

THE ANALYSIS OF SINGLE CELLS*

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There are a number of problems which would be furthered if means were available for analyzing single mammalian cells or other structures of comparable size. In the central nervous system, for example, there exists a wide variety of large and small single cell bodies (0.0001 to 0.05 γ dry weight) which are enmeshed in nervous tissue of completely different histological structure. The isolation and analysis of these individual cell bodies might be helpful in understanding the chemistry of the nervous system. For some time it has been possible to make rough measurements of a few enzymes in large single nerve cell bodies (1). However, the methods were unsatisfactory in regard to precision, and fragments of tissue of known composition which were equal in size to single nerve cell bodies gave low results. After considerable study the rather interesting reasons for the inaccuracies have been found. Consequently it is now quite simple to obtain reproducible and accurate values for a number of enzymes with samples of 0.01 γ dry weight and it is more than likely that samples 100 times smaller may be analyzed for several enzymes at least.

Methods will be presented for measuring GDH,¹ glutamic-aspartic transaminase, and phosphoglucisomerase in samples of 0.005 to 0.02 γ dry weight, together with analyses of single anterior horn cell bodies, dorsal root ganglion cell bodies, and some neighboring structures of the rabbit nervous system. In addition a method for measuring MDH in samples of 0.00001 γ dry weight will also be presented as an illustration of inherent analytical possibilities. The standard error of this last method is equivalent to about 15,000 molecules of MDH.

Analytical Methods

Isolation of Material to Be Analyzed—All of the tissue samples were obtained from frozen-dried sections prepared as described before (2). Ice crystal artifacts were kept to a minimum by quick freezing and scrupulous avoidance of temperatures higher than -10° until the sections were dry,

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¹ The abbreviations used include glutamic dehydrogenase, GDH, malic dehydrogenase, MDH, tris(hydroxymethyl)aminomethane, Tris, oxidized and reduced diphosphopyridine nucleotide DPN⁺ and DPNH, respectively.

in order that cell bodies could be clearly visualized. Sections were cut at 20 to 25 μ for the same reason, even though this usually cut off parts of the larger cell bodies. Dissection was accomplished free hand at room temperature under about 90 \times magnification by teasing out with metal needles which were ground to a fine blade-shaped point. Dorsal root ganglion cell bodies are very easily visualized. The capsule was teased off and analyzed separately; however, the capsular material was probably contaminated with some of the surrounding nervous tissue. Anterior horn cell bodies were harder to see, but usually could be dissected cleanly because of a distinct line of cleavage. After dissection they presented the characteristic thorn-like appearance. The success of dissection was frequently confirmed by staining. Only occasionally were tags of surrounding neuropile found to be attached.

Reaction Tubes and Pipettes—The tubes for incubation were 1.6 to 1.8 mm. bore and 50 mm. long and each tip was carefully drawn to a truncated cone 6 to 8 mm. long with a 0.4 to 0.6 mm. bore at the narrow end. This end was sealed off with a hot flame to avoid a thread-like prolongation of the lumen which could trap material and make cleaning difficult. All tubes were examined under a microscope and rejected if they did not meet specifications. The cleaning of such tubes has been described (3).

Constriction pipettes (4) were used throughout. For introduction of the reagent into the bottom of the cone-tipped tubes the pipette tips had to be straight and not over 0.2 mm. in outer diameter. With the smallest pipettes (0.1 to 0.2 μ l.) the bore was large enough to place the constriction not more than 2 or 3 mm. from the tip, thus keeping the capillary force to a magnitude easily overcome during delivery (3).

Weighing of Samples—The balance that was used is of the fish pole variety previously described (2) but is 100 times more sensitive. The quartz fiber is 1 cm. long mounted in a glass tube of 8 mm. bore and 20 mm. length. The glass pan measures 0.2 \times 0.2 mm. and weighs about 0.02 γ . The sensitivity is about 0.0001 γ corresponding to a displacement of 0.005 mm. This displacement is measured with a micrometer ocular with 0.05 mm. rulings, mounted in the eye piece of a wide angled binocular microscope (2 \times objective and 9 \times oculars). The load limit is about 0.05 γ . Displacement is not linear with load and the weight has to be calculated from a curve. This curve is established by weighing individual crystals of quinine bromide which are dissolved in 1 ml. of 0.1 N H_2SO_4 . The solutions are compared fluorometrically with standards prepared on a macro scale. (Quinine sulfate crystals are unsuitable, being flat and therefore very hard to remove from the pan.) Unfortunately dust may collect on the fiber and change the rest point and calibration. If the accumulation becomes excessive, the dust may be removed by carefully drawing a 0.5 μ l. droplet

of water along the fiber from back to front. The droplet of water is placed on the tip of a fine pipette or rod. It is probably unnecessary to coat the fiber with platinum, but the balance case should contain a generous amount of radium. Cool illumination is provided by a fluorescent light focused onto the pan from some distance.

Samples are manipulated on and off the balance with a hair point (2) which is sharpened with a razor blade to about $10\ \mu$ at the tip.

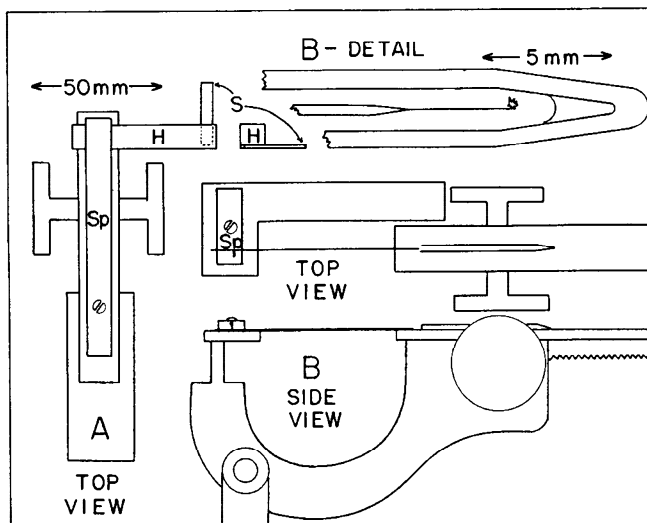


FIG. 1. The rack and pinion device (A) is for manipulating the sample holder (H) while loading samples (S) onto quartz fiber balance. The spring (Sp) holds the holder in place. The rack and pinion device (B) is for transferring samples into the reagent in the bottom of the incubation tubes. Sp holds quartz or glass needle in position. The needle is 8 or 10 cm. long, 0.5 to 1 mm. in diameter, and tapered abruptly to a short tip 10 or 20 μ in diameter. "B detail" shows the sample just before delivery into the reagent. Either device may be constructed from an old microscope stand.

A great convenience but not a necessity is a mechanical device for holding the plate of samples (Fig. 1). This is provided with a rack and pinion to introduce the tip of the plate and the samples directly into the case under the balance pan.

Addition of Sample to Buffer-Substrate Reagent—As will be discussed below a most critical step is the bringing together of dry sample and buffer-substrate solution. The procedure adopted was first to introduce the complete substrate reagent (0.2 to 1 μ l.) into the tip of the incubation tube and to insert the dry sample directly into the reagent. Each tube is then placed in an ice bath until all samples are ready for incubation. A simple mechan-

ical loading device is required (Fig. 1). The incubation tube is laid in a groove supported on a rack and pinion which is so arranged that the tube may be racked horizontally onto a stationary needle until the needle tip reaches the bottom. While observing the process through a low power wide angle microscope, the sample is placed with a fine tipped hair point onto the needle tip to which it readily adheres. The tube is racked along until the sample is introduced into the middle of the meniscus of the buffer-substrate in the bottom of the tube. The whole loading process requires less than 1 minute.

Preventing Evaporation—When the incubation volume is very small and the incubation time is prolonged, a substantial amount of water may evaporate or distill onto the upper wall of the tube, even though the tube is well capped. Therefore, with volumes of 0.5 μ l. or less and incubation times longer than 30 minutes, each sample is covered with a 3 to 5 mm. column of redistilled hexane. In this case the tubes are not covered individually during incubation, but a sheet of aluminum foil is placed over the rack of tubes to keep out dust. After incubation, the hexane is withdrawn with a fine pipette and the last traces are removed by drawing air through the same pipette with the tip held just above the aqueous layer. If the reaction has been stopped as usual by chilling in an ice bath, each tube is warmed momentarily with the fingers to hasten hexane evaporation. A trace of hexane if left may cause turbidity in subsequent steps. With a hexane protective layer 0.2 μ l. samples are not detectably changed in volume when incubated for as long as 24 hours, although much of the hexane disappears.

Glutamic-Aspartic Transaminase—The enzyme is allowed to act on a mixture of α -ketoglutarate and aspartate in the presence of DPNH and an excess of purified pig heart MDH. Oxalacetate produced is immediately reduced to malate and the DPN⁺ formed is measured fluorometrically. A similar method has been described previously with malate instead of DPN⁺ measurement (5). The method proposed is, however, much more sensitive, since final concentrations as low as 10^{-8} M DPN⁺ may be easily measured (5). LaDue *et al.* (6) used the same enzyme system for transaminase assay but measured the decrease in DPNH absorption at 340 m μ . This is several thousand fold less sensitive than the fluorometric method.

Each dry sample weighing 0.005 to 0.03 γ is added to 1 μ l. of reagent consisting of 8 mM α -ketoglutarate, 40 mM aspartate, 4 mg. per cent pyridoxal phosphate, 1 mM DPNH, 20 mM nicotinamide, 0.05 per cent bovine plasma albumin, and purified MDH all in 0.1 M Tris at pH 7.9. The MDH preparation and the concentration used are unchanged from the previous method (5).

Each tube with sample is capped with Parafilm and placed in a rack in

an ice bath together with blanks and standards (0.2 mM DPN⁺ prepared in the complete reagent). No mixing is required and is undesirable. The set of tubes is incubated for 30 minutes at 38° and returned to the ice bath and at once 1 μ l. of 0.5 N HCl is added to each tube to stop the reaction and destroy the remaining DPNH. Fluorescence is developed by the addition of 20 μ l. of 7 N NaOH followed by standing for 60 minutes at room temperature. An aliquot of 20 μ l. is added to 1 ml. of water in a 3 ml. fluorometer tube and the fluorescence is measured (5).

Glutamic Dehydrogenase—The method for measuring this enzyme is a simple micro modification of a published procedure (5). For samples of 0.005 to 0.02 γ dry weight the incubation volume is reduced to 0.5 μ l., with a reagent made in 0.05 per cent crystalline bovine albumin with 0.1 mM DPNH. Incubation is carried out as described for transaminase. The reaction is stopped with 0.25 μ l. of 0.6 N HCl which is followed by 5 μ l. of 7 N NaOH. After 60 minutes a 5 μ l. aliquot is added to 50 μ l. of water in a fluorometer tube of 3 to 3.2 mm. bore. The fluorescence is read by using a special adapter (3). Standards consist of 0.5 μ l. aliquots of 0.03 mM DPN⁺ prepared in complete buffer-substrate reagent. Standards and blanks are incubated and otherwise treated exactly like the samples.

Phosphoglucoisomerase—This is a micro modification of a method to be published.² The reagent consists of a 90 mM solution of glucose-6-phosphate and 0.05 per cent bovine albumin in 0.1 M Tris, pH 8.

The dry sample weighing 0.005 to 0.03 γ is added to 0.5 μ l. of reagent and incubated for 30 minutes at 38° as described for transaminase. After incubation 50 μ l. of a reagent which consists of a fresh mixture of 40 volumes of 20 N H₂SO₄ with 1 volume of 0.4 per cent resorcinol-1 per cent thiourea in glacial acetic acid are added. The samples are mixed well, capped loosely with aluminum foil, and heated for 20 minutes at 60°. The samples are read in micro cells (7) in the Beckman spectrophotometer at 500 m μ . Since both blanks and samples change somewhat with time, the samples are read as rapidly as possible and enough blanks and standards (3 mM fructose made in the complete buffer substrate) are included to permit some to be read both before and after the tissue samples. If significant changes are observed in the blank and standard readings, the tissue values are adjusted accordingly. Fructose has been found to give 125 per cent as much color as fructose-6-phosphate on a molar basis. The standards (0.5 μ l.) are therefore equivalent to 1.87×10^{-9} mole of fructose-6-phosphate. The color obtained with resorcinol in H₂SO₄ differs from that produced with resorcinol in HCl (8) by being (a) stable to light, (b) stronger

² Buell, M. V., Lowry, O. H., Kappahn, J. I., and Roberts, N. R.

in absorption with an absorption maximum at a shorter wave-length, and (c) less influenced by slight changes in acidity.

Malic Dehydrogenase—MDH is allowed to reduce oxalacetate with DPNH and the DPN⁺ formed is measured fluorometrically (5). Since brain is very rich in MDH, and the enzyme is measured in the faster direction, and since DPN⁺ may be readily measured at a dilution of 10^{-8} M, the method is exceedingly sensitive in terms of the amount of brain required. The procedure is described for brain homogenate, since it has not yet been applied to dry tissue samples.

The buffer-substrate reagent consists of 0.5 mM oxalacetate (fresh), 0.25 mM DPNH, and 0.05 per cent crystalline bovine albumin in 0.1 M Tris at pH 8.6.

A 0.25 μ l. volume of ice-cold buffer-substrate reagent containing 5 to 25×10^{-5} γ of brain (1 to 5×10^{-5} γ dry weight) is placed in the bottom of an incubation tube in an ice bath and covered with several mm. of hexane. A rack of tubes is incubated for 60 minutes at 38° and returned to the ice bath. The hexane is removed as described above. The DPNH is destroyed with 1 μ l. of 0.2 N HCl with gentle buzzing to mix. Fluorescence is developed by the addition of 5 μ l. of 8 N NaOH with incubation for 60 minutes at room temperature. A 5 μ l. aliquot is added to 50 μ l. of water in a fluorometer tube and the fluorescence is read as in the case of GDH above.

Because of the sensitivity of the analytical method for DPN⁺ measurement, it was possible to determine the velocity of brain MDH with very low levels of oxalacetate and DPNH. The respective Michaelis constants were found to be 0.046 and 0.045 mM. The pH optimum in Tris buffer is about 8.2 with little difference through the range pH 7.8 to 8.6.

The MDH activity of brain homogenates is about 40 moles per kilo wet weight per hour at 38°. This is about 4 times that found when the reaction is measured in the reverse direction at pH 10 (9).

The oxalacetate must be sufficiently fresh to be low in pyruvate, as otherwise lactic dehydrogenase would interfere. However, since the K_m for brain lactic dehydrogenase (rabbit) is 0.15 mM, a 5 per cent decomposition of the 0.5 mM oxalacetate substrate would give a pyruvate concentration (0.025 mM) such as to measure only 15 per cent of lactic dehydrogenase activity present. In average brain this would constitute only a 3 per cent positive error in MDH activity.

Macro- versus Micro-Analyses

Before single cell bodies could be successfully analyzed it was necessary (1) to find out how to analyze brain homogenates containing a mass of tissue equal to a single cell body and (2) to analyze correctly dry tissue sam-

ples equal to single cell bodies in size from a brain area of known composition. Different kinds of difficulties were encountered in these two steps.

Brain Homogenates—Given methods of sufficient sensitivity it was found that the same enzyme activities could be obtained with 0.05 γ samples of brain (0.01 γ dry weight) as with macro samples (5 γ), provided sufficient inactive protein was present (crystalline bovine albumin) and provided evaporation during incubation could be controlled (Table I). Albumin is particularly important for GDH, for example (Table I), or for any enzyme

TABLE I

Measurement of Four Enzyme Activities in Rabbit Brain Homogenates

Wet weights are given. Note that the weights for MDH are given in millimicrograms (0.001 γ). The activities are recorded as moles of substrate converted per kilo wet weight per hour. Hexane was used during incubation with MDH micro samples and the last four isomerase samples.

Isomerase		GDH		Transaminase		MDH	
Weight	Activity	Weight	Activity	Weight	Activity	Weight	Activity
γ		γ		γ		m μ gm.	
13.3	23.6	2.83	1.20	4.51	6.88	23	42
0.136	24.7	0.029	1.16	0.046	7.16	0.21	37
0.136	23.7	0.029	1.21	0.046	6.92	0.21	34
0.136	22.9	0.029	1.25	0.046	7.02	0.21	22
0.136	24.2	0.029	1.23	0.018	6.77	0.21	44
0.136	24.1	0.029	0.70*	0.018	6.77	0.21	42
11.0	26.3†	0.029	0.82*	0.018	6.80	0.053	32
0.069	25.6†	0.029	0.84*	0.011	6.80	0.053	36
0.069	25.5†	0.029	1.03‡	0.011	6.62	0.053	35
0.069	25.2†	0.029	0.84‡	0.011	6.75	0.053	36
0.069	25.5†	0.029	0.86‡	0.011	6.36	0.053	35

* Bovine albumin concentration reduced from 0.05 to 0.01 per cent.

† These values were obtained with another brain homogenate.

‡ Samples mixed vigorously before incubation; the rest are unmixed.

if measured in a very small sample or at a very high dilution. MDH activity with 0.0002 γ samples was only half as great in 0.01 per cent albumin as in 0.05 per cent albumin (not shown). Isomerase values were about one-third the proper level and very erratic if albumin was omitted.

Violent agitation is also detrimental (see GDH in Table I), presumably because of surface denaturation.

It is difficult to analyze micro samples and truly macro samples by the same procedures for comparison. Thus to analyze 1 mg. of brain, wet weight, for MDH under the conditions of Table I would require a liter of reagent per sample.

Dry Tissue Sections—When analytical techniques which gave consistent and proper values with homogenates were applied to small dry tissue sections (0.005 to 0.03 γ dry weight), the results were discordant and low. Mixing was found to be particularly dangerous when working with dry sections because of the danger of trapping the section on the wall of the tube above the bulk of the fluid. Mixing has therefore been avoided entirely until after incubation. Diffusion provides sufficient mixing because of the small dimensions of the fluid volume and because the dry sample is added to a ready mixed reagent. Therefore the liquid phase, at least, is homogeneous and trouble would occur only through depletion of substrate in the vicinity of the sample which in its entirety is as small as the usual particle of an ordinary homogenate.

There proved, however, to be more serious troubles than mixing. Results were particularly bad with sections which had been stored at -20° in tubes ready for analysis. This was baffling, since it had been shown that whole dry sections or large (2 γ) pieces could be stored for months or years at -20° with little or no loss in most enzyme activities. The trouble was finally traced to the condensation of moisture onto the minute sample from the 0.1 ml. of air in the reaction tube. This occurred when the samples were placed in a deep freeze for storage or when placed in an ice bath (as was customary) prior to addition of substrate reagent. Moisture even distilled onto the sample if fluid was placed nearby on the tube or held near the sample on the tip of a pipette. Under all these circumstances a small droplet of fluid instantly collected around the sample and the enzyme activity decreased 10 to 75 per cent depending on the enzyme, the temperature, and the time interval before addition of substrate reagent. Phosphoglucoisomerase was used for most of the tests, but other enzymes were similarly affected. It is not clear why the moisture was so harmful, since the enzymes tested are comparatively stable in water homogenates. Samples were placed in 0.05 μ l. droplets of water, Tris buffer at pH 8, or 0.05 per cent bovine serum albumin. Full activity was subsequently obtained with either buffer or albumin present, whereas with water alone the activity was a third of that obtained with larger specimens. This suggests that either CO_2 from the air or alkali from the glass may have been harmful or surface denaturation may have occurred. Coating the glass tubes with silicone (Dri-Film) was not helpful. The phenomenon was not observed with larger samples, since there was insufficient moisture in the air of the reaction tubes to flood them. (A 0.1 ml. volume of air at 30° with 50 per cent humidity contains only about 0.005 μ l. of water.)

Regardless of the full explanation the difficulty could be avoided by reversing the usual practice and adding the freshly dissected sample to the

reagent already in the tube as described above. This also avoided the danger of trapping the sample on the pipette tip.

With the revised procedure 0.01 γ dry samples gave reproducible enzyme activities. The mean values were within 20 per cent of those obtained with much larger specimens from the same part of the brain (Table II). The

TABLE II

Measurement of Three Enzyme Activities of Large and Small Dry Brain Samples

The samples were all obtained from the zona radiata (chiefly dendrites) of Ammon's horn of a single rabbit (20 μ sections). The macro samples were incubated in 50 to 100 times larger volumes of reagent than were the micro samples. The activity is recorded as moles per kilo dry weight per hour.

Transaminase			GDH		Isomerase	
Weight		Activity	Weight	Activity	Weight	Activity
	$m\mu gm.$		$m\mu gm.$		$m\mu gm.$	
Average....	1470	26.8*	1560	2.01*	1100	149*
S.e.m.		1.4		0.12		4
	14.0	28.3	12.1	2.77	21.6	124
	9.6	26.6	13.4	2.63	22.4	125
	9.2	24.6	16.4	2.32	16.5	120
	12.6	22.9	13.4	2.38	14.0	100
	11.9	28.4	12.6	2.64	18.6	139
	7.8	29.9	9.1	2.39	19.5	124
	12.2	26.5	11.1	2.49	21.5	139
	9.1	30.0	15.6	2.42	19.8	121
	9.3	28.6	12.9	2.53		
	11.6	29.8	14.7	2.13		
Average....	10.7	27.6	13.1	2.47	19.2	124
S.e.m.		2.3		0.18		11

* Only the averages (six to eight determinations each) are recorded for these macro-analyses.

larger standard deviations for the smaller sections do not necessarily indicate greater inaccuracies, since there must be histological differences between each of the small samples (20 \times 50 \times 50 μ) even though they were taken from as uniform an area of the brain as possible. The higher average values for GDH with the small samples may reflect better access of substrate to enzyme in the smaller incubation volume. The isomerase values are still a little low on the single cell scale, but the method should nevertheless be useful.

Analytical Results

The large cell bodies, in comparison to whole brain, are about average in glutamic-aspartic transaminase, somewhat low in GDH, and quite low in phosphoglucisomerase (Table III). The data for the first two enzymes are consonant with the values for these enzymes in the cell body layers of

TABLE III

Activity of Three Enzymes in Single Large Cell Bodies and Related Structures

The activities are recorded as moles of substrate converted per kilo dry weight per hour.

	Phosphoglucisomerase		Glutamic-aspartic transaminase		Glutamic dehydrogenase	
	Weight	Ac-tivity	Weight	Ac-tivity	Weight	Activity
	γ		γ		γ	
Dorsal root ganglion cells (type A*).....	0.010 (11)†	39 ±4‡	0.010 (7)	27.9 ±1.5	0.009 (6)	1.98 ±0.25
Dorsal root ganglion cells (type B*).....	0.010 (10)	29 ±3	0.011 (4)	31.3 ±2.2	0.010 (5)	3.04 ±0.20
Capsules of dorsal root ganglion cells.....	0.011 (8)	18 ±4	0.008 (5)	4.9 ±0.6	0.008 (5)	1.22 ±0.13
White fibers within dorsal root ganglion.....	0.025 (6)	11 ±3	0.018 (5)	2.4 ±0.2	0.022 (6)	0.26 ±0.02
Anterior horn cell bodies.....	0.010 (13)	24 ±3	0.010 (10)	26.0 ±1.2	0.008 (6)	2.86 ±0.24
Ammon's horn stratum radiata.....	0.016 (6)	121 ±2	0.014 (7)	25.6 ±0.4	0.016 (7)	2.30 ±0.19
Whole brain homogenate.....	0.014 (4)	127 ±1	0.015 (4)	33.8 ±0.2	0.007 (4)	5.1 ±0.1

* Type A cells in comparison with type B cells were larger and somewhat less dense in appearance and in stainability (thionine).

† The figures in parentheses represent the number of samples.

‡ Standard error of the mean.

cerebellum and Ammon's horn (9, 10).² These cell body layers were also relatively low in isomerase, although not as low as the single large cell bodies. Perhaps the higher values in the cell body layers are due to contamination with other nervous elements richer in this enzyme, since these layers must contain at least 30 or 40 per cent of non-cell body material.

The differences between the cell bodies themselves are of interest in view

of the known differential sensitivity of various cell bodies to various toxic agents and neurotropic viruses.

Fuller discussion of these data is probably premature.

SUMMARY

1. Procedures are presented for measuring GDH, glutamic-aspartic transaminase, and phosphoglucisomerase in single large cell bodies and other structures of similar size (0.005 to 0.03 γ dry weight), together with a description of the difficulties to be avoided.

2. The activities of these three enzymes are recorded for anterior horn cell bodies, dorsal root ganglion cell bodies, and adjacent structures in the dorsal root ganglion of the rabbit. Isomerase is especially low in cell bodies; GDH is about half that of average brain. Substantial differences are found in the composition of the two types of cell body.

3. A method for MDH is presented which requires only 10^{-5} γ of dry brain, *i.e.* one-two thousandth the mass of a large single cell body.

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