THE QUANTITATIVE HISTOCHEMISTRY OF BRAIN

II. ENZYME MEASUREMENTS*

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It was desired to measure a variety of enzyme activities in very small amounts of brain (10 γ wet weight or less) as part of a study of the quantitative histochemistry of brain. Existing analytical methods were insufficiently sensitive. Accordingly, suitable microprocedures were elaborated for measuring acid and alkaline phosphatase, adenosinetriphosphatase, cholinesterase, aldolase, and fumarase on the desired scale. With the resultant methods certain of the enzymes may be measured in less than 1 γ of brain (wet weight). Similarly sensitive procedures for lactic, malic, and glutamic dehydrogenase will be presented elsewhere.[1] Robins *et al.* have published a comparable method for nucleoside phosphorylase (1).

All the procedures have been validated for use with frozen-dried material obtainable for histochemical purposes. Although in general existing methods have been used as a basis, the drastic reduction in sample size has necessitated numerous alterations. The fumarase method is based on malate measurement by an unpublished method of Dr. John Speck[2] and may be of general interest. The aldolase method of Sibley and Lehninger (2) has been studied and certain changes proposed.

The quantitative measurement of enzymes in whole tissues would be distorted if there were inhibitory or stimulating substances present in the crude incubation mixture. To test this possibility, each enzyme under study has been partially purified from brain and returned to crude brain homogenates. Neither serious inhibition nor stimulation has been encountered as yet, perhaps because the enzymes are measured in all cases at rather high tissue dilutions. This evidence does not of course rule out the possibility of inactive forms of the enzymes or validate the methods for other tissues.

The methods are first presented with comment limited chiefly to matters

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[1] Strominger, J. L., and Lowry, O. H., in preparation.

[2] Kindly supplied through Dr. Morris E. Friedkin.

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involved in the conduct of analyses. Following this, information is given concerning the six enzymes as they exist in rabbit brain, together with evidence as to the validity of the six methods.

Methods

The techniques for obtaining suitable frozen-dried material of 0.1 to 10 γ dry weight (0.5 to 50 γ fresh weight) and the general method of manipulation and measurement on the necessary scale have been described (3, 4) and need not be repeated.

Alkaline Phosphatase—The reagent consists of 8 mM disodium *p*-nitrophenyl phosphate and 2 mM $MgCl_2$ in 0.05 M glycine buffer[3] at pH 10.0. The complete reagent may be preserved for some time if frozen. The substrate as obtained from the Sigma Chemical Company, St. Louis (at present about 70 per cent disodium *p*-nitrophenyl phosphate), may be used directly because of virtual absence of free nitrophenol. The stability of this product is also much better than that of earlier preparations (5). The procedure is adapted from a method designed for use with serum. The original paper gives additional details (5).

The frozen-dried sample, representing 10 to 40 γ of wet brain, is transferred to a pointed tube of 2 to 2.5 mm. inner diameter which is placed in a rack in ice water, and 10 μl. of ice-cold reagent are added. All tubes in the rack are mixed without warming and are capped with Parafilm. At zero time the rack is placed in a water bath at 38°. After exactly 60 minutes (or other appropriate interval) the rack is replaced in ice water, and to each tube are added 2 μl. of 30 per cent trichloroacetic acid (TCA).[4] (Since the reagent is easily hydrolyzed by acid, the following steps are carried out rapidly.) After centrifuging at 3000 r.p.m. for 15 minutes, preferably cold, 10 μl. of supernatant fluid are transferred to a tube of 3 or 4 mm. bore and immediately diluted with 40 μl. of 0.15 N NaOH and read at 410 mμ within a few hours (molar extinction 17,500). The absorption peak is actually at λ 400 mμ, but at this wave-length the unhydrolyzed

[3] Although the data supporting this method were obtained with glycine buffer, it has now been found that a superior buffer for alkaline phosphatase is 2-amino-2-methyl-1-propanol (Distillation Products Industries, Rochester, New York) which has a pK of 9.9 and which does not inhibit alkaline phosphatase even in 1 M concentration. Approximately twice as much enzyme activity is found with this buffer as with glycine, and also a far better buffered medium is possible.

[4] If protein is not to be determined, it is possible to omit the protein precipitation and merely add 40 μl. of 0.1 N NaOH and read. In this case standards are obtained by adding to extra blanks 2 μl. of 1 mM *p*-nitrophenol in water, with a 4 per cent volume correction. Without protein removal a slight turbidity from the tissue contributes a little to the sample reading. In the case of brain homogenates, this is equivalent to about 3 mM of nitrophenol per kilo of tissue. Protein precipitation may also be omitted if desired in the measurement of acid phosphatase.

reagent gives a considerably higher blank value. Standards are provided by incubating extra blanks and substituting 2 μl. of 1 mM *p*-nitrophenol in 30 per cent TCA for the TCA used otherwise. With 60 minutes incubation the standard is thus equivalent to a splitting of 2×10^{-9} mole of substrate per hour.

If desired, the protein may be measured on the TCA precipitate as previously described (6), and this often furnishes a convenient basis of calculation.

Acid Phosphatase—This is measured almost exactly as described for alkaline phosphatase except that a 30 minute incubation is sufficient for 10 to 40 γ of brain and the volume of final alkali may be increased to 100 μl. if desired because of the greater activity. In this case the concentration of standard in TCA is increased to 2 mM, which with a 30 minute incubation is equivalent to 4×10^{-9} mole hydrolysis per hour.

The reagent consists of a mixture of equal parts of (*a*) 16 mM *p*-nitrophenyl phosphate and (*b*) buffer at pH 5.3 (0.075 M disodium succinate, 0.025 M succinic acid, 5 mM $MgCl_2$). Because the substrate is not very stable at pH 5, the two reagent components are not mixed until just before use.[4]

Adenosinetriphosphatase—The reagent consists of a mixture of equal parts of (*a*) 5 mM adenosinetriphosphate (ATP) (*e.g.*, "chromatographed" sodium salt from the Sigma Chemical Company) and (*b*) buffer containing per liter 0.03 M tris(hydroxymethyl)aminomethane (7), 0.03 M 2-amino-2-methyl-1,3-propanediol (7), 0.03 M HCl, and 2 mM $MgCl_2$. The final reagent pH is 8.4 $\pm$ 0.1. This may be kept frozen until the blank value becomes too high.

Aside from the use of a different reagent, the procedure given for alkaline phosphatase may be followed in detail as far as the point of transfer of the aliquot of supernatant TCA extract. However, a 30 minute incubation period is usually sufficient with 10 to 40 γ of brain. The liberated inorganic phosphate is measured within an hour or less by adding to the 10 μl. aliquot 100 μl. of molybdate-ascorbic acid reagent with very prompt and thorough mixing. Samples are read at 870 mμ after 15 to 45 minutes, but all are read at the same interval ($\pm$ 10 minutes) after molybdate addition (since ATP is slowly split by molybdate).

The molybdate reagent is prepared by mixing (*a*) 1 ml. of 2.5 per cent ammonium molybdate, (*b*) 23 ml. of acetate buffer (0.1 M acetic acid, 0.065 M sodium acetate), and, just before use, (*c*) 1 ml. of 1 per cent ascorbic acid (fresh or frozen).

Standards are conveniently prepared by running extra complete blanks and substituting 2 μl. of 10 mM KH_2PO_4 in 30 per cent TCA for the TCA used with samples and blanks. With a 30 minute incubation, such a standard would be equivalent to 40×10^{-9} mole hydrolysis per hour.

If protein determination is to be made on the TCA precipitate, it is necessary to wash the acid precipitate twice with 5 per cent TCA to remove substrate which would give a large blank with the Folin phenol reagent. Such washing requires great care and good lighting to avoid loss of precipitated protein.

Cholinesterase—The principle of this method is similar to that of the method of Croxatto *et al.* (8). Acetylcholine is split in a solution of 1.5 mM sodium barbital containing phenol red. The acid dissociation constants of phenol red and barbital are within 0.1 pH unit of each other. Therefore, as the liberated acetic acid converts a stoichiometric amount of sodium barbital into free diethylbarbituric acid, a much smaller but almost proportionate amount of alkaline phenol red is converted into the acid form. A colorimetric reading at 561 mμ, in which only the alkaline form is measured, becomes therefore a direct measure of the enzyme action. Phenol red at the concentration used was shown not to be inhibitory.

The stock buffer consists of 0.06 per cent phenol red in 0.03 M sodium barbital. This is diluted exactly 1:20 on the day of use with CO_2-free redistilled water.[5] The substrate is 0.6 per cent (0.025 M) acetylcholine bromide (Distillation Products Industries), which is stored frozen in small quantities to avoid repeated thawing and is discarded if appreciable hydrolysis occurs (low initial readings).

The reaction tubes (40 to 50 mm. long) are made from carefully selected uniform 3 mm. Pyrex tubing with a bore of about 1.5 mm. and a tolerance of 3 per cent. These are used in a special adapter block for the Beckman spectrophotometer (4).

The rack of tubes containing the sections for analysis (10 to 30 γ of brain) is placed in a desiccator which is evacuated, and air is readmitted through a soda lime trap or from the outdoors. This is to avoid CO_2 as much as possible. In a room low in CO_2 (*i.e.*, where people have not been working), 10 μl. of the dilute phenol red buffer are added to each sample with gentle tapping to disperse the sections. (A small soda lime tube is inserted in the rubber tube attached to pipettes used for adding buffer or substrate.) Each tube is at once covered with a tight fitting rubber cap cut from the end of a vial stopper. It is well to remove possible CO_2 from the rubber caps by evacuation before use. After a 10 or 15 minute foreperiod, 1 μl. of substrate is added to each tube at zero time, and the sample is buzzed, with care to prevent scratching the bottom half. The rubber cap is replaced, and readings are made at λ 561 mμ at 2, 17, 32, 47 minutes, etc. Before each reading the tube is gently tapped.

[5] Somewhat higher activity is obtained by substitution of 1 mM $MgCl_2$ in 0.1 N NaCl for the distilled water. The supporting data given in this paper were obtained without these additions.

The procedure is standardized by reading a few tubes after adding a trace of NaOH (R_1) and a slight excess of acetic acid (R_2). The difference in these two readings, $\Delta R_{std.}$, is equivalent to $10 \times 10^{-6} \times 1.5 \times 10^{-3} = 15 \times 10^{-9}$ mole of acetic acid liberation (or actually 15.8×10^{-9} mole, since the phenol red is about 0.08 mM). In any tube a color change (corrected for blank) of 32 per cent of $R_{std.}$ indicates the liberation of 5×10^{-9} mole of acetic acid. The time t in minutes to reach 32 per cent of $R_{std.}$ is calculated by interpolation. The rate of enzyme activity is $5 \times (60/t) \times 10^{-9}$ mole per hour. This calculation is preferable to one based on the color change at a fixed period of time, since the readings depart from linearity after a third or a half of the color is gone. The eventual lack of linearity results from (*a*) the fact that the pK of phenol red is about 0.1 unit more alkaline than that of barbital and (*b*) the pH is changing away from the optimum for the enzyme. However, the rate of enzyme activity does not fall below 90 per cent until more than half the color is gone.

Temperature control is not critical, but correction to a single temperature may be made by use of Table IV. Usually a dozen samples may be run concurrently.

If *protein* determination is desirable, the samples are precipitated with 1 μl. of 66 per cent TCA, 10 μl. of supernatant fluid are removed, and two washings are performed with 10 μl. of 5 per cent TCA to get rid of phenol red which inhibits color development with the Folin phenol reagent. (This leaves 2 ± 0.2 μl. of TCA behind.) The color reaction and readings are carried out in the cholinesterase tubes. The procedure previously described (6) is used except that the reagent volumes and compositions are changed to (*a*) 2 μl. of 2.3 N NaOH, (*b*) 10 μl. of 3 per cent Na_2CO_3-0.5 per cent sodium tartrate-0.025 per cent $CuSO_4 \cdot 5H_2O$, and (*c*) 4 μl. of the Folin phenol reagent (1.0 N in acid). The smaller final volume (18 μl.) compensates for the shorter light path. For greater accuracy, each tube may be finally rinsed and read with water to provide a cell correction. Appropriate protein standards may be provided by 1 μl. volumes of 50 to 200 mg. per cent serum protein plus 1 μl. of 6 per cent TCA.

Fumarase—This determination depends on the measurement of malate formed. The malate estimation is adapted from an unpublished method of the late Dr. J. F. Speck, which was based on a qualitative test of Eegriwe (9). Because it is a fluorescence method, the sensitivity greatly exceeds the present requirements.

The substrate reagent is 0.02 M fumaric acid in 0.04 M Na_2HPO_4 (pH 6.8 ± 0.1). It is stored frozen to prevent bacterial growth. With this substrate the reaction is carried out exactly as described for alkaline phosphatase, except that a 30 minute incubation is sufficient for 5 to 25 γ of brain. Unless protein is to be determined, precipitation with acid may be omitted.

Instead, as soon as possible after the incubated tubes are returned to ice water, an aliquot of 8 μl. is added to 1 ml. of the fluorescence reagent in a 3 ml. fluorometer tube (selected 3 ml. Pyrex test-tube). The samples are mixed immediately to stop the enzyme action. The fluorescence reagent consists of a fresh mixture of 1 volume of 56 mg. per cent of β-naphthol in 0.004 N NaOH with 25 volumes of 7:1 sulfuric acid (875 ml. of concentrated H_2SO_4 and 125 ml. of water). The stock β-naphthol may be preserved frozen for a long time and is discarded when it turns quite yellow. The viscous final reagent is conveniently handled with a syringe pipette (Mr. Herman Ruf, 5023 192nd Street, Flushing, New York) but a steel needle (even a stainless steel needle) is not safe to use.

Because of the viscosity, vigorous mixing with a large buzzer (4, 5) is required, *after which* each tube is stoppered with an aluminum foil-covered cork, and, within an hour, heated for 30 minutes in a shallow boiling water bath. After cooling exactly to room temperature and removal of the stoppers, the samples are buzzed again and read in a fluorometer (*e.g.*, Farrand) with a primary filter of Corning glass No. 5860 (B1 primary, which isolates the Hg line at λ 365 mμ) and a secondary filter of Corning glass Nos. 5543 and 3387 (transmission chiefly at λ 465 mμ).

The readings may be made either against quinine in 0.1 N H_2SO_4 or, since the fluorescence is reasonably stable to light, against one of the standards. These are conveniently provided by replacing the regular substrate reagent with 10 μl. of 0.5, 1.2, and 5 mM malic acid prepared in substrate reagent. The standards are carried through the entire operation together with the sample and blanks. With a 30 minute incubation a 1 mM standard would be equivalent to 20×10^{-9} mole of malic formed per hour.

If protein determinations are required, these may be performed on a TCA precipitate (2 μl. of 66 per cent TCA are added after incubation and 10 μl. of the supernatant fluid are used for malate measurement). No washing of the protein precipitate is necessary.

Notes on Fumarase Method—The fluorescence is linear with final concentrations up to about 10^{-5} M. With higher levels it is necessary to calculate from a curve. Samples may stand an hour and probably much longer before heating without change in final reading. After heating, no change in fluorescence was detected at the end of 3 hours. The same readings were obtained with samples heated 30, 45, and 60 minutes at 100°. The sulfuric acid concentration is critical. Substitution of 6:1 and of concentrated sulfuric acid gave fluorescence readings that were 86 and 43 per cent, respectively, of those obtained with 7:1 sulfuric acid. At a lower temperature (60°) the reaction is very slow and probably incomplete. The reagent does not remain reactive after it has been once heated for 30 minutes at 100°. Only a 20 per cent fluctuation is permissible in the prescribed (optimal) concentration of β-naphthol.

The blank reagent fluorescence (chiefly from β-naphthol) is appreciable, being equivalent to about 2×10^{-9} per mole per ml., roughly the activity of 1 γ of brain in an hour. The blank is minimized by suitable secondary fluorometer filters since the blank and malate have different fluorescence spectra (Fig. 1). If necessary, the blank may be reduced by developing fluorescence in a smaller volume and diluting with 7:1 sulfuric acid to read. Weaker acid as a diluent gives lower readings.

The malate reaction is rather specific. According to Speck, the following give less than 1 per cent as much fluorescence as malate per mole: acetate, malonate, succinate, fumarate, *cis*-aconitate, α-ketoglutarate, gly-

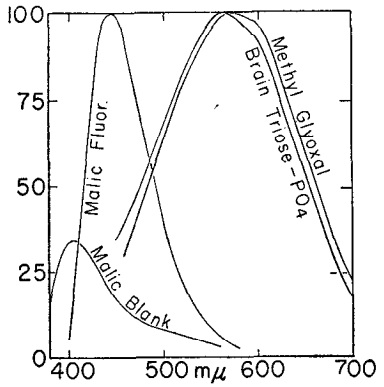

FIG. 1. Fluorescence and absorption spectra in fumarase and aldolase methods. Fluorescence spectrum of condensation product of 10^{-5} M malic acid with β-naphthol and of β-naphthol blank. Absorption spectra of dinitrophenylhydrazine derivatives of methylglyoxal and of triose phosphates in aldolase measurement ("brain"). The ordinate is per cent of maximal fluorescence or optical density.

cine, alanine, adenine, uracil, and xylose. The following give 1 to 3 per cent as much fluorescence as malate per mole: glucose, fructose, lactate, citrate, pyruvate, oxalacetate, aspartate, and glutamate.

Aldolase—The substrate reagent is a fresh mixture of equal parts of 0.02 M magnesium fructose-1,6-diphosphate (HDP) (Schwarz Laboratories, Inc., 230 Washington Street, Mt. Vernon, New York) and 0.12 M hydrazine of pH 8.6 ± 0.2. Each component may be preserved for long periods if frozen. The hydrazine is either prepared from hydrazine sulfate with 1.5 moles of NaOH per mole of hydrazine or from hydrazine hydrate with 0.5 mole of HCl per mole of hydrazine. The latter prepared from 100 per cent hydrazine hydrate (Fairmount Chemical Company, Inc., 136 Liberty Street, New York) has given lower blank values.

With this substrate the procedure for alkaline phosphatase may be followed in detail, except that a 30 minute incubation is sufficient for 10 to 50 γ of brain. Although it is necessary to add TCA after incubation, it is

unnecessary to centrifuge unless protein measurement is required, since the protein will not interfere at the high final dilution. An aliquot of 10 μl. of the TCA supernatant fluid is placed in the bottom of a 1 ml. tube (6 $\times$ 70 mm., A. S. Aloe Company, St. Louis) and 10 μl. of 0.6 N NaOH are added with gentle tapping to be sure the two small volumes mix completely. After 30 $\pm$ 5 minutes at room temperature, 15 μl. of 0.1 per cent dinitrophenylhydrazine in 2 N HCl are added with careful mixing. (This reagent seems to keep indefinitely at 4°. Unless crystal-clear, it is centrifuged before use.) After 30 to 60 minutes at room temperature, 300 μl. of a mixture of 1 volume of 1 N NaOH and 2 volumes of ethylene glycol monomethyl ether (methyl Cellosolve) are added with thorough mixing by buzzing (total volume 335 μl.). After another 30 to 45 minutes a reading is made at λ 570 mμ.

A molar extinction coefficient of 58,000 for the splitting of HDP has been obtained. For most purposes this will suffice as a basis of calculation. (For example, with a 30 minute incubation an optical density of 0.580, corrected for blank, would indicate splitting of $(0.580/58{,}000) \times (0.335/1000) \times (10/12) \times (60/30) = 5.6 \times 10^{-9}$ mole of HDP split per hour.)

Since the extinction coefficient is somewhat empirical (see below), it may be necessary for some purposes to have an independent standardization. This may be made on the basis of alkali-labile P (2) or by the following procedure, which is 10 times more sensitive and which appears to have other advantages: 2 ml. of substrate reagent are incubated with 100 μl. of 1:10 brain homogenate (for example) for 45 minutes to split about 2 mM of HDP per liter. The reaction is stopped with 500 μl. of 25 per cent TCA and centrifuged. A blank is similarly treated. To 100 μl. of the supernatant TCA extract is added 1 ml. of the blank. Aliquots of 10 μl. of this diluted sample are analyzed by the regular procedure above. Another 100 μl. aliquot of the original TCA extract is diluted with 100 μl. of water and 2 ml. of 1 N HCl in a long, slender tube. A standard is similarly prepared with 100 μl. of the blank, 100 μl. of 4 mM dihydroxyacetone (Nutritional Biochemicals Corporation, Cleveland), and 2 ml. of 1 N HCl. Both samples are heated for 1 hour in boiling water to convert all trioses to methylglyoxal (10). Precautions are taken to prevent evaporation or to restore any lost volume, or a small portion is heated in a sealed tube. Aliquots of 20 μl. of sample, standard, and appropriate blank are treated with 15 μl. of 0.1 per cent dinitrophenylhydrazine in 0.5 N HCl, and, after 30 minutes at room temperature, 300 μl. of alkaline methyl Cellosolve are added as in the regular procedure. The standard dihydroxyacetone reading is used to calculate the total triose present, and from this the molar extinction coefficient by the regular procedure may be determined. For methylglyoxal from dihydroxyacetone by this procedure ϵ = 53,000 was

observed, which is equivalent to 106,000 for HDP. Hydrazine inhibits the conversion to methylglyoxal and must be kept low (2 or 3 mM) during this step.

Notes on Aldolase Method—This method, which originated with Sibley and Lehninger (2), has been studied, with the result that certain changes have been made which have permitted adaptation to a small scale and perhaps increased the reproducibility. There are five steps concerned.

Incubation—The buffer originally recommended (tris(hydroxymethyl)-aminomethane) has been omitted, since hydrazine is itself a good buffer in the required pH range. The hydrazine concentration is not critical during incubation.

Acidification (with TCA)—For some obscure reason acidification is necessary before the following step in order to achieve full color.

First Alkaline Treatment—This is the most critical step. Everything indicates that methylglyoxal or a close relative is formed at this point from both triose phosphates in about 60 per cent yield. In the absence of hydrazine, triose phosphates are converted in alkali to lactic acid (10). Apparently hydrazine traps the intermediate. With the present procedure no color at all is produced from dihydroxyacetone phosphate if hydrazine is omitted. Hydrazine, alkali, and time are all interdependent in the production of maximal color. The reaction is slowed by increasing the hydrazine and decreasing the alkali. Color is diminished by too much alkali. With more hydrazine more latitude in alkali concentration is permissible. With 0.025 M hydrazine at this step, as suggested, the net alkali concentration may vary from 0.06 to 0.15 M without serious error. After the recommended time interval, the ultimate color slowly decreases, owing presumably to conversion to lactic acid.

Reaction with Dinitrophenylhydrazine—This step is not critical. The reaction is 95 per cent complete in 10 minutes under the prescribed conditions. The product is stable for at least an hour and probably much longer.

Final Color Development—The organic solvent is necessary to stabilize the color, particularly with small volumes. Otherwise, precipitation may occur with serious and capricious fading. The final *net* alkali concentration may vary from 0.15 to 0.5 N without causing trouble. Fading and high blanks result from too little alkali.

Free dihydroxyacetone and glyceraldehyde give low and non-proportional amounts of color in the present analytical procedure. For dihydroxyacetone approximate values are $\epsilon = 2800$ without alkaline treatment, $\epsilon = 9000$ with alkaline hydrazine treatment, $\epsilon = 14{,}000$ with the complete procedure. For glyceraldehyde values about half of these were obtained. In contrast, dihydroxyacetone phosphate[6] gave an $\epsilon = 28{,}000$, or about

[6] Prepared by Dr. T. Baranowski in the Department of Biochemistry.

half that observed for two triose phosphates formed from HDP. Therefore, glyceraldehyde phosphate must also yield a comparable amount of color, although Sibley and Lehninger concluded that most of the color comes from dihydroxyacetone phosphate. The spectra from methylglyoxal and from the triose phosphates in the present analytical procedure are not quite identical (Fig. 1). This leaves the question open as to the identity of the product obtained from triose phosphate in alkali in the presence of hydrazine.

General Characteristics of Enzymes and Methods

Effects of pH and Activators (Fig. 2)—The pH optimum for acid phosphatase is more alkaline when Mg is present than without it (pH 5.9 compared to pH 5.3). Unpublished evidence indicates this is due to the presence of two phosphatases, one of which has a marked requirement for Mg, with an optimum of about 6.5. Under the analytical conditions prescribed actually both enzymes are measured together. Reasonably good separate measurements of the two enzymes may be obtained with (*a*) succinate buffer, pH 5.0, without Mg, and (*b*) collidine buffer (0.05 M), pH 6.8, with 5 mM Mg. Alkaline phosphatase activity is increased about 30 per cent by Mg.

The pH properties of the rest of the enzymes are very similar to those reported by others for the same enzymes in other tissues. The relative pH independence of aldolase activity from pH 7.2 to 9.5 may be noted. Since the pH optimum for ATPase occurs where the two buffers used in Fig. 1 overlap, a mixture containing both has been prescribed. Doubling the concentration of either buffer caused only a 7 per cent decrease in enzyme activity.

ATPase of rabbit brain is inactive without a divalent cation (Fig. 3), and it is 50 per cent activated by 2×10^{-4} M Mg. Ca activation (11) is only a third that of Mg (or Mn) and Ca appears to compete with Mg and inhibit the activity when both are present. Gore (12) observed similar behavior for guinea pig brain, except that Ca inhibited even in the absence of Mg. We observed this also with rabbit brain when a certain ATP preparation was used which gave substantial activity without added Mg. That is, Ca may always activate unless another more effective activator is already present. NaCl and KCl at 0.075 and 0.15 M were moderately inhibitory at pH 8.4, although at acid pH small amounts of K were stimulating, as Gore has found. The possibility that two enzymes are present has not been ruled out.

Enzyme Kinetics—Well defined Michaelis constants were obtained for acid and alkaline phosphatase and for fumarase (Fig. 4). These are, respectively, 1.58, 0.89, and 1.83 mM. The fumarase constant is a little

lower than that found at the same pH by Massey (2.5×10^{-3}) for crystalline pig heart enzyme (14).

The K_s for brain aldolase is very small. No decrease in initial velocity

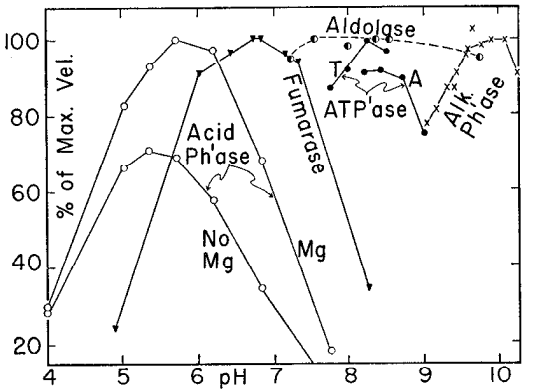

FIG. 2. The effect of pH on five brain enzymes. All incubated 30 minutes at 38°. *Alkaline phosphatase*, 500 γ of brain incubated in 109 μl. of 0.05 M glycine buffer with 5 mM Mg; *acid phosphatase*, 150 γ incubated in 60 μl. of 0.05 M succinate-0.05 M collidine buffer with and without 5 mM Mg; *ATPase*, 490 γ of brain incubated in 210 μl. with 1 mM Mg in either 0.05 M tris(hydroxymethyl)aminomethane buffer (curve *T*) or 2,2-aminomethyl-1,3-propanediol (curve *A*); *fumarase*, 224 γ of brain incubated in 108 μl. of 0.05 M phosphate buffer except pH 4.9, which contained 0.15 N acetate as well; *aldolase*, 37 γ of brain incubated in 46 μl. of 0.06 M hydrazine buffer.

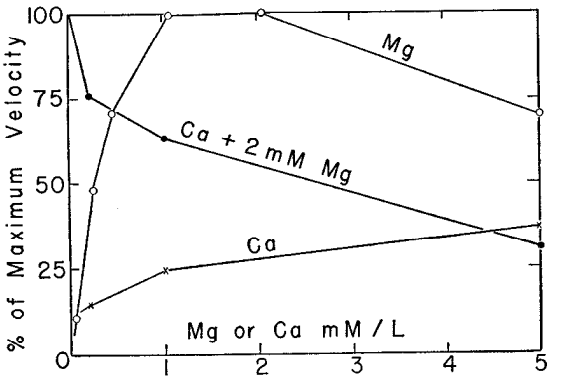

FIG. 3. Effect of Mg and Ca on activity of brain ATPase. 490 γ of brain in 210 μl.; 30 minutes incubation. Ordinate, fraction of maximal activity.

was observed with 10^{-4} M substrate, from which it seems likely that the K_s is not more than 10^{-5} M. Meyerhof found no decrease in the initial velocity of skeletal muscle aldolase activity with 5×10^{-4} M substrate (15).

The K_s for brain ATPase is so low as to make its measurement difficult.

It is probably considerably less than 10^{-4} M. Adenosinediphosphate (ADP) is quite inhibitory to brain ATPase. An enzyme preparation sufficiently free of myokinase to have little action on ADP was tested with various combinations of ATP and ADP. Values obtained for the ratio K_s: K_i (inhibitor constant) were 3.0, 3.2, 2.6, and 3.1; *i.e.*, the affinity of the enzyme for ADP is 3 times greater than it is for ATP. This probably

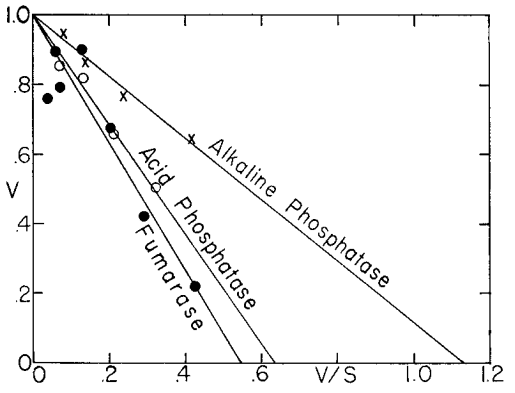

FIG. 4

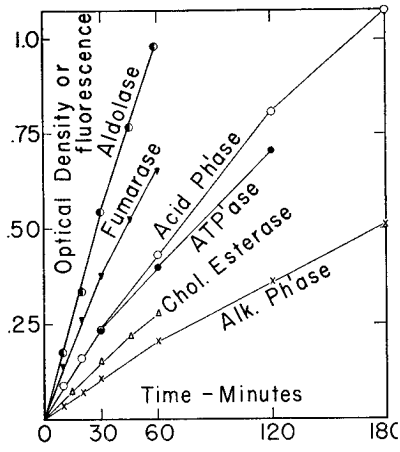

FIG. 5

FIG. 4. Substrate-velocity relationship for three brain enzymes. The plot is made according to the method of Eadie (13). The intercept on the abscissa is $V_{max.}/K_s$. Since the maximal velocity is here set at 1.0 for each enzyme and S is expressed in millimoles per liter, the intercept is the reciprocal of K_s in millimoles per liter.

FIG. 5. Activity of six brain enzymes with time. All except cholinesterase incubated at 38°. *Alkaline phosphatase*, incubated with 17.8 γ of brain in 13.7 μl., color with 61 per cent aliquot at 59 μl.; *acid phosphatase*, same incubation conditions but color at 110 μl.; *ATPase*, 490 γ of brain in 210 μl., color with 45 per cent aliquot at 1.15 ml.; *cholinesterase*, 14 γ of brain at 25° in 11.4 μl.; *aldolase*, 12.2 γ of brain in 13.3 μl., color with 74 per cent aliquot at 331 μl.; *fumarase*, 25.3 γ of brain in 13.3 μl., 74 per cent aliquot with fluorescence measured at 1 ml.; for fumarase a scale reading of $0.5 = 5 \times 10^{-9}$ mole hydrated.

accounts for some of the decrease in velocity with time in longer incubations (Fig. 5). Although both AMP and inorganic P inhibit brain ATPase, they are only about one-tenth as inhibitory as ADP.

The K_s for brain cholinesterase has been thoroughly investigated by others. The level prescribed here is about optimal.

Enzyme Activity with Time and Amount of Brain—Figs. 5 and 6 serve as guides to the limitations of the proposed procedures in regard to incubation time and amount of brain. It is to be noted that the recorded range of time and brain greatly exceeds that recommended. Of the six enzymes,

ATPase activity falls off most with increasing product formation. The decrease of acid phosphatase activity with time is due to instability of the enzyme. In the case of alkaline phosphatase the fall after an hour is in part due to some instability of the enzyme at pH 10 and in part to failure of the glycine buffer to maintain the alkaline pH with longer incubation and such small volumes.[3]

Reproducibility and Recovery Experiments with Partially Purified Enzymes—The reproducibility obtained by the proposed procedures is of the order of 5 per cent, expressed as the coefficient of variation (Tables I and II).

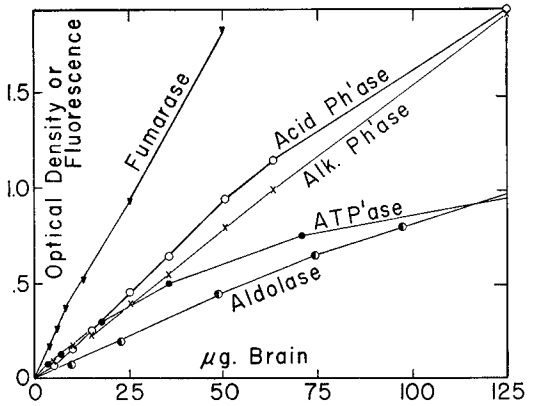

FIG. 6. Activity of five brain enzymes with different amounts of brain. All incubated at 38°. *Alkaline phosphatase*, incubated 60 minutes at 12.6 μl. volume, color with 86 per cent aliquot at 60 μl.; *acid phosphatase*, 30 minutes at 12.6 μl. volume, color with 86 per cent aliquot at 109 μl.; *ATPase*, 60 minutes at 13.7 μl. volume, color with 61 per cent at 110 μl. volume; *aldolase*, 30 minutes at 59 μl., color with 72 per cent aliquot at 1.3 ml.; *fumarase*, 30 minutes at 13.3 μl. volume, fluorescence with 40 per cent aliquot at 1 ml.; for fumarase a scale reading of 1.0 = 4×10^{-8} mole hydrated.

Each enzyme was partially purified from *rabbit brain* and returned to a crude homogenate to test for recovery (Table III). Four of the six enzymes are predominantly insoluble in brain, and no great success was obtained in making them soluble. However, by freeing them of soluble enzymes and other soluble substances, it was possible to test for inhibitory or accelerating soluble materials in crude homogenates.

Fumarase was purified 20-fold by fractionation with $(NH_4)_2SO_4$ at pH 7.5. The material was precipitated several times between 2.0 and 2.5 M. The preparation used to test recovery had a turnover number of about 1000. Summation of activity within analytical limits was observed when this preparation was added to crude homogenate (Table III).

Aldolase was purified 23-fold by fractionating at pH 7.5 with $(NH_4)_2SO_4$.

Four precipitations were made between the following salt molarities, 1.8 and 2.5, 2.0 and 2.4, 1.8 and 2.2, 1.9 and 2.2. The turnover number of the final preparation was about 250 (tested in dilute crystalline bovine albumin to protect the dilute enzyme). The activity was enhanced or protected about 10 per cent when added to crude homogenate (Table III).

Although most of the *acid phosphatase* activity of brain is insoluble, a preparation with twice the activity of original brain was obtained from an acetone powder (prepared at $-12°$) by extraction of the powder and pre-

TABLE I

Reproducibility of Analysis for Acid and Alkaline Phosphatase

Samples of rabbit brain water homogenate, each containing 9.7 γ of brain, were analyzed for both enzyme activity and protein.

Alkaline phosphatase				Acid phosphatase			
Substrate split (60 min.)	Protein	Enzyme activity		Substrate split (30 min.)	Protein	Enzyme activity	
10^{-9} mole	γ	*mole per kg. wet weight per hr.*	*mole per kg. protein per hr.*	*10^{-9} mole*	γ	*mole per kg. wet weight per hr.*	*mole per kg. protein per hr.*
0.57	0.87	0.058	0.65	0.98	0.86	0.202	2.29
0.59	0.87	0.060	0.68	0.92	0.93	0.191	1.99
0.57	0.85	0.058	0.67	0.97	0.81	0.200	2.39
0.52	0.82	0.053	0.64	0.94	0.84	0.193	2.24
0.55	0.87	0.056	0.63	0.95	0.86	0.196	2.21
0.56	0.92	0.056	0.59	0.92	0.91	0.191	2.13
0.54	0.82	0.055	0.66	0.92	0.79	0.191	2.37
0.53	0.83	0.054	0.63	0.90	0.87	0.186	2.09
0.54	0.91	0.055	0.59	0.92	0.86	0.190	2.14
0.54	0.84	0.055	0.64	0.92	0.82	0.191	2.27
Average.....		0.056	0.64			0.193	2.21
S.d.....		0.002	0.03			0.005	0.14

cipitation with $(NH_4)_2SO_4$ between 1 and 1.5 M. A 15 to 20 per cent enhancement of the activity of this purified material was observed when added to crude homogenate (Table III). This enhancement may possibly be due to a cofactor, since Dr. Byron Wenger (personal communication) has obtained definite evidence of such a factor for the acid phosphatase of chick nervous system.

Alkaline phosphatase activity is at least 85 per cent insoluble and resisted attempts to put it in true solution (alkaline pH, acetone treatment, sonic disintegration). However, it was freed from soluble materials by extracting successively with tris(hydroxymethyl)aminomethane buffer at pH 7.2, 2 M KCl, and water. The residue was homogenized in a small glass grinder.

Part of the sample was dispersed with a sonic vibrator and reprecipitated with 2 M $(NH_4)_2SO_4$ with little loss in activity (but also without making the enzyme truly soluble). Both preparations were added to crude brain homogenate with satisfactory recovery of activity (Table III).

TABLE II

Reproducibility of Analysis for Four Enzymes

Rabbit brain water homogenate used except as noted. Values reported as moles of substrate split or hydrated per kilo of brain per hour, except that in the case of *sections* the values are recorded as moles per kilo of protein per hour. The actual amounts of substrate transformed in each sample were 20×10^{-9} mole of fumarate in 30 minutes, 11×10^{-9} mole of hexosediphosphate in 30 minutes, 5×10^{-9} mole of acetylcholine, and 6 and 11×10^{-9} mole of ATP in 60 and 120 minutes, respectively.

	Fumarase	Aldolase	Cholinesterase		ATPase
	18 γ brain	34 γ brain	14 γ brain	Sections*	10 γ brain
	2.19	0.60	0.34	4.2	0.63
	2.45	0.59	0.36	4.8	0.65
	2.28	0.62	0.33	4.3	0.61
	2.29	0.60	0.33	4.6	0.65
	2.15	0.61	0.36	4.2	0.60
	2.31	0.61	0.35	4.4	0.56†
	2.24	0.62	0.37	4.0	0.56†
	2.43	0.62	0.32	4.0	0.58†
	2.27				0.61†
	2.16				0.57†
Average....	2.28	0.61‡	0.34	4.3	§
S.d.....	0.10	0.01	0.018	0.28	0.02

* Frozen-dried sections of cortex containing an average of 4.4 γ of protein.

† Incubated 120 minutes; the other samples in this column were incubated 60 minutes.

‡ A macro sample (665 γ) gave 0.63 mole per kilo per hour.

§ Average 0.63 and 0.57 mole per kilo per hour at 60 and 120 minutes. Macro samples (250 γ) gave 0.62 and 0.56 at corresponding incubation times.

Adenosinetriphosphatase of a brain homogenate in 0.02 M Na Veronal was separated from soluble impurities by centrifuging at 18,000 r.p.m. after discarding the precipitate at 1000 r.p.m. The high speed precipitate was washed twice with 2 M KCl and twice with water and used for testing the recovery of activity when added to whole brain homogenate (Table III). Summation of activity was satisfactory.

Cholinesterase was "purified" 2-fold by discarding the precipitate obtained on centrifuging (3000 r.p.m.) a brain homogenate at pH 10 (0.5 N NH_4OH, 0.05 N ammonium acetate) and collecting the precipitate obtained

after neutralizing with KH_2PO_4 and adding $(NH_4)_2SO_4$ to 0.7 M. The activity of this preparation and that of whole brain homogenate were additive within analytical limits when the two were combined (Table III)

TABLE III

Recovery of Activity of Partially Purified Enzymes When Added to Whole Brain Homogenates

The analytical conditions are those described in the text, except that the scale was increased 10- to 50-fold. Homogenates were made in water.

Cholinesterase				Fumarase				Aldolase			
Brain	Purified enzyme	Hydrolysis		Brain	Purified enzyme	Hydration		Brain	Purified enzyme	Hydrolysis	
		Observed	Calculated			Observed	Calculated			Observed	Calculated
mg.	*mg.*	*μM per hr.*	*μM per hr.*	*γ*	*γ*	*μM per hr.*	*μM per hr.*	*γ*	*γ*	*μM per hr.*	*μM per hr.*
10.2	0	3.25		59	0	0.122		147	0	0.128	
19.2	0	6.78		103	0	0.198		258	0	0.226	
0	0.209	1.39		162	0	0.291		0	1.52	0.100	
0	0.425	2.56		0	0.30	0.136		0	2.66	0.182	
10.2	0.209	4.56	4.64	0	0.53	0.204		147	1.52	0.240	0.228
10.2	0.209	4.69	4.64	59	0.53	0.333	0.326	147	2.66	0.336	0.318
				103	0.30	0.316	0.334	258	1.52	0.344	0.326
Acid phosphatase*				Alkaline phosphatase				Adenosinetriphosphatase†			
490	0	50						36	0	40	
980	0	96						72	0	78	
0	24	51		203	0	12.8		102	0	104	
0	48	104		0	15	24.8		143	0	134	
490	24	109	101	203	15	37.4	37.6	0	1.74	23	
490	0	53		294	0	19.1		0	4.35	68	
0	24	59		0	12‡	25.2		36	1.74	60	61
490	24	126	112	294	12‡	46.7	44.3	36	4.35	98	100
490	0	40						72	1.74	98	95
0	21	68						72	4.35	131	126
490	21	119	108								

* The results of three experiments conducted at different times are recorded.

† Calculated from a curve, since activity is not strictly linear with amount of enzyme.

‡ This enzyme preparation was made soluble with sonic vibration and reprecipitated with $(NH_4)_2SO_4$ (see the text).

Temperature Coefficient—The six enzymes have very different temperature coefficients (Fig. 7). Cholinesterase is least sensitive to temperature, having $Q_{10} = 1.21$ between 10–38° (*cf.* Vahlquist (16)). Aldolase is most temperature-sensitive, with $Q_{10} = 4.1$ between 4–15°. These data are con-

sonant with those of Herbert *et al.* (17) for muscle aldolase. The energy of activation for brain fumarase at pH 6.7 was found to be 6600 calories between 25–38° and 14,600 calories between 4–15°. For crystalline pig heart fumarase at pH 6.6, Massey (14) found 6800 and 10,400 calories, respectively; *i.e.*, agreement only at the higher temperature range.

Two practical aspects of the temperature characteristics of these six enzymes appear in Table IV. (1) To measure each enzyme with a precision

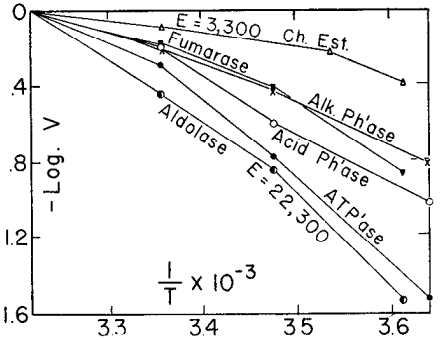

FIG. 7. Effect of temperature on the activity of six brain enzymes. Experimental conditions as described under "Methods," except for acid phosphatase, which was studied at pH 5 to 6 in the absence of Mg.

TABLE IV

Effect of Temperature on Activity of Brain Enzymes

	ATPase	Cholinesterase	Acid phosphatase	Alkaline phosphatase	Aldolase	Fumarase
Change in velocity at 25–38°, % *per degree*	5	1.5	3.5	3.5	7.6	3.5
Velocity at 4°, % *that at 38°*	4	40	11	18	3	14

of 3 per cent, the permissible temperature range varies from less than 0.5° for aldolase to 3° for cholinesterase. (2) The proposed methods for all of the enzymes except cholinesterase involve initial and final exposure of substrate to enzyme at 4°, which is not counted in the incubation time. To keep the 4° activity to less than 5 per cent of the 38° (1 hour) activity, the permissible exposure at 4° would be 90 minutes for aldolase but only 15 minutes for alkaline phosphatase.

Freezing, Drying, and Storage—In homogenates acid phosphatase is quite unstable. The activity disappears at 38° on a die away curve, with a half time of about 4 hours. The other five enzymes do not present any particular problem in this regard, except that dilute homogenates (1:100) are difficult to preserve.

All six enzymes withstand freezing and drying (Table V). In fact, several of them increase in apparent activity by this treatment, owing presumably to better disruption of the tissue. They are also all stable for at least 6 hours at room temperature, permitting ample time for dissection of frozen-dried sections. Aldolase and acid phosphatase are the least stable upon longer storage; cholinesterase is the toughest.

TABLE V

Stability of Enzymes of Brain to Freezing, Drying, and Storage

Values recorded as per cent of non-stored frozen-dried samples. The homogenates were made in water.

	Period of storage	Temperature of storage	ATPase	Aldolase	Fumarase	Acid phosphatase	Alkaline phosphatase	Cholinesterase
	days	°C.						
Fresh homogenate	0		84	86	96	79	102	105
Frozen homogenate	0		70	92	100	84	105	101
Frozen-dried homogenate	0		(100)	(100)	(100)	(100)	(100)	(100)
" "	0.25	25		95	94	98	104	101
" "	1	25	99	90	91	82	99	99
" "	7	25	88	64	81	66	89	94
" "	1	4	101			111	102	
" "	6	4	106			107		
" "	14	−20	113*	97	111	103*	101*	100
" "	120	−20	126†	90		99‡	102‡	
" sections	16	−20	109			90	107	
" "	400§	−20		115	111	100		103

* 6 days.

† 37 days.

‡ 67 days.

§ This is a comparison of samples from the stratum oriens of Ammon's horn from two different rabbits, one analyzed within 2 weeks, the other after storage of the dry sections for more than a year.

At −20° all six enzymes are amazingly stable. There is no demonstrable loss in activity after 1 year in dried sections (Table V). Therefore a given set of sections may be stored and sampled for various analyses almost indefinitely. The increase in ATPase activity with storage is of interest. Hunter (18) and others have found striking increase in ATPase activity in liver mitochondria under various kinds of rough treatment.

SUMMARY

1. Methods are described for measuring six enzymes with as little as 5 or 10 γ of brain.

2. Adenosinetriphosphatase and acid and alkaline phosphatase are meas-

ured with conventional substrates (ATP and nitrophenyl phosphate) by ncubation at a 10 μl. volume and final color measurement at 50 to 100 μl.

3. Cholinesterase is measured by the color change from acetylcholine nydrolysis in 10 μl. of a buffer-indicator pair (barbital and phenol red) naving nearly the same pK. An almost linear relation between enzyme activity and color change is achieved.

4. Fumarase is measured by determination of malate by an unpublished sensitive method of Dr. John Speck. This fluorometric procedure has sensitivity far in excess of the present needs.

5. Aldolase measurement is based on the macroprocedure of Sibley and Lehninger. Certain changes were required on the micro scale and may also be helpful for macro work.

6. The six methods have a coefficient of variation of about 5 per cent with 10 or 20 γ of brain.

7. All six enzymes were partially purified or separated from soluble components of rabbit brain and added to crude brain homogenates to test for summation of activity. No inhibition was observed, but slight enhancement was found for acid phosphatase and aldolase.

8. All six enzymes withstand freezing, drying, and storage up to a year at $-20°$.

9. The effects of variation of pH, time, temperature, substrate concentration, and amount of enzyme have been investigated.

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