Variation II, based on the previously ether-insoluble colour that becomes ether-soluble with methoxycarbonyl chloride, also gives high results. Bunyan & Price (1960) used this in its original form with no correction for destruction during hydrolysis (Bruno & Carpenter, 1957), and report values for six samples coded WM and FM that also appear in Table 3; their results are similar to ours and the samples have the same ranking in each case.

In view of the wide differences between the mean values obtained for different samples, the reproducibility of the procedure, as calculated from a statistical analysis of a large number of results, seems adequate.

## SUMMARY

1. The Sanger reaction with 1-fluoro-2:4-dinitrobenzene for the determination of the free $\epsilon$-amino groups of lysine units in purified proteins has been applied to animal-protein foods in an attempt to measure nutritional damage which may occur through the formation of enzyme-resistant linkages with $\epsilon$-amino groups during processing.

2. The dinitrophenyl-proteins were hydrolysed with acid without separation from the digest, and the hydrolysates were ether-extracted. The measure of $\epsilon$-dinitrophenyl-lysine was based on the further decrease in colour in the aqueous layer after digestion with methoxycarbonyl chloride and re-extraction with ether. This procedure gave a nil value for zein and the expected value for insulin.

3. Twenty-four materials, prepared in most cases as human or animal foods, gave a wide range of values from 1·5 g./16 g. of nitrogen for feather meal to 8·4 and 8·6 g./16 g. of nitrogen for freeze-dried cod fillets and spray-dried blood meal respectively. Recovery of added $\epsilon$-dinitrophenyl-lysine was 92%.

4. Samples that had been heated and had shown decreased nutritional value in feeding tests also showed lower 'available lysine' values in the chemical procedure.

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# The Intracellular Distribution of Glycolytic and other Enzymes in Rat-Brain Homogenates and Mitochondrial Preparations

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It has been shown (Hers, Berthet, Berthet & de Duve, 1951) that, although most of the liver hexokinase is soluble, a proportion of the hexokinase of various mammalian tissues is associated with subcellular particles (Long, 1952; Crane & Sols, 1953), and the latter workers have shown that with brain almost all of the enzyme is particulate. Glycolytic activity has been reported for various brain mitochondrial preparations (Du Buy & Hesselbach, 1956; Gallagher, Judah & Rees, 1956; Balázs & Richter, 1958; Abood, Brunngraber & Taylor, 1959). It seemed of interest therefore to determine the

distribution of each enzyme of the glycolytic chain in brain fractions and to examine the homogeneity of brain mitochondrial preparations.

## METHODS

*Special chemicals and reagents.* The following chemicals were obtained from the sources indicated: glucose, glucose 6-phosphate (barium salt), phenyl butyrate, collidine and triethanolamine (British Drug Houses Ltd.) (the collidine and triethanolamine were both fractionally distilled before being converted into hydrochlorides for use as buffers); sodium pyruvate and acetylcholine chloride (Roche Products Ltd.); 2-deoxyglucose (L. Light and Co. Ltd.); ethylenediaminetetra-acetic acid (EDTA; Hopkin and Williams Ltd.; the potassium salt was used at all times); bovine-serum albumin (fraction V) (The Armour Laboratories); 2:3-diphosphoglyceric acid (barium salt; Schwarz Laboratories Inc., U.S.A.); DL-glyceraldehyde 1-bromide 3-phosphate (dioxan addition compound); and 'cyclohexylammonium dihydroxyacetone phosphate dimethyl ketal' (California Corporation for Biochemical Research, U.S.A.) (free dihydroxyacetone phosphate was prepared from its complex by conversion into free acid on a deionizing column followed by hydrolysis of the ketal at 40° for 4 hr.); heavy water ($D_2O$) (Imperial Chemical Industries Ltd.); Ficoll (Atkieselsekabt Pharmacia, Copenhagen; the compound is a high-molecular-weight carbohydrate of unspecified composition; a 20% soln. was dialysed against cold distilled water for 20 hr. before use); adenosine triphosphate (ATP), disodium salt (Sigma Chemical Co., U.S.A.); reduced diphosphopyridine nucleotide (DPNH), disodium salt; adenosine diphosphate (ADP), trisodium salt; barium salts of fructose 6-phosphate, phosphopyruvic acid, DL-2-phosphoglyceric acid and DL-3-phosphoglyceric acid; and an equimolar solution (0·1 M) of sodium triose phosphate esters (C. F. Boehringer und Söhne, Mannheim, Germany). Barium salts were converted into free acids by using Amberlite IR-120 resin, and the acids were neutralized with KOH: an arbitrary allowance of 30% was made for loss by adsorption on the resin. Purified enzymes were obtained as follows: glucose 6-phosphate dehydrogenase (Sigma Chemical Co., U.S.A.) (anhydrous type III was substantially free of hexokinase but contained some phosphoglucose isomerase); all other purified enzymes were supplied as suspensions in ammonium phosphate solution (C. F. Boehringer und Söhne, Mannheim, Germany).

Butyrylcholine perchlorate was prepared according to Aldridge (1953). Triphosphopyridine nucleotide (TPN) was supplied by Mr C. J. Threlfall and was about 80% pure.

*Fractionation procedure.* Brains from two decapitated rats (mature females of the Porton strain) were homogenized in 0·3 M-sucrose with a smooth glass tube and a Perspex pestle with all-round clearance of 0·005 in. (Aldridge, 1957). The method of preparing washed fractions by centrifuging, and the nomenclature applied to them, have been defined by Aldridge & Johnson (1959). For most experiments, the homogenate was divided by centrifuging at 20 000 *g* for 30 min. into one washed fraction (20.particles.30), containing cell debris, nuclei, mitochondria and some microsomes, and a supernatant (S) containing soluble proteins and some microsomes. In a few cases a more extensive fractionation was carried out giving cell debris plus nuclei (N), mitochondrial ($M_1$ and $M_2$), microsomal (P) and supernatant (S) fractions. The prefixed numbers are $10^{-3} \times$ the sedimentation force (*g*) and the suffix is the sedimentation time in minutes. Each particulate fraction was washed once by resuspending in 0·3 M-sucrose and sedimenting under the same conditions as those used for preparing the fraction: the washings were added to the supernatant before separation of the next fraction.

*Fractionation in dense media.* A homogenate was prepared as above. An unwashed nuclear fraction (1.N.10) was discarded, and an unwashed mitochondrial pellet (13.M.15), was obtained from the remainder; the fraction was subfractionated on a density gradient by one of two methods with the Spinco model L centrifuge.

In procedure A, which was used for preliminary studies, 4·0 ml. portions of the material suspended in 0·3 M-sucrose were layered over 1·4 ml. portions of a dense medium in each of three centrifuge tubes fitting the swing-out head (SW 39) of the Spinco. The tubes were centrifuged at maximum speed (40 000 rev./min.) for 1 hr. (145 000 *g* at the bottom of the tubes). Some material was sedimented into a pellet at the bottom of the tube, and some was retained at the interface. The fractions were carefully separated with a Pasteur pipette and resuspended in EDTA solution (potassium salt, 2 mM, pH 7·0); the clear liquid above each layer of particles was removed separately and retained: it was shown that the clear liquids contained very little of the enzymes being studied.

Procedure B was devised to enable larger quantities to be handled in one tube, and to avoid the difficulties associated with an interface. The crude mitochondrial pellet was suspended evenly in about 20 ml. of a suitable dense medium and placed in a 30 ml. centrifuge tube to fit the no. 30 angle head of the Spinco; the remaining tube-space was filled with 0·3 M-sucrose to prevent collapse of the walls. The tube was then centrifuged at maximum speed (30 000 rev./min. 105 000 *g* at the bottom of the tube) for 1 hr. The particles separated into a packed pellet at the bottom and a particle layer at the top of the dense solution, while a small proportion, having the same density as the solution, remained suspended; no soluble or particulate enzymes migrated into the 0·3 M-sucrose. Since the centrifugal field was not directly down the axis of the tube, a small amount of the dense particles which should have been sedimented to the bottom, adhered to the side wall of the tube: as the subfractionation was a quite arbitrary process, it was found convenient to combine all the unsedimented material into one subfraction, and the sedimented particles including those adhering to the wall into the other subfraction. Although having certain advantages, this method obviously cannot be adapted to the separation of more than two subfractions, whereas, if sufficiently delicate sampling devices and assay procedures are available, procedure A can be used to separate multiple subfractions.

Most studies with density gradients have utilized high concentrations of sucrose (Kuff & Schneider, 1954), but colloidal silica (Mateyko & Kopac, 1959), high-molecular-weight polymers (Thomson & Klipfel, 1958; Holter & Moller, 1958) and heavy water (Beaufay, Bendall, Baudhin, Wattiaux & de Duve, 1959) have also been used. The aim of the later procedures has been to decrease the possible damaging effects of high concentrations of sucrose. It is almost impossible to obtain full activity of succinic dehydrogenase in brain mitochondria, which have been sedimented

through 1·0 M-sucrose, even if the particles are subsequently suspended in water for 2 hr.: the latter, or similar treatment to cause damage to the mitochondrial membrane, was necessary and effective for the assay of succinic dehydrogenase in mitochondria (Aldridge & Johnson, 1959). The use of heavy water to increase the density of the medium is based on the presumption that, under the working conditions, exchange of the water within the particles for heavy water is not rapid. Beaufay *et al.* (1959) have shown that this is probably so for liver-mitochondria preparations, but is less true for lysosomes. Of the available high-molecular-weight polymers, Ficoll, used by Holter & Moller (1958), seems to have the required negligible osmotic pressure in solution combined with stability and moderate viscosity; this material, dissolved in 0·3 M-sucrose, was chosen for most experiments with procedure B. Sucrose solutions were usually used in procedure A where the stickiness of Ficoll solutions caused particles retained at the interface to cohere, forming a mass difficult to disperse.

### *Spectrophotometric assay methods*

Enzyme activity was determined by the rate of change of concentration of reduced pyridine nucleotide, as shown by extinction measurements at 340 mμ. Where it was necessary to couple the reaction being assayed to a reaction involving pyridine nucleotide, purified enzymes were added in excess. Tissue suspensions were stored at 0°, and diluted to a suitable volume just before assay. Tubes containing all the assay-medium components except coupling enzymes and tissue were prepared: 0·05 ml. of coupling enzyme was added where necessary, followed immediately by 0·1 ml. of the dilute tissue suspension, making a final volume of 3·0 ml. The mixture was tipped into a 1 cm. quartz cell, and optical readings were taken against a blank at 20–30 sec. intervals over a period of about 8 min. with a Unicam SP.700 spectrophotometer. The blank contained the basic medium plus tissue but omitted substrate, DPNH and coupling enzymes. Preliminary control experiments were performed with each assay to show that the coupling enzyme was not contaminated with the enzyme being assayed, and that the substrate was not reacting with DPNH by any route other than that being studied. A suitable control was then run in parallel with each determination and consisted of the basic medium plus DPNH and tissue suspension; substrate and coupling enzymes were omitted. The control recorded oxidation of DPNH by endogenous tissue enzymes and substrates, and by air. Direct reading of the sample against control was inconvenient. In all except the α-glycerophosphate dehydrogenase and phosphofructokinase assays, tissue suspension equivalent to less than 1 mg. of original brain was needed, and the control rate was less than 10% of the sample rate. After sample readings had been corrected for control changes, the extinctions were plotted against time and the rate was measured from the best straight line. For some, the rate was irregular during the first 100–150 sec., and then became linear until most of the pyridine nucleotide had been used. Rates of change of extinction of 0·05/100 sec. over the range 0·6–0·2 were convenient in most cases, and were linear with respect to concentration of enzyme. Temperature was uniform during each experiment and varied during the series over the range 19–23°. Specific activities of whole homogenate are reported ±deviation for two determinations, and ±standard deviation for more than two. One extinction unit is an extinction of 1·00 under the described conditions.

The enzymes of the glycolytic chain are grouped below according to the final reaction to which they have been coupled.

*Assay coupled to glucose 6-phosphate dehydrogenase.* Phosphoglucose isomerase was assayed by conversion of the glucose 6-phosphate produced into 6-phosphogluconic acid with concomitant reduction of TPN. The method is essentially that of Slein (1955). The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine buffer (50 mM), KCl (10 mM), $MgCl_2$ (10 mM), EDTA (2 mM), potassium fructose 6-phosphate (3 mM), TPN (0·2 mM), glucose 6-phosphate dehydrogenase coupling enzyme and tissue. Slow rates were measured in order that the minimum amount of coupling enzyme should be used; the control rates due to phosphoglucose-isomerase contamination of the coupling enzyme were then 40% of the total rates measured, making the method of only moderate accuracy. The specific activity of whole homogenate was 5 extinction units/100 sec./g. of brain (one experiment).

*Assays coupled to α-glycerophosphate dehydrogenase.* Phosphofructokinase, aldolase, triose phosphate isomerase and α-glycerophosphate dehydrogenase were assayed by oxidation of DPNH accompanying the conversion of dihydroxyacetone phosphate into α-glycerophosphate.

(*i*) Assay of phosphofructokinase. The method is based on that of Racker (1947), but with increased ATP concentration and addition of cysteine to stabilize the enzyme.

The 3 ml. reaction medium contained (in final concentration and at pH 7·6) ammonium phosphate (3 mM), $MgCl_2$ (2 mM), cysteine hydrochloride (7 mM), potassium fructose 6-phosphate (7 mM), ATP (0·44 mM), DPNH (80 μM), aldolase and a mixture of α-glycerophosphate dehydrogenase and triose phosphate isomerase coupling enzymes, and tissue. The higher ATP concentration enabled maintenance of a steady rate for a longer period, and also discouraged any tendency for contaminating glyceraldehyde phosphate dehydrogenase to divert the coupling reaction with reduction of DPN. The control rate was high (25% of the total), probably owing to the comparatively large amounts of tissue needed: addition of 3 mM-KCN did not decrease the control rate. The enzyme is unstable but was preserved for 1–2 hr. in sucrose solution at 0° in the presence of 0·5% of albumin buffered with 0·05 M-glycylglycine at pH 7·0. The rate plots were erratic and not reliable to more than 10%; a rate of change of extinction of not more than 0·04/100 sec. gave the best results. The specific activity of whole homogenate was 15±1 extinction units/100 sec./g. of brain (two experiments).

(*ii*) Assay of aldolase. The method is essentially that of Baranowski & Niederland (1949), but the medium chosen was identical with that used in the assay of phosphofructokinase, except that the substrate was sodium hexose diphosphate, and α-glycerophosphate dehydrogenase and triose phosphate isomerase were used as coupling enzymes. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, and the control rate was less than 10% of the sample. The crude enzyme was stable in sucrose homogenates for at least 24 hr. at 0°. The specific activity of whole homogenate was 45±1 extinction units/100 sec./g. of brain (two experiments).

(*iii*) Assay of triose phosphate isomerase. The method is essentially that reported by Beisenherz (1955), but with increased reactant concentrations to allow a longer duration

of steady reaction. The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine buffer (50 mM), DL-glyceraldehyde 1-bromide 3-phosphate (0·6 mM, D-form, dioxan complex), DPNH (80 μM), α-glycerophosphate dehydrogenase coupling enzyme, and tissue. (The bromide is rapidly hydrolysed in solution but neither the bromide ion nor the dioxan interfered with the assay.) Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, and control rates were negligible. Rates were unaltered in the presence of potassium iodoacetate (3 mM), showing that there was no interference from glyceraldehyde phosphate dehydrogenase. Such high rates of reaction were obtained that tissue dilutions were performed with 0·3 M-sucrose containing 0·5% of albumin to hinder denaturation of the enzyme at the great dilutions necessary. The specific activity of whole homogenate was 2320 ± 120 extinction units/100 sec./g. of brain (two experiments).

(*iv*) Assay of diphosphopyridine nucleotide-dependent α-glycerophosphate dehydrogenase. The method is that described by Beisenherz, Bücher & Garbade (1955), and the medium is that used in the triose phosphate isomerase assay, except that no coupling enzyme is necessary. The substrate, dihydroxyacetone phosphate (0·6 mM), is accompanied by either an equimolar amount of glyceraldehyde 3-phosphate or by methanol according to the source of supply (see Special chemicals and reagents): these additions cause no interference, for the control rate was zero both in the presence and absence of iodoacetate (3 mM), and each substrate reacted at a similar rate in the presence of a limiting amount of purified enzyme. This sensitive assay method was used to show the virtual absence of the enzyme in brain homogenates of both male and female rats of several strains. The specific activity of whole homogenate was 0–4 extinction units/100 sec./g. of brain (six experiments).

*Assays coupled to lactic dehydrogenase.* Lactic dehydrogenase, phosphopyruvate kinase, enolase and phosphoglyceric acid mutase were assayed by the oxidation of DPNH accompanying conversion of pyruvate into lactate. There was little likelihood that the reversal of glycolysis would cause interference by means of the phosphoglyceraldehyde dehydrogenase reaction since this would require ATP, and these media contain ADP in three cases and none contain ATP.

(*i*) Assay of lactic dehydrogenase. The method is that described by Kornberg (1955). The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine or ammonium phosphate buffer (50 mM), sodium pyruvate (0·33 mM), DPNH (80 μM) and tissue. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration. Occasionally, in phosphate buffer, a distinct decrease in rate occurred after 3–4 min.: this was not a reproducible phenomenon and was not observed in triethanolamine buffer. Control rates were zero. About 50% loss of activity in the concentrated homogenate had occurred after 48 hr. at 2°. The specific activity of whole homogenate was 173 ± 1 extinction units/100 sec./g. of brain (four experiments).

(*ii*) Assay of phosphopyruvate kinase. The method is that described by Bücher & Pfleiderer (1955). The 3 ml. reaction medium contained (in final concentration and at pH 7·4) triethanolamine buffer (50 mM), KCl (75 mM), $MgCl_2$ (8 mM), ADP (0·23 mM), potassium phosphopyruvate (0·8 mM), DPNH (80 μM), lactic dehydrogenase coupling enzyme, and tissue. Phosphate buffer was unsuitable because of the insolubility of magnesium phosphate, and in collidine buffer the reaction rate was low, erratic and not proportional to the concentration of enzyme. Rates of change of extinction up to 0·12/100 sec. which were linear with respect to tissue concentration could be obtained with triethanolamine buffer, and control rates were negligible. The concentrated enzyme was stable in sucrose homogenates for at least 24 hr. at 2°. The specific activity of whole homogenate was 57 ± 4 extinction units/100 sec./g. of brain (two experiments).

(*iii*) Assay of enolase. The only published spectrophotometric assays depend on the absorption of light at 240 mμ by phosphopyruvate, and are not suited to crude tissue preparations, nor to more than brief reaction periods. A suitable method was devised with the same medium as for phosphopyruvate kinase, but with DL-2-phosphoglyceric acid (0·7 mM, D-isomer) as substrate: phosphopyruvate kinase and lactic dehydrogenase were used as coupling enzymes. Rates of change of extinction up to 0·12/100 sec. were linear with respect to tissue concentration, and the control rates were zero. The specific activity of whole homogenate was 50 ± 5 extinction units/100 sec./g. of brain (two experiments).

(*iv*) Assay of phosphoglyceric acid mutase. The principle of the method was outlined by Sutherland, Posternak & Cori (1949). The present method used the same medium as for phosphopyruvate kinase, but with potassium 3-phosphoglycerate (7 mM) as substrate, potassium 2:3-diphosphoglycerate (10 μM) as cofactor, and enolase, phosphopyruvate kinase and lactic dehydrogenase as coupling enzymes. The cofactor was virtually free of 2-phosphoglycerate. Rates of change of extinction which were linear with respect to tissue concentration could be obtained only up to 0·07/100 sec., but this may have been due to the sparing use of coupling enzymes. The control rates were zero. The specific activity of whole homogenate was 120 ± 3 extinction units/100 sec./g. of brain (two experiments).

*Assays coupled to glyceraldehyde 3-phosphate dehydrogenase.* 3-Phosphoglyceric acid kinase and glyceraldehyde phosphate dehydrogenase are assayed by causing a reversal of glycolysis, and measuring the oxidation of DPNH accompanying the conversion of 1:3-diphosphoglyceric acid into glyceraldehyde 3-phosphate. This reaction is less subject to interference from other enzymes in crude tissue preparations than is the forward reaction. In the dehydrogenase assay, one coupling enzyme is involved before the reaction step being studied: 1:3-diphosphoglyceric acid is generated *in situ*.

(*i*) Assay of glyceraldehyde phosphate dehydrogenase. The method is essentially that of Beisenherz *et al.* (1953), as improved by Wu & Racker (1959), except that triethanolamine buffer was used, and EDTA was included to prevent inactivation of the enzyme by heavy-metal impurities. The 3 ml. reaction sample contained (in final concentration, and at pH 7·4) triethanolamine buffer (50 mM), $MgCl_2$ (10 mM), EDTA (3 mM), ATP (3 mM), potassium 3-phosphoglycerate (5 mM), DPNH (80 μM), and phosphoglyceric acid kinase coupling enzyme, and tissue. A moderate rate of DPNH oxidation was observed in a preliminary control containing substrate and tissue but without coupling enzymes (see Methods). This was eliminated by 3 mM-potassium iodoacetate, and not inhibited by 0·3 mM-potassium oxalate [a lactic dehydrogenase inhibitor (Neilands, 1954)]. The

preliminary control rate was therefore not subtracted from the overall rates of reaction measured, as it appeared to be due to the reaction to be studied proceeding slowly with the aid of endogenous coupling enzyme. The method was very sensitive, but addition of α-glycerophosphate dehydrogenase and triose phosphate isomerase would increase the observed rates if greater sensitivity still were desired. Endogenous α-glycerophosphate dehydrogenase in most tissue samples other than brain would cause a variable interference best eliminated by adding a definite excess of purified enzyme; the necessity does not arise with brain, which has very small amounts of this enzyme. Tissue suspensions were diluted with 0·3 M-sucrose containing 2 mM-EDTA. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, but a definite decrease in rate occurred after 3–4 min., the slower rate being then maintained until the DPNH was consumed; the faster rate was chosen as significant. Control rates were zero. Even at very high dilutions the enzyme was stable in the presence of EDTA at 0° for at least 1 hr. The specific activity of whole homogenate was 241 ± 4 extinction units/100 sec./g. of brain (two experiments).

(*ii*) Assay of 3-phosphoglyceric acid kinase. The method is identical with that for glyceraldehyde phosphate dehydrogenase, except that glyceraldehyde phosphate dehydrogenase is used as the coupling enzyme instead of phosphoglyceric acid kinase. Rates of change of extinction up to 0·10/100 sec. were linear with respect to tissue concentration, and the control rates were zero. The specific activity of whole homogenate was 200 ± 8 extinction units/100 sec./g. of brain (two experiments).

### *Other assays*

*Chemical assay of hexokinase.* This was based on the original method of Crane & Sols (1953, 1954*b*), with 2-deoxyglucose as substrate in a phosphate buffer. A number of variations were studied in order to obtain rates linear with respect to tissue concentration and to time for 30 min.; high ATP concentrations (10 mM) were desirable, and, although triethanolamine was not inhibitory in the presence of phosphate, reaction did not occur in triethanolamine buffer alone; other buffers were not tried. Tissue suspensions (0·2 ml.) containing EDTA (2 mM) were assayed in a medium (final vol. 1·0 ml.) containing potassium phosphate (20 mM), $MgCl_2$ (8 mM), KF (10 mM), EDTA (2 mM), 2-deoxyglucose (4 mM) and ATP (10 mM). Best results were obtained at pH 6·5–7·0: activity was high at pH 7·4, but results were more erratic, possibly owing to the decreased stability at this pH (Crane & Sols, 1954*a*). Incubation was for 30 min. at 30°, and the reaction was stopped by adding 1 ml. of 0·3 N-$Ba(OH)_2$, followed by 1 ml. of 5% $ZnSO_4$ soln. The volume was made up to 5 ml. and, after centrifuging, a 2 ml. sample of the clear supernatant was analysed for reducing sugar with the alkaline Shaffer–Somogyi reagent 60 (1933) containing smaller quantities of tartrate as recommended by Somogyi (1952) for use with the arsenomolybdate reagent of Nelson (1944); determinations were made in triplicate and zero-time controls were run. The specific activity of whole homogenate was 51 ± 4 mg. of 2-deoxyglucose consumed/30 min./g. of brain (eleven experiments).

*Assay of cholinesterase.* The manometric method was that described by Aldridge & Johnson (1959), in a medium containing (in final concentration) NaCl (0·13 M), $MgCl_2$ (35 mM), $NaHCO_3$ (31 mM) and equilibrated at 37° with $CO_2 + N_2$ (5:95). True cholinesterase and pseudocholinesterase were determined from the ratio of the rates of hydrolysis of acetylcholine (14 mM) and *n*-butyrylcholine (30 mM) by using the substrate ratios for the enzymes of rat brain determined by Davison (1953). With acetylcholine as substrate, the specific activity of whole homogenate was 11 700 ± 1200 μl. of $CO_2$/hr./g. of brain (seven experiments).

*Assay of esterase.* The manometric method was the modified one described by Aldridge & Johnson (1959). The medium contained (in final concentration) NaCl (0·13 M), $MgCl_2$ (35 mM), $NaHCO_3$ (31 mM), bovine-serum albumin (1·5%, w/v) and phenyl butyrate (23 μl./flask; final volume 4 ml.), equilibrated at 37° with $CO_2 + N_2$ (5:95). The specific activity of whole homogenate was 20 000 ± 1750 μl. of $CO_2$/hr./g. of brain (three experiments).

*Assay of succinic dehydrogenase.* The manometric method was that of Aldridge & Johnson (1959). Suspensions of ruptured particles in hypotonic solution were assayed at 37° and pH 7·5 in a medium containing (in final concentration) potassium phosphate (12·5 mM), $MgCl_2$ (3·5 mM), EDTA (0·6 mM), potassium succinate (22·5 mM), $NaHCO_3$ (25 mM) and $K_3Fe(CN)_6$ (12·5 mM), equilibrated with $CO_2 + N_2$ (5:95). The specific activity of whole homogenate was 11 500 ± 900 μl. of $Co_2$/hr./g. of brain (four experiments).

*Determination of protein.* The biuret procedure was used as described by Aldridge & Johnson (1959).

## RESULTS

### *Distribution of glycolytic enzymes among particulate and supernatant fractions*

When a whole brain homogenate is crudely separated into a washed particles fraction (20.particles.30) and an almost clear supernatant, the distribution of glycolytic enzymes is as shown in Table 1: hexokinase is very different from all the rest which are predominately in the supernatant fraction. So little DPN-dependent α-glycerophosphate dehydrogenase could be detected, even after freezing and thawing of samples and addition of $MgCl_2$ (40 mM) to the assay medium, that the results are hardly significant; little activity was found in brains from adult female rats of two strains, males of a third strain, and in brains from young females. The distribution of three enzymes after more complete fractionation is shown in Table 2. Most of the lactic dehydrogenase and phosphofructokinase associated with particles was found in the mitochondrial fractions. Although about 75% of the hexokinase was in the mitochondrial fractions, about 15% could not be sedimented by forces of 145 000 *g* for 60 min. No more hexokinase could be obtained in solution after freezing and thawing the mitochondria and resedimenting the particles.

### *Subfractionation of mitochondrial preparations*

Previous work (Aldridge & Johnson, 1959) has shown that a typical (13.M.15) brain mitochondrial preparation contains about 80% of whole-homogenate succinic-dehydrogenase activity, 40% of

cholinesterase and 35% of esterase. It was believed that the latter two enzymes were associated with non-mitochondrial membranous material. Table 2 shows that about 20% of the phosphofructokinase and lactic dehydrogenase and 75% of the hexokinase are also present in such a preparation, and it was of interest to determine the extent of association of these enzymes with each other.

*Fractionation in* 0·3M-*sucrose.* Previous work (Aldridge & Johnson, 1959) showed that the cholinesterase and esterase of a 13.M.15 preparation were much less readily sedimented than was the succinic dehydrogenase. It has now been shown that, when a 13.M.15 fraction is subfractionated by centrifuging in 0·3M-sucrose at 2500 *g* for 15 min., hexokinase and succinic dehydrogenase are not separated significantly, the amount of sedimented and unsedimented enzymes being 54 and 46% for both; recovery was 103% for hexokinase and 94% for succinic dehydrogenase.

*Fractionation in dense media.* Whichever procedure was used to subfractionate the cream-coloured mitochondrial suspension according to density, two obviously distinct sets of particles were obtained: the light layer was almost white, and the heavy pellet was mainly tan-coloured, being covered by more or less of the white material according to the conditions of separation. The extent of separation differed, not only according to the density of the medium, but also according to its nature and whether procedure A or B was used. Thus with procedure A most of the white material was retained in the upper layer when a sucrose lower-layer concentration was 40% (w/v) (*d*, 1·14 g./ml.): in this case the cholinesterase content of the top layer was 80% and of the bottom layer 7% (two experiments). The same effect could be obtained with a medium of Ficoll (17–20%, w/v) in 0·3M-sucrose (*d*, 1·08 g./ml.), the cholinesterase distribution being 84–94% at the top and 3–4% at

Table 1. *Distribution of glycolytic enzymes between washed particulate* (20.*particles*.30), *and supernatant fractions of rat-brain homogenates*

Fraction designations and assay conditions were described under Methods. Assays were performed in duplicate, except for those of hexokinase which were in triplicate. Mean values are stated ±deviation for two experiments, and ±standard deviation for more than 2.

| Enzyme | No. of expts. | Percentage of total recovered activity in fraction (20.particles.30) | Supernatant | Percentage of homogenate activity recovered |
|---|---|---|---|---|
| Hexokinase | 3 | 84±1 | 16±1 | 95±10 |
| Phosphoglucose isomerase | 1 | 14 | 86 | 115 |
| Phosphofructokinase | 2 | 24±4 | 76±4 | 77± 9 |
| Aldolase | 2 | 28±3 | 72±3 | 101± 1 |
| Triose phosphate isomerase | 2 | 15±7 | 85±7 | 91±12 |
| α-Glycerophosphate dehydrogenase (DPN-dependent) | 6 | Very low activity mainly in supernatant | | |
| Glyceraldehyde phosphate dehydrogenase | 2 | 18±1 | 82±1 | 92± 7 |
| Phosphoglyceric acid kinase | 2 | 18±1 | 82±1 | 100± 3 |
| Phosphoglyceric acid mutase | 2 | 7±0 | 93±0 | 105± 3 |
| Enolase | 2 | 8±1 | 92±1 | 106± 3 |
| Phosphopyruvate kinase | 2 | 25±1 | 75±1 | 111± 4 |
| Lactic dehydrogenase | 3 | 23±1 | 77±1 | 94± 4 |

Table 2. *Detailed distribution of glycolytic enzymes among washed fractions of rat-brain homogenates*

Fraction designations and assay conditions were as described under Methods. Assays were performed in duplicate, except for hexokinase which were in triplicate. The poor recoveries for phosphofructokinase are due to the instability of fractions, and results are of low accuracy.

| Enzyme | Total recovered activity in fraction (%) 1.N.10 | 4.$M_1$.15 | 13.$M_2$.15 | 145.P.60 | S | Percentage of homogenate activity recovered in fractions |
|---|---|---|---|---|---|---|
| Hexokinase | 9 | 76 | | 15 | | 87 |
| | | | 83 | | 17 | 93 |
| Phosphofructokinase | 5 | 14 | 6 | 3 | 72 | 145 |
| | 3 | 11 | 7 | 4 | 75 | 68 |
| Lactic dehydrogenase | 3 | 22 | | 5 | 70 | 93 |

the bottom (two experiments). This difference in densities required to achieve similar distribution did not appear to be due to failure to reach equilibrium within the hour of centrifuging since extending the time did not alter the effect. It seems that the effective density of the particles was drastically increased by contact with a high concentration of sucrose (40%, w/v; 1·2M).

When 0·3M-sucrose in $D_2O$–$H_2O$ (70:30, v/v) mixture was used for one experiment under procedure B, the density was 1·11 g./ml. to allow for some $D_2O$ exchange into the particles. The allowance was not sufficient and all the particles were sedimented. In the view of Beaufay *et al.* (1959) this may indicate damage of the particle membranes which renders them more permeable and subject to water-exchange; also, the oxidative phosphorylation systems in isolated brain mitochondria, prepared by the present homogenizing technique, are less stable than in liver mitochondria (Aldridge, 1957).

When 20% Ficoll in 0·3M-sucrose was used in procedure A, only 3% of the cholinesterase was sedimented, whereas in procedure B about 20% was sedimented; this was believed to indicate hindrance of transit of particles through the layer accumulating at the interface in procedure A.

The results of six fractionations in different media with procedure B are shown in Table 3. An unsatisfactory aspect is the tendency to obtain excessively high recovery values for succinic dehydrogenase; it was difficult to measure the full activity of the original particles suspended in Ficoll, and repeated freezing and thawing were necessary. A small separation of hexokinase and succinic dehydrogenase occurred in Expts. 2 and 3, but not in the rest. Attempts to obtain separation at higher densities were hindered by the difficulty of handling the more viscous medium. Lactic dehydrogenase, cholinesterase and esterase were clearly associated with the light fraction, in contrast with hexokinase and succinic dehydrogenase, which were mainly in the heavy fraction. A small separation of cholinesterase and esterase was achieved by a double fractionation by using procedure A; a 20.particles.30 preparation was fractionated by using 6% Ficoll in 0·3M-sucrose, and the heavy fraction obtained was refractionated by using 10% Ficoll in 0·3M-sucrose. The distribution in the light, medium and heavy fractions respectively was, for esterase, 22, 17 and 61%, and, for true cholinesterase, 36, 21 and 43%. Although the activity was low and the measurements were not accurate, the pseudocholinesterase appeared to be mainly in the heaviest fraction.

Table 3. *Distribution of enzyme activity between light and heavy subfractions of rat-brain mitochondrial preparations*

A 13.M.15 preparation was subfractionated by procedure B in 0·3M-sucrose media containing additions as noted. F, Ficoll (%, w/v); $D_2O$ (%, v/v). Cholinesterase results are not corrected for the small pseudocholinesterase content.

| Expt. no. | 1 | 2 | 3 | 4 | 5* | 6 |
|---|---|---|---|---|---|---|
| Additions to 0·3M-sucrose | 15% F | 20% F | 20% F | 18–20% F | 70% $D_2O$ | 20–22% F |
| | Percentage of total recovered activity in light fraction | | | | | |
| Hexokinase | 20 | 54 | 54 | 31 | 20 | 33 |
| Succinic dehydrogenase | 14 | 32 | 39 | 26 | 19 | 31 |
| Cholinesterase | 72 | 75 | 81 | 81 | 75 | 69 |
| Esterase | — | 70 | 74 | 78 | 69 | — |
| Lactic dehydrogenase | — | — | — | — | — | 74 |
| Protein | — | 70 | 77 | 75 | 61 | 68 |
| | Percentage of total recovered activity in heavy fraction | | | | | |
| Hexokinase | 80 | 46 | 46 | 69 | 80 | 67 |
| Succinic dehydrogenase | 86 | 68 | 61 | 74 | 81 | 69 |
| Cholinesterase | 28 | 25 | 19 | 19 | 25 | 31 |
| Esterase | — | 30 | 26 | 22 | 31 | — |
| Lactic dehydrogenase | — | — | — | — | — | 26 |
| Protein | — | 30 | 23 | 25 | 39 | 32 |
| | Percentage of 13.M.15 activity recovered in fractions | | | | | |
| Hexokinase | 100 | 87 | 104 | 101 | 100 | 113 |
| Succinic dehydrogenase | 94 | 118 | 125 | 92 | 113 | 126 |
| Cholinesterase | 107 | 111 | 103 | 102 | 100 | 95 |
| Esterase | — | 93 | 94 | 96 | 95 | — |
| Lactic dehydrogenase | — | — | — | — | — | 101 |
| Protein | — | 93 | 94 | 101 | 92 | 97 |

* All the particles sedimented in this experiment; the upper layer of the packed pellet was removed and treated as a light layer.

## DISCUSSION

The whole-homogenate fractionation experiments reveal a sharp difference between the location of hexokinase and the rest of the glycolytic enzymes (Table 1). A possible association of particulate hexokinase with that portion (about 20%) of the other glycolytic enzymes which were found in mitochondrial preparations is unlikely, for simple subfractionation of such preparations into light and heavy portions shows a great divergence of hexokinase and lactic dehydrogenase (Table 3): the latter is taken as typical of the remaining glycolytic enzymes. The fact that hexokinase could not be separated significantly from succinic dehydrogenase does not entirely prove identity of location, but is highly suggestive. To apply more extensive separative methods is not easy as brain hexokinase is comparatively unstable. In the experiment with heavy water the two enzymes remained together, indicating a similar response of their particulate 'hosts' to water exchange. The separation of lactic dehydrogenase from hexokinase and succinic dehydrogenase, and its association with cholinesterase, suggest that lactic dehydrogenase may be associated with nerve fragments, and it seems reasonable to suppose that at least some of the particulate lactic dehydrogenase represents cytoplasm entrapped in axons which have not been completely broken. A few histological studies of the subfractions show the presence of many small silver-staining nerve fibres in the light fraction and fewer coarser fragments in the heavy fraction. The fact that about 80% of the mitochondria, as measured by succinic dehydrogenase, appear to be in the heavy fraction containing only 40% of the protein indicates that there must be much non-mitochondrial material present in the original 13.M.15 pellet, and other histological studies confirm that this is so. The presence of lysosomes and other granules has been reported in brain mitochondrial preparations (Beaufay, Berleur & Doyen, 1957; Walaszek & Abood, 1957; Hebb & Whittaker, 1958; Whittaker, 1958). The gross contamination of brain mitochondrial preparations by other particles has also been shown in electron micrographs of fractions prepared in 0·88 M-sucrose (Petrushka & Giuditta, 1959).

These subfractionation studies suggest that the reports of glycolysis in brain mitochondria (Du Buy & Hesselbach, 1956; Gallagher *et al.* 1956; Balázs & Richter, 1958; Abood *et al.* 1959) reflect the limitations of the separative techniques rather than realities *in vivo*. It should also be realized that in any glycolytic studies the hexokinase content of brain mitochondrial preparations relative to other glycolytic enzymes is about four times that of whole-brain homogenate, whereas clear supernatant has a relative hexokinase content of approximately one-fifth of that of whole homogenate: this tends to vitiate attempts to elucidate glycolytic rate-controlling steps of physiological significance. M. K. Johnson (unpublished work) has found that the glycolytic activity of clear supernatant from rat-brain homogenate is about one-third of that of a whole homogenate, and is trebled by addition of only 60% of the equivalent brain mitochondria. This is in accord with the work of Rossiter (1957). The low rate presumably reflects the activity of the small amount of soluble hexokinase in the clear supernatant: addition of brain mitochondria makes good the deficiency of hexokinase. Whether the soluble hexokinase is a physiological reality or an artifact of homogenization is not clear. Attempts to liberate any more hexokinase from the mitochondria by freezing and thawing were not successful. Crane & Sols (1953) failed to liberate hexokinase into solution by homogenizing brain in water or potassium phosphate buffer instead of sucrose. It is tempting to speculate that the soluble hexokinase is involved in basal metabolism of glucose and that the higher rates of glycolysis required for active metabolism are controlled in some way by access to the soluble enzymes of glucose 6-phosphate formed in the mitochondria.

It is difficult to attach much significance to the variation of content of the mainly soluble glycolytic enzymes in the particulate fraction; this was as low as 8% for phosphoglyceric acid kinase and enolase, and as high as 28% for aldolase. In one experiment in which brain was homogenized by using a Nelco blade homogenizer, the particles contained only 10% of the lactic dehydrogenase instead of 23%. This supports the view that the particulate enzyme is entrapped within poorly broken fragments rather than adsorbed on the surfaces.

The absence or very low activity of the DPN-dependent α-glycerophosphate dehydrogenase is in accord with the report by Green, Needham & Dewan (1937) that such an enzyme could not be found in ox brain, but in disagreement with Boxer & Shonk (1960), who report levels in rat brain about one-quarter of that of the lactic dehydrogenase activity. These workers used a similar assay procedure but give insufficient details for a comparison with the present work. I found no large change according to age, sex or breed of rat, nor was purified muscle α-glycerophosphate dehydrogenase inactivated when diluted with cold brain homogenate and stored for 2 hr. at 0°. Green (1936) also reported the presence of an active particulate non-DPN-dependent α-glycerophosphate dehydrogenase in rat brain, and this has been shown to be associated with mitochondrial particles (Ringler & Singer, 1958).

## SUMMARY

1. Homogenates of rat brain have been fractionated by centrifuging in 0·3 M-sucrose, and mitochondrial preparations have been subfractionated in dense media.

2. Part (15%) of the hexokinase could not be sedimented, but 75% was present in the mitochondrial fractions and could not be separated from succinic dehydrogenase. The hexokinase in the mitochondrial fraction was not liberated by freezing and thawing.

3. Each of the other glycolytic enzymes was present mainly (70–90%) in the clear cytoplasmic fraction.

4. Only traces of DPN-dependent α-glycerophosphate dehydrogenase could be detected in brain homogenates.

5. The particulate portions having lactic-dehydrogenase and phosphofructokinase activity were mainly associated with the mitochondrial fraction.

6. Substantial separation of the cholinesterase, esterase and lactic dehydrogenase from the succinic dehydrogenase and hexokinase of brain-mitochondrial preparations was achieved: a light fraction containing 70–80% of the former three enzymes and a heavy fraction containing 70–80% of the latter two enzymes could be obtained.

7. A slight separation of cholinesterase and esterase was achieved by a double subfractionation of brain particles.


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