picture of the biochemical matrix which is responsible for the functioning of the whole. By altering and exploiting the general procedure of Linderstrøm-Lang and his collaborators, it is in fact practical to obtain samples as small as 0.2 γ wet weight from any desired region of the brain (1). In brief, frozen sections are dehydrated at $-30°$, and the desired regions are cut from the dry section under a dissecting microscope. The isolated fragments are ready for direct analysis for any constituent stable to freezing and drying at $-30°$.

In order to carry out the analytical aspects of the problem, microchemical procedures have been devised which are not only sufficiently sensitive but are also simple to perform. The reduction in the number and complexity of analytical maneuvers has tended to decrease the danger of errors at the small scale of operations and has permitted larger numbers of analyses, a requirement for histochemical studies, without sacrifice in precision.

This paper describes the general procedures and simple tools which are necessary for all of the methods. Also given are directions for measuring chloride (4×10^{-10} mole), riboflavin (3×10^{-5} γ or 10^{-13} mole), and several phosphorus fractions in 10 γ of brain or other tissue. (These methods are either new or contain new features which might find use on a larger scale as well.) Paper II describes procedures for measurement of a number of enzymes with 10 γ or less of brain (2). Methods for measuring protein (3), cholesterol,[1] and phospholipide fractions[2] on the necessary micro scale have also been developed. Elsewhere will be found data obtained by these methods on the histochemistry of Ammon's horn (4) and cerebellum (5).

* Supported in part by a grant from the American Cancer Society through the Committee on Growth of the National Research Council.

[1] Albers, R. W., and Lowry, O. H., in preparation.

[2] Robins, E., McCaman, R. E., and Lowry, O. H., in preparation.

Microchemical Tools, Laboratory Aids, and Procedures for General Use

Reaction Tubes—With a few exceptions the tubes used are smaller than any available commercially, and they are made from standard Pyrex tubing which is selected for size and cut in lengths twice as long as the tube needed. Each piece is made into two tubes in a single operation with a small hot flame. A pointed tip facilitates the removal of supernatant fluid from precipitates, but the tube tip must not be so sharp as to trap part of the sample or to make mixing difficult. The tops are fire-polished, but only very lightly to prevent narrowing of the orifice.

Tubes of 2 to 2.5 mm. bore and 5 or 6 cm. long are most used. These are made from 4 mm., outer diameter, tubing, but should be selected on the basis of the inner diameter. Less frequently tubes are required with bores of 1.5 to 1.8 mm. or of 3 mm. which are made from tubing 3 and 5 mm. outer diameter respectively. Tubes larger than these can be bought ready-made, *e.g.*, serological tubes (soft glass) 6 × 50 mm. (0.7 ml. volume), Kimble Glass Company, No. 45060, and 7 × 75 mm. (1 ml. volume) (A. S. Aloe Company, St. Louis). When fluorometry or colorimetry is to be performed directly in the tube, it must be more carefully made, as described below.

Clean tubes are of course absolutely essential. Tubes smaller than 4 mm. bore are a problem to clean because of the difficulty in removing wash fluids. Therefore, the following scheme was devised to clean many hundreds of tubes at a time, both inside and out. The dirty tubes are packed into beakers (200 ml. tall form, without lip, Corning No. 1040) until they cannot tip over. The tubes are filled with half concentrated nitric acid by covering with acid and centrifuging for a few seconds at a *very low speed* in a centrifuge designed to hold a 250 ml. centrifuge bottle (International Equipment Company, Boston). The beakers of acid-filled tubes are heated for at least 15 minutes in a pan of nearly boiling water in a hood. The excess acid is decanted, and the tubes are transferred, bottom up, into another beaker which has been fitted with a false bottom of stainless steel screen raised half an inch from the bottom of the beaker. The tubes can be transferred without getting them twisted, if, for example, a 50 ml. Erlenmeyer flask is used (base toward the tubes) to keep them moving as a group. The tubes are now centrifuged empty, and the nitric acid is drawn off from under the screen with suction through a slender glass tube or a long stainless steel needle, after which the tubes are tipped back into the original beaker and filled with rinse water in the centrifuge. Usually, tubes are rinsed three times with glass-distilled water with the help of the centrifuge. They are heated in a boiling water bath during the second rinse. After the final rinse, by centrifuging a little longer than usual the tubes will become nearly dry, and it will be unnecessary to risk contamina-

tion from heating in an oven. The tubes are stored in dust-proof glass jars.[3]

Pipettes—All of the pipettes used are Lang-Levy constriction pipettes (6). For the narrow tubes, it is necessary to construct the pipettes with a very short bend at the end of a very narrow tip (Fig. 1). Although larger pipettes are available commercially (Carlsberg Laboratory, Copenhagen, Denmark, Microchemical Specialties Company, Berkeley, California, and Arthur H. Thomas Company, Philadelphia), most of them must be specially made. Directions given earlier may be helpful (7).

With pipettes of 1 μl. or less, especially with this slender construction, surface tension may make it difficult to deliver the sample. In this event, the pipettes are coated with Silicone by rinsing the clean, dry pipette up to the constriction successively with Desicote (Beckman Instruments, Inc., South Pasadena, California), benzene, and alcohol. If it becomes necessary to clean the pipette with fuming nitric acid (the agent of choice if rinsing is not sufficient), the pipette may need to be recoated. To remove the Silicone permanently, pipettes are rinsed with 10 N NaOH a few times. With Silicone coating, pipettes as small as 0.02 μl. have been used successfully and appear to have a precision of better than 1 per cent.

Calibration of Pipettes—Pipettes too small to calibrate easily by weight of delivered water (less than 20 μl.) may be calibrated colorimetrically as follows: A pipetteful of 0.6 per cent *p*-nitrophenol is added to an exactly measured amount of 0.01 N NaOH of about 1000 times the volume of the pipette. The reading at λ 400 mμ in the Beckman spectrophotometer is compared to that of two standards prepared *the same day* by adding, respectively, 1.000 and 1.100 ml. of the 0.6 per cent *p*-nitrophenol to 1 liter volumes of 0.01 N NaOH. The volume of the pipette is calculated by interpolation if the optical densities are not linear. Calibration to within 0.3 per cent is easily attainable. Other pigments might be used if, like *p*-nitrophenol, they are highly colored, are not absorbed on glass, are sufficiently soluble, and if the optical density is nearly linear with concentration.

Mixing Samples—With slender tubes it is difficult to mix well enough by tapping with the finger. Tubes of any size from 1 to 15 mm. in diameter can be thoroughly mixed by what may be called "buzzing" (8). Smaller tubes are held at an angle against a flattened nail rotating at high speed (5000 to 10,000 r.p.m.) in the chuck of a commercial high speed hand grinder. The eccentric motion of the nail imparts a violent whirling motion to the liquid in the tube without spilling. Alternatively, a commercial

[3] Tubes for measuring protein are filled with 0.5 N NaOH and heated in boiling water for 15 minutes to remove molybdic and tungstic acids before nitric acid cleaning.

massage vibrator will serve quite well for tubes of 4 to 6 mm. bore. For larger tubes, a bigger rod turning more slowly (3000 to 5000 r.p.m.) is used. In general the tubes must be not over one-third full to prevent spillage.

Colorimetry—Many of the methods are based on colorimetric measurement in the Beckman spectrophotometer adapted to volumes of 40 μl. or less (9).

In certain cases it is necessary or more convenient to measure reactions in tubes rather than square cuvettes; *e.g.*, when CO_2 from the air must be avoided, as in the measurement of cholinesterase (2), or with many timed reactions such as the reduction of diphosphopyridine nucleotide (DPN) by substrate in measuring lactic and malic dehydrogenases (10). Tubes are especially useful when temperature control is important. Adaptation for tubes may readily be accomplished with the Beckman spectrophotometer by substituting for the standard carriage holder compartment a block of black plastic of the same dimensions, drilled and channeled to hold a tube in position (Fig. 1). Tubes are made as described for fluorometer tubes (below) from selected tubing. With tubes of 1.5 mm. bore as little as 8 μl. volume is sufficient. (Since the light path in this case is only 1.5 mm., the colorimetric sensitivity is not greater than that obtained with 50 μl. and a 1 cm. light path.) Care is exercised in cleaning and handling such tubes not to scratch the optical portion near the bottom.

The most favorable position for reading each tube is indicated by a mark with a diamond point or carborundum wheel. Tubes are selected on the basis of readings with water and with a colored solution. Reproducibility is more important than the absolute reading with a colored solution, since most measurements are made by difference.

Fluorometry—The fluorometer used is the Farrand instrument (Farrand Optical Company, Inc., New York). The general construction has been described (11). This sensitive instrument may be further adapted to volumes smaller than the regular 1 ml. A block of wood replaces the standard tube carriage. This block is drilled to receive a brass rod which is in turn drilled to receive tubes of desired size. Details are given in Fig. 1. Every part is coated with India ink (some black paints are fluorescent). In addition, scattering of incident light to the phototube is further decreased by extending the baffles within the instrument and sealing off any possible communication between light source and phototube.

The benefit of these changes may be illustrated by comparing the instrumental performance with volumes of 10 μl. and 1 ml. (1.8 mm. versus 8 mm. bore tubes). The sensitivity is increased about 30-fold by the 100-fold reduction in volume, and the ratio of sample to blank reading is almost unchanged. With riboflavin, for example, at 10 μl. with full sensitivity 1

scale division (10^{-9} ampere) is equivalent to about 5×10^{-13} gm. of riboflavin (6×10^{8} molecules). The blank with distilled water is about 20 divisions.

The fluorometer tubes are made from tubing as uniform and free from flaws and striae as possible. Lengths or portions of lengths with close tolerances both inside and out are selected. Pieces 10 cm. long are cut in two with a flame which is so small that the glass near the cut is not softened

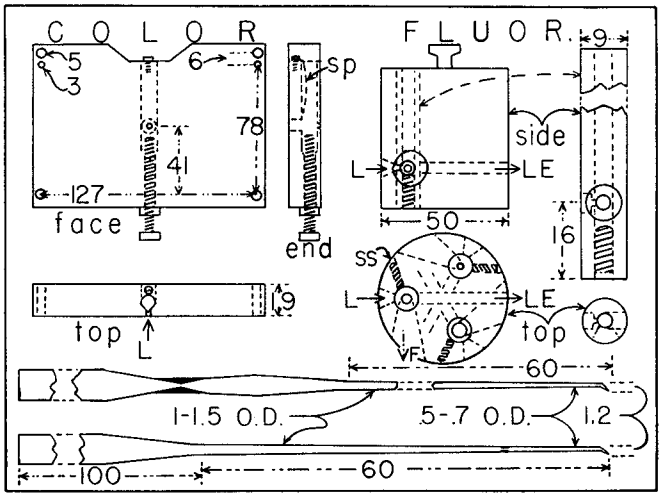

FIG. 1. Micro pipettes (large and small) for use with tubes of 2 mm. bore, adapters to hold small tubes in Beckman spectrophotometer ("color"), and Farrand fluorometer. The dimensions are in mm. *Sp* is a flat bronze spring, *L* the entering light, *LE* the light exit, *F* the path of fluorescent light toward phototube, *SS* a set-screw to hold the 9 mm. brass insert in place. This insert is shown separately at the extreme right. A set-screw at an angle (not shown) is also provided to hold the larger block in place when the most favorable orientation is located.

and thereby distorted. The bottom should be nearly flat and of the same thickness in all tubes.

The finished tubes after thorough cleaning are checked in the fluorometer first with water and then with quinine in 0.1 N H_2SO_4, and unsatisfactory tubes are discarded. A scratch mark is used to indicate the most stable position.

Individual Chemical Methods

Riboflavin

This is measured by its fluorescence in a fairly standard manner (*cf.* (12)), except that the final volume for measurement is reduced to 10 μl.

To the dry sample, representing 10 to 25 γ wet weight in a pointed tube of 2 to 2.5 mm. bore, are added 7 μl. of 5 per cent trichloroacetic acid (TCA). The sample is buzzed gently, capped, and centrifuged, and 5 μl. of the supernatant fluid are transferred to a fluorometer tube 5 cm. long made from Pyrex tubing of 1.8 mm. bore.

The tube, capped with Parafilm, is allowed to stand overnight at 38° to hydrolyze the flavin-adenine dinucleotide (12). (To prevent evaporation the tube, or rack of tubes, is placed in a covered glass jar with a little water in the bottom.) Shortly before reading, 5 μl. of 0.45 M K_2HPO_4 are added in the darkened fluorometer room, and the tube is carefully wiped with a cloth washed and dampened with distilled water. Only a few tubes are neutralized at a time, since the sensitivity to light is much less before neutralization. After reading, the blank is obtained by adding 1 μl. of 2.5 per cent $Na_2S_2O_4$ (of good activity) in 2.5 per cent ice-cold $NaHCO_3$ (preserved in a tube in ice water for not more than an hour). Blanks and standards are run separately. Mixing after hydrosulfite addition must be by tapping instead of buzzing to prevent reoxidation.

After use, the tubes are rinsed with water to remove hydrosulfite before acid cleaning. Tubes not used for some time ought to be rinsed twice with distilled water (in the centrifuge) just before use to keep the blank as low as possible.

Comment on Riboflavin Determination—Because of the high dilution riboflavin gives the same reading when added to a brain sample (as internal standard) as when added to a blank. If desired, flavin mononucleotide and dinucleotide may be measured separately by determining the fluorescence of an unhydrolyzed TCA aliquot (12).

With the amounts of riboflavin in 10 or 20 γ of brain the standard error is about 1×10^{-12} gm. (Table I) or 2×10^{-15} mole. The change in reading of the blank on addition of hydrosulfite is equivalent to not more than 1 or 2×10^{-12} gm. of riboflavin. The total reading of the blank before hydrosulfite may be equivalent to 10 or 20×10^{-12} gm. of riboflavin. Higher readings would indicate contaminated tubes or reagents or a faulty optical system.

Chloride

This method is based on the precipitation of chloride with silver from a nitric acid tissue extract and the measurement of the excess silver colorimetrically with 5-(*p*-dimethylaminobenzylidene)rhodanine.

Because of the extreme danger of contamination with chloride, including HCl from laboratory air, the analyses need to be conducted, at least until the final step, in a room completely free of Cl-containing fumes. The tubes are thoroughly rinsed in the same room and either used immediately after cleaning or preserved in hermetically sealed jars.

To the dry tissue sample equal to 10 to 25 γ of fresh brain (4 to 10 $\times$ 10^{-10} mole of Cl) in a pointed tube of 2 mm. bore are added 2.5 μl of 0.75 N HNO_3.[4] This is centrifuged after 10 or 15 minutes at 3000 r.p.m. and capped with Parafilm. To avoid contamination with chloride from sweat, the tops of the tubes are never touched with the fingers.

A 2 μl. aliquot of the supernatant fluid is transferred to another similar pointed tube, and 0.5 μl of 2 mM $AgNO_3$ in 0.1 N HNO_3 is added. The sample is tapped or buzzed gently, capped with Parafilm, and after 30 to 60 minutes centrifuged for 20 minutes at 3000 r.p.m. A 2 μl. aliquot, which contains the excess silver, is added to 50 μl. of the rhodanine reagent

TABLE I

Measurement of Riboflavin in Rabbit Brain Homogenate

Homogenate samples equivalent to 25 γ (Brain A) and 20 γ (Brain B) were precipitated with trichloroacetic acid. An aliquot equal to 40 per cent of each sample was used for each analysis. The instrument was set at a different sensitivity for each series.

32.4 $\times$ 10^{-12} gm. standards	10 γ samples, Brain A		38.2 $\times$ 10^{-12} gm. standards	8.4 γ samples, Brain B	
Net galvanometer reading	Net galvanometer reading	Riboflavin	Net galvanometer reading	Net galvanometer reading	Riboflavin
		10^{-12} gm.			*10^{-12} gm.*
31.3	34.7	36.1	67.2	50.7	28.8
31.6	39.0	40.1	67.2	48.8	27.7
31.0	37.5	39.1	67.4	50.4	28.0
29.3	37.1	38.6	67.3	50.0	28.4
	37.5	39.1	67.4		
	37.4	39.0	67.5		

previously placed in a tube of 3 or 4 mm. bore (pointed tip undesirable). Each tube is immediately mixed by tapping. Vigorous mixing or buzzing is avoided because this might favor precipitation of the silver-rhodanine complex. The color is read after 30 to 60 minutes at the absorption maximum, λ 470 mμ. The rhodanine reagent is prepared by mixing within a few hours of use 4 ml. of 1 N H_2SO_4, 1 ml. of 10 per cent gum arabic,[5] and

[4] If protein is to be measured on the precipitate, the tubes should be kept chilled in ice water, preferably centrifuged cold, the supernatant fluid removed as soon as possible, and the precipitate dissolved in alkali at once by adding 5 μl. of 1.1 N NaOH. The protein determination is completed as described (3) with 50 μl. of Reagent D and 5 μl. of diluted Folin's reagent. Since even brief exposure to nitric acid reduces somewhat the color produced, standards are treated in the same manner as the unknowns or a suitable correction factor is applied. With brain, the color decrement is about 10 per cent.

[5] Gum arabic "photo purified" (Mallinckrodt Chemical Works, St. Louis) purified

250 μl. of a 50 mg. per cent solution in methyl Cellosolve of *p*-dimethylaminobenzylidenerhodanine (*p*-dimethylaminobenzalrhodanine, No. 2748, Distillation Products Industries, Rochester, New York).

The stock rhodanine solution in methyl Cellosolve is not completely stable. It is kept at 4° and discarded if an optical density of much less than 2 is obtained with the final reagent plus an excess of Ag; *e.g.*, 1 ml. of final reagent plus 10 μl. of 10 mM $AgNO_3$.

Standards and blanks are prepared by substituting 2.5 μl. of 0, 0.2, 0.3, and 0.4 mM NaCl in 0.75 N HNO_3 for the 0.75 N HNO_3 used with the tissue samples. Calculation is made from a curve which is nearly linear.

Tubes with AgCl precipitate are rinsed (via the centrifuge) with 1 per cent (of concentrated) NH_4OH before the usual acid cleaning process.

Comment on Chloride Method—The reagent *p*-dimethylaminobenzylidenerhodanine has been used since 1928 (13) for the detection of Ag and as an indicator in Ag titrations, but its use for quantitative measurement of Ag (14) has been criticized (15) on the grounds of lack of proportionality between Ag and color. It has been found, however, that by substituting 0.8 N H_2SO_4 as solvent for 0.04 N HNO_3, as originally proposed (14), and by introducing gum arabic to prevent precipitation the color is nearly proportional to Ag concentration over a wide range (Fig. 2). Since the molar extinction coefficient based on silver is about 33,000, the sensitivity for Ag, and hence for Cl, is quite great.

When silver is in excess, the molar extinction coefficient with recrystallized rhodanine was found to be 16,000 (based on rhodanine). From this it would appear that the composition of the colored product approximates $AgRh_2$. When Ag and rhodanine concentrations are varied, the color produced (Fig. 2) is that predicted from the equations

$$Ag + Rh_2 \rightleftharpoons AgRh_2\downarrow, \text{ and } Ag \times Rh_2 = k = 1.11 \times 10^{-10}$$

That is, the colored product $AgRh_2$ acts as though it were out of solution (actually a dispersed aggregate), and the dissociation constant has the form of a solubility product. Furthermore, the data are compatible with a reaction between silver and a rhodanine dimer rather than with single rhodanine molecules. In Fig. 2 the curves are theoretical, based on this equation, and the points are observed. Feigl (13) reported that the precipitate from organic solvents had the composition AgRh. We have found, when Ag is precipitated with rhodanine from methyl Cellosolve, that with a large Ag excess the precipitate contains about 1 mole of rhodanine per mole of Ag,

further by ethanol fractionation. To 100 ml. of a 20 per cent solution in water are added 120 ml. of 95 per cent ethanol. The precipitate is discarded. The filtrate or supernatant fluid (turbid) is precipitated with half its volume of ethanol, and the precipitate is dried with ethanol and ether.

whereas with an excess of rhodanine the precipitate contains 2 moles of rhodanine per mole of Ag, which agrees with the colorimetric data.

Since the silver-rhodanine complex has a dissociation constant as low as the AgCl solubility product, there is little danger from chloride contamination in the last step, in which rhodanine is in good excess, provided the

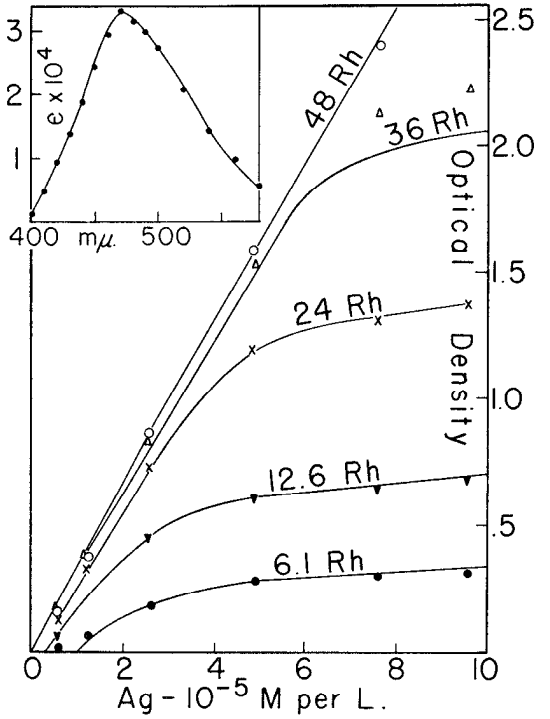

FIG. 2. Relationship between color and silver concentration with different levels of rhodanine. The points are experimental; the curves are calculated from the equation Ag (free) $\times$ Rh_2 (free) = 1.11 $\times$ 10^{-10}; *i.e.*, the optical density is equal to 33,000 $\times$ $AgRh_2$ = 33,000 (Rh_2 (total) $-$ Rh_2 (free)). In the *inset* is the absorption spectrum of the silver-rhodanine complex.

rhodanine reagent is placed in the final tube before the silver aliquot. This has been confirmed by deliberately adding small amounts of chloride after the silver and rhodanine have been mixed. In spite of the small AgRh dissociation constant, rhodanine will not remove silver from precipitated AgCl. Apparently the AgCl aggregate and the rhodanine (which is probably present as a large micelle) react extremely slowly with each other.

Color development is 80 per cent complete in 5 minutes and 98 per cent complete in 30 minutes. After an hour there is only slight change for 24

hours. The blank is quite stable. An acidity of about 1 N is most favorable for a low blank and the development of stable, proportionate color. The exact acidity is not critical, but with higher acidity the Ag-rhodanine dissociation constant increases. Nitric acid is unsatisfactory because it causes high blanks and low readings with Ag.

It is necessary to precipitate the Cl at a concentration of 10^{-4} M or over because of the magnitude of the AgCl solubility product (10^{-10}) and the need to keep the absolute Ag excess low (25 to 100 per cent is optimal). Consequently the precipitation volume is much smaller than the volume for color development. Precipitation is satisfactory in 0.5 to 2 N HNO_3.

As little as 4×10^{-10} mole of Cl (0.015 γ) is measured satisfactorily by the micromethod and essentially the same value is obtained as by the macro-Volhard method for brain (Table II). The sensitivity is about 25 times greater than that for the method used by Westfall *et al.* (17) in their studies of glomerular fluid, or by Linderstrøm-Lang *et al.* (18) and others. The use of 0.75 N nitric acid as a deproteinizing agent for chloride and other inorganic measurements has been validated previously (19).

The colorimetric method may be readily adapted to a larger scale, and, when the expected chloride is approximately known, the precision can be considerably increased by keeping the silver excess small and the concentrations of silver and chloride high.[6]

Phosphorus Fractions

With the Fiske-Subbarow method for phosphate, the molar extinction coefficient is about 4000 at the maximal wave-length (820 mμ). By increasing the sulfuric acid concentration from 0.5 to 1 N (to keep the blank small) and heating at 100° for 30 minutes, Fiske and Subbarow were able to increase the visual color 3- or 4-fold.[7] Actually, at λ 820 mμ the increase is more than 6-fold. This procedure has recently been further explored (21). The reaction is not suited to micro work, since there is a tendency to reoxidation of the reduced phosphomolybdate by air (22) and because heating to 100° would result in serious change in volume. It has been found that by substituting 1 per cent ascorbic acid for the sulfite and aminonaphtholsulfonic acid of the Fiske-Subbarow reagent, the formation of the highly colored second stage reduction product is complete in 2 hours at 38°. Since the ϵ_{820} is 25,000 (Fig. 3), it is possible to measure phosphate at a final concentration of 4×10^{-6} M (0.003 γ of P or 10^{-10} mole in 25 μl.) with reasonable precision. This is approximately the sensitivity obtained

[6] Smith, C. A., Lowry, O. H., and Wu, M.-L., in preparation.

[7] Dr. C. H. Fiske, personal communication. Benedict and Theis achieved a similar degree of color development by heating with hydroquinone and bisulfite in 1.8 N H_2SO_4 (20).

by Schaffer *et al.* (16) by a more elaborate procedure. Ammon and Hinsberg (23) used 0.02 per cent ascorbic acid to reduce phosphomolybdic acid, but obtained chiefly the first stage, low colored reduction product.

The ashing procedure for organic phosphate fractions is adapted from a method described for measuring the acid-insoluble phosphate of white blood cells and platelets (24). Directions are given for measuring five different fractions on a single sample. If only acid-soluble P and lipide P are to be measured, a considerably smaller sample is sufficient.

To a dried sample equal to about 20 γ of original brain in a 2 to 2.5 mm. bore pointed tube, in a rack in an ice bath, are added 12 μl. of 5 per cent

TABLE II

Reproducibility of Proposed Chloride Method and Comparison with Macro-Volhard Titration

A rabbit brain was homogenized with water and analyzed in 1 gm. quantities for chloride by a Volhard (thiocyanate) titration of a nitric acid extract (19). There were found to be 38.7 mM of Cl per kilo of fresh brain. Aliquots of the same homogenate equivalent to 10 and 20 γ of brain were analyzed by the proposed procedure. The values are recorded as millimoles per kilo, wet weight.

10 γ brain		20 γ brain	
37.0	37.2	36.4	37.4
39.4	37.0	36.4	39.8
39.0	39.0	37.5	37.8
41.2	38.6	36.9	39.7
37.0	37.4	33.2	
Average			37.6
S.d.			1.7

TCA (from redistilled acid). The tube is capped with Parafilm. After buzzing and allowing to stand for 5 to 15 minutes, the samples are mixed again and centrifuged, preferably cold. They are returned to ice water, and two separate 5 μl. aliquots are transferred from each tube into tubes of the same size as the original.

At once 20 μl. of absolute alcohol are added to the original tube which contains 0.1 M potassium acetate, and the sample is buzzed.[8] The speed and chilling to this point are to prevent breakdown and solution of ribonucleic acid. After adding the alcoholic acetate, the sample may be allowed to stand for some hours, perhaps days, in the ice box.

Inorganic Plus Labile P—To one 5 μl. aliquot of the TCA extract are

[8] The acetate is added to neutralize the trichloroacetic acid; otherwise some protein would be dissolved and lost in the alcohol.

added 40 μl. of a reagent made by mixing, in this order, 1 ml. of 10 N H_2SO_4, 6 ml. of H_2O, 1 ml. of 2.5 per cent ammonium molybdate, and 1 ml. of 10 per cent ascorbic acid. Each sample is mixed promptly after addition of the reagent and is capped with Parafilm. The entire rack of tubes, including appropriate blanks and standards also prepared in TCA, is incubated in an oven at 38° for 2 hours and is read at λ 820 mμ. Standards consisting

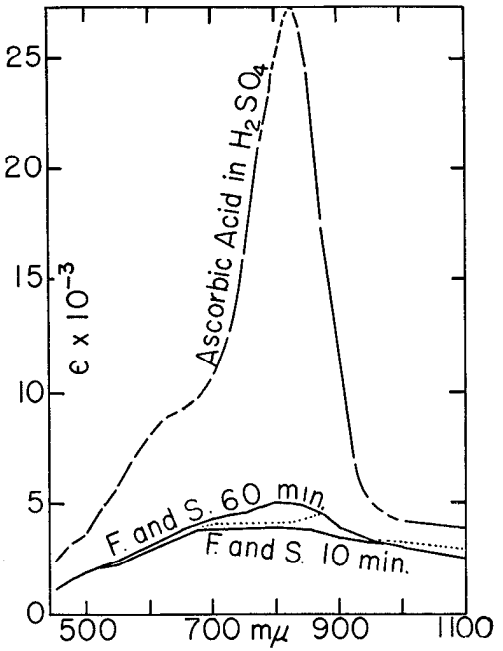

FIG. 3. Absorption spectra obtained with phosphate determined by the Fiske-Subbarow method at 10 and 60 minutes and by the proposed method (ascorbic acid, 1 N H_2SO_4). Also shown (dotted curve) is the spectrum of phosphomolybdate reduced by ascorbic acid at pH 4.

of 5 μl. of 0.1 mM KH_2PO_4 (0.5×10^{-9} mole) in 5 per cent TCA and blanks of 5 μl. of TCA are treated like the samples.

Acid-Soluble Phosphorus—To the second 5 μl. aliquot of TCA extract are added 5 μl. of ashing mixture consisting of 0.8 N $HClO_4$ in 10 N H_2SO_4. (To prepare, mix 65 ml. of 70 per cent $HClO_4$ (1.18 gm. of $HClO_4$ per ml.) with 500 ml. of 20 N H_2SO_4 and dilute to 1 liter.) A group of samples is dried for 2 hours, or until all the water is evaporated, in a metal rack in an oven at 95° (measured near the rack). The rack is transferred to another oven at 165° (measured near the rack) for 2 hours to ash. To each sample are added 50 μl. of 0.25 per cent ammonium molybdate in 0.1 N sodium

acetate, to which has been added just before use 1 ml. of 10 per cent ascorbic acid per 10 ml. of solution. (After addition of the ascorbic acid this reagent is kept in ice water and used within 1 hour.) Each sample must be *mixed immediately and thoroughly.* The rack of samples capped with Parafilm is left in an incubator at 38° for 2 hours and is read at λ 820 mμ.

The final volume may be increased or decreased to measure greater or lesser quantities of phosphorus, provided the given relationship of ashing mixture to ascorbic acid-molybdate reagent is maintained. Standards and blanks are prepared as for inorganic P and are carried through the whole procedure.

Lipide Phosphorus—The lipides are removed from the TCA precipitate by successive extractions with 20 μl. of 0.1 N potassium acetate in absolute alcohol (see above) and absolute alcohol. After each solvent addition the tubes are capped with Parafilm and centrifuged at 3000 r.p.m., and most of the solvent is removed with a 20 μl. constriction pipette under a good light, with great care to avoid disturbance of the precipitate. During the final alcohol extraction the tubes, capped with Parafilm, are heated 10 minutes at 60° in a shallow water bath. The extracted solvents are pooled from each sample in a flat bottomed tube of 3 mm. bore, which is kept chilled in a rack in an ice bath to avoid creeping of the solvent up the tube. The combined solvents are evaporated by directing a stream of filtered N_2 into the tube through a glass capillary while the tube, held in a spring clip, is lowered into a beaker of near boiling water. The stream of N_2 must be rapid enough to vaporize the alcohol before it can creep up the walls of the tube. A convenient substitute for the water bath is a very small electric heater (*e.g.* "hotspotter" Fisher Scientific Company, New York). The tube is held at an appropriate distance above the heating element. A centrifugal device for evaporating a number of samples at once, with a very substantial saving of time, will be described.[1]

After evaporation, to each tube are added 20 μl. of 0.4 N $HClO_4$ in 5 N H_2SO_4 (ashing mixture for acid-soluble phosphorus diluted 1:1). The sample is buzzed to wet the walls where lipide may have dried. The water is driven off, the sample is ashed, and color is developed as described for acid-soluble phosphorus, except that 100 μl. of the ascorbic acid-molybdate reagent are used instead of 50 μl. If the samples do not appear to be completely ashed (dark colored), 5 μl. of fuming HNO_3 may be added to each tube (cold) and the sample heated at 165° for another half hour.

Blanks are prepared by evaporating 20 μl. of acetate alcohol plus 20 μl. of alcohol in each tube. Standards are prepared by adding to extra blanks 5 μl. of 0.4 mM KH_2PO_4 (2×10^{-9} mole) in water. Blanks and standards are treated just like the samples thereafter.

Some of the acid-soluble P is carried into the lipide P sample, since only

10 of 12 μl. of the original TCA extract were removed from the tube prior to lipide extraction. A suitable deduction is made (16 per cent of the acid-soluble P).

Nucleic Acid P—The nucleic acid is made soluble as described by Schneider (25) except for the substitution of $HClO_4$ for TCA and a longer heating period at lower temperature to decrease the danger of evaporation. To the residue after lipide extraction are added 12 μl. of 0.3 N $HClO_4$, and the tube is capped with a *very tight fitting* rubber cap (cut-off vial stopper) and heated at 75° ± 5° for 1 hour. The tube is buzzed, centrifuged, and 10 μl. of the supernatant fluid are evaporated with 5 μl. of 10 N sulfuric acid (no $HClO_4$). The further analytical steps are as given for measurement of acid-soluble P. Standards of 12 μl. of 0.1 mM KH_2PO_4 (1.2×10^{-9} mole) in 0.3 N $HClO_4$ are carried through every step of the procedure.

Residual Phosphorus—To the residue after nucleic acid extraction are added 5 μl. of 0.8 N $HClO_4$ in 10 N H_2SO_4, and the sample is ashed and treated as above with 50 μl. of ascorbic acid-molybdate reagent for color development. It may be necessary to complete the wet ashing with fuming nitric acid (see above). The standards consist of 5 μl. of 0.1 mM KH_2PO_4 in water. The values must be corrected for one-sixth of the nucleic acid P which is included.

Comment on Phosphorus Determination—The final sulfuric acid concentration for color development is 0.9 N plus enough extra acid (0.1 N) to acidify the sodium acetate of the ascorbic acid-molybdic acid reagent. All of this acid is incorporated in the ashing mixture. The small amount of perchloric acid also present is largely volatilized during wet ashing, and there is not enough in any event to disturb the analysis. There is at least a 10 or 15 per cent tolerance in final acidity. The rate of color development decreases as the acidity is increased, whereas the blank becomes appreciable if acidity is not great enough.

The sodium acetate is added to the molybdate reagent to neutralize the ascorbic acid, since there exists a danger zone from pH 0.6 to 3 in which ascorbic acid will reduce molybdic acid in the absence of phosphate (26). It is for this same reason that mixing must be rapid and thorough when the ascorbic acid-molybdate is added; otherwise there would occur local regions of intermediate acidity, with resulting high readings.

The precision of measurement is satisfactory with as little as 3 mμgm. of P (Table III). The reproducibility of analyses of aliquots of brain homogenates is adequate (Table IV). A comparison was made between analyses of the same brain homogenate on a micro scale (18 γ of brain) and analyses made on a macro scale (2 mg. of brain). The average values found for micro- and macrodeterminations were, respectively, acid-soluble P 30.6 and 31.6 mM per kilo, lipide P 56.2 and 59.8 mM per kilo, nucleic acid P plus residual P 13.4 and 11.1 mM per kilo.

Silicate does not give color under the conditions prescribed, which is fortunate, considering the necessity of handling small quantities of phosphorus in glass vessels with relatively large surfaces. Arsenate gives one-fifth as much color as phosphate, on a molar basis.

Certain of the more labile phosphate compounds are hydrolyzed to some degree and therefore give color. Phosphocreatine and acetyl phosphate

TABLE III

Precision of Phosphate Measurement

Known amounts of inorganic P (2.5 to 17.5 mμgm.) were carried through the complete ashing procedure and measured in a final volume of 48.6 μl.

P present	Optical density	P found	P present	Optical density	P found
10^{-10} *mole*	$cm.^{-1}$	10^{-10} *mole*	10^{-10} *mole*	$cm.^{-1}$	10^{-10} *mole*
0.78	0.041	0.80	2.80	0.146	2.84
0.78	0.041	0.80	2.80	0.149	2.90
0.78	0.042	0.82	2.80	0.149	2.90
1.91	0.099	1.93	5.53	0.274	5.32
1.91	0.101	1.97	5.53	0.279	5.43
1.91	0.101	1.97	5.53	0.279	5.43

TABLE IV

Reproducibility of Acid-Soluble and Lipide P Measurements with Brain

Aliquots of rabbit brain homogenate equivalent to 28 γ of brain were analyzed. Lipide was extracted with alcohol, followed by isopropyl ether. Subsequently isopropyl ether has been found unnecessary.

Acid-soluble P	Lipide P	Acid-soluble P	Lipide P
mM per kg.	*mM per kg.*	*mM per kg.*	*mM per kg.*
38	61	35	63
36	54	31	65
37	63	35	60
36	60	35	60

are of course split 100 per cent. Other compounds were found to give the following amounts of color relative to equimolar quantities of inorganic phosphate: glucose-1-phosphate 54 per cent, adenosinetriphosphate 17 per cent of labile P, inorganic pyrophosphate 12 per cent, glucose-6-phosphate none, and fructose diphosphate none. Weil-Malherbe and Green (27) have studied the catalytic splitting of organic phosphate compounds by molybdic acid in more detail.

A homogenate was prepared from rabbit brain with no special care to prevent autogenous splitting of phosphocreatine and adenosinetriphosphate. A TCA filtrate of this homogenate was analyzed for inorganic P

by the proposed procedure, and by the pH 4 method (26), in which inorganic P is measured in the presence of very labile organic P compounds The observed values by the three procedures were, respectively, 15.2, 14.6 and 14.0 mM per kilo of fresh brain. The true inorganic P of brain is of course lower than any of these values.

SUMMARY

1. General analytical procedures and tools are described for making various determinations with as little as 10 γ of brain or other tissue.

2. The measurement of the riboflavin in 10 γ of brain (3×10^{-5} γ of riboflavin) is described.

3. A method for measuring chloride (4×10^{-10} mole) in 10 γ of tissue is presented. This involves the precipitation of chloride with silver at a volume of 2.5 μl., followed by measurement of excess silver with 5-(*p*-dimethylaminobenzylidene)rhodanine.

4. A more sensitive means of measuring phosphate is given, together with directions for the determination of five phosphorus fractions from 20 γ of brain.

BIBLIOGRAPHY

1. Lowry, O. H., *J. Histochem. and Cytochem.*, **1,** 420 (1953).
2. Lowry, O. H., Roberts, N. R., Wu, M.-L., Hixon, W. S., and Crawford, E. J., *J. Biol. Chem.*, **207,** 19 (1954).
3. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., *J. Biol. Chem.*, **193,** 265 (1951).
4. Lowry, O. H., Roberts, N. R., Leiner, K. Y., Wu, M.-L., Farr, A. L., and Albers, R. W., *J. Biol. Chem.*, **207,** 39 (1954).
5. Robins, E., and Smith, D. E., *Res. Publ., Assn. Res. Nerv. and Ment. Dis.*, **32,** 305 (1953).
6. Levy, M., *Compt.-rend. trav. Lab. Carlsberg, Série chim.*, **21,** 101 (1936).
7. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, **164,** 321 (1946).
8. Bessey, O. A., Lowry, O. H., Brock, M. J., and Lopez, J. A., *J. Biol. Chem.*, **166,** 177 (1946).
9. Lowry, O. H., and Bessey, O. A., *J. Biol. Chem.*, **163,** 633 (1946).
10. Strominger, J. L., and Lowry, O. H., *Federation Proc.*, **11,** 295 (1952).
11. Lowry, O. H., *J. Biol. Chem.*, **173,** 677 (1948).
12. Bessey, O. A., Lowry, O. H., and Love, R. H., *J. Biol. Chem.*, **180,** 755 (1949).
13. Feigl, F., *Z. anal. Chem.*, **74,** 380 (1928).
14. Schoonover, I. C., *J. Res. Nat. Bur. Standards*, **15,** 377 (1935).
15. Allen, J. A., and Holloway, D. G., *Nature*, **166,** 274 (1950).
16. Schaffer, F. L., Fong, J., and Kirk, P. L., *Anal. Chem.*, **25,** 343 (1953).
17. Westfall, B. B., Findley, T., and Richards, A. N., *J. Biol. Chem.*, **107,** 661 (1934).
18. Linderstrøm-Lang, K., Palmer, A. H., and Holter, H., *Z. physiol. Chem.*, **231,** 226 (1935).
19. Lowry, O. H., and Hastings, A. B., *J. Biol. Chem.*, **143,** 257 (1942).
20. Benedict, S. R., and Theis, R. C., *J. Biol. Chem.*, **61,** 63 (1924).

21. Griswold, B. L., Humoller, F. L., and McIntyre, A. R., *Anal. Chem.*, **23,** 192 (1951).
22. Bessey, O. A., and Lowry, O. H., *J. Biol. Chem.*, **155,** 635 (1944).
23. Ammon, R., and Hinsberg, K., *Z. physiol. Chem.*, **239,** 207 (1936).
24. Bessey, O. A., Lowry, O. H., and Brock, M. J., *J. Biol. Chem.*, **168,** 197 (1947).
25. Schneider, W. C., *J. Biol. Chem.*, **161,** 293 (1945).
26. Lowry, O. H., and Lopez, J. A., *J. Biol. Chem.*, **162,** 421 (1946).
27. Weil-Malherbe, H., and Green, R. H., *Biochem. J.*, **49,** 286 (1951).