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QUANTITATIVE HISTOCHEMICAL ANALYSIS OF GLYCOLYTIC INTERMEDIATES AND COFACTORS WITH AN OIL WELL TECHNIQUE[1, 2]

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Quantitative histochemical measurements of glycolytic intermediates and cofactors in samples of the order of 0.1 μg dry weight have been made possible by the use of enzymatic fluorometric methods in combination with the technique of enzymatic cycling. In order to prevent evaporation of the necessarily small volumes of fluid and to avoid exposure to CO_2 in the air, incubations are carried out under oil in wells drilled in Teflon. The general technique for conducting analyses in oil wells is described together with specific procedures for measuring glycogen, glucose, glucose 6-phosphate, uridine diphosphoglucose, fructose diphosphate, lactate, adenosine triphosphate, phosphocreatine and inorganic phosphate. The potential of the technique is illustrated by measurements of these compounds in each of nine discrete layers of the retina.

The chemical analytical techniques of quantitative histochemistry have improved to the point where it is possible to select, weigh and analyze single tissue cells for a wide variety of enzymes. Information gained by such analysis of the enzyme pattern indicates local functional capacity but not actual activity of the enzymes and enzyme systems. For the latter, measurements are needed of the levels of the intermediates of the metabolic pathways concerned, under whatever physiologic or pathologic circumstance is being considered.

To measure the substrate or product of an enzyme in living material requires much greater sensitivity than to measure the enzyme itself. For instance, the hexokinase in 1 μg brain can form in 1 hr 10,000 times more glucose-6-P[3] than is normally present in 1 μg brain. Fortunately, in the case of any metabolite or enzyme that can be coupled with a pyridine nucleotide system, sensitivity can be increased sufficiently to demonstrate as little as 10^{-15} moles of an intermediate or product of an enzyme. This is achieved by enzymatic assays in combination with the method of enzymatic cycling. Oxidized or reduced pyridine nucleotides are employed as catalysts of an enzymatic oxidation-reduction cycle and the rate of the reaction serves as a measure of the nucle-o tide. For the analysis of TPN^+ or TPNH, glucose-6-P is oxidized to 6-P-gluconate by means of glucose-6-P dehydrogenase and α-ketoglutarate is converted to glutamate by means of glutamic dehydrogenase. The two products 6-P-gluconate and glutamate accumulate during the catalytic reaction and either one can be determined finally by enzymatic fluorometry. A similar system is available for DPN^+ and DPNH. By use of these cycling systems, Gatfield *et al.* (2) were able to measure some of the more abundant metabolites in 1-μg dry samples from individual layers of cerebellum.

It is highly desirable, however, to extend such measurements to smaller samples and to less abundant metabolites. To do so it is necessary to work with fluid volumes too small for ordinary micro-test tubes. The simplest solution appears to be to work with very small aqueous droplets under oil (5). This principle has been employed earlier for measurements of absorbance, fluorometric determinations of enzymes and microbiologic assays (3).

What follows is a general description of an "oil well technique" together with specific procedures for the measurement of nine metabolites

[1] In honor of Dr. Ralph D. Lillie.

[2] Supported in part by Research Grants 5 RO1 NB 00434 and 1 RO1 AM 1059 from the National Institutes of Health and P-78 from the American Cancer Society.

[3] The abbreviations used are: creatine phosphate, creatine-P; dihydroxyacetone phosphate, dihydroxyacetone-P; glucose 6-phosphate, glucose-6-P; glucose 6-phosphate dehydrogenase, G6PDH; glutamic dehydrogenase, GDH; inorganic phosphate, P_i; lactic dehydrogenase (beef heart), LDH; 6-phosphogluconate, 6-P-gluconate; diphosphopyridine nucleotide, DPN; triphosphopyridine nucleotid›, TPN; reduced forms of pyridine nucleotides, DPNH and TPNH; uridine diphosphoglucose, UDP-glucose.

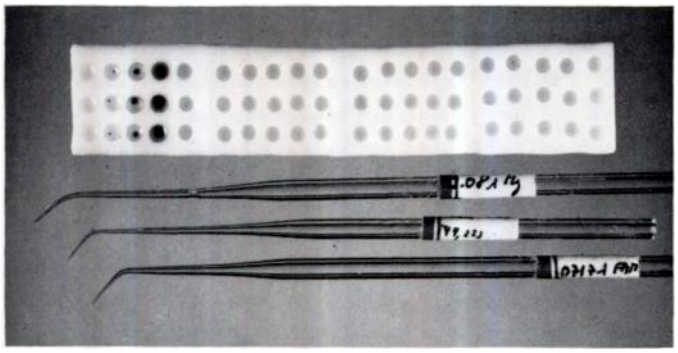

FIG. 1. Teflon oil well rack and constriction pipettes. The scale can be judged by the length of the rack (12 cm). Several of the wells are filled with oil and some contain droplets of three sizes 0.07, 1.3 and 9.1 μl, which have been colored for better visualization. These were introduced into the wells with the pipettes shown below the rack.

in 0.1-μg samples from the individual layers of retina.

MATERIALS

Oil wells (Teflon racks and glass tubes): Teflon racks (Fig. 1), each providing a set of 60 wells, are designed as follows. Small holes of 3-mm inner diameter are drilled 4 mm deep in a Teflon block measuring 5 x 20 x 120 mm. Visibility of very small specimens and minute droplets can be enhanced by drilling the holes deep enough so that only a very thin translucent flat bottom remains. Teflon racks are also constructed to hold reagents. A convenient size is 5 x 15 x 70 mm with six to eight holes of 6-mm diameter and 4 mm deep. In some instances little glass cups of a size comparable to the Teflon wells have been fused onto glass rods and used instead of the Teflon wells.

Siliconizing of glass tubes: Whereas Teflon is water-repellent as such, glass has to be siliconized to avoid spreading of small reagent droplets. This can be accomplished in two ways. Silicone SC-87 (Dri-Film, General Electric) is put into the cups using an eye dropper and sucked out again after 10 min. After three washings with acetone to remove excess silicone the tubes are ready for use. The glass surface can also be rendered sufficiently water-repellent by short contact of the tubes with silicone vapor. For this purpose SC-87 is spread on a Petri dish and placed in a desiccator together with clean dry tubes assembled in a beaker. The desiccator is evacuated and opened several times in order to facilitate contact of the inner glass surfaces with the Silicone vapor. The tubes can then be used without prior rinsing with acetone. However, since HCl is liberated when SC-87 reacts with the glass, rinsing may be mandatory for certain applications. The benefit of a Silicone film is somewhat limited, since the coating does not withstand completely the treatment with hot alkali and acid which is necessary in the course of the assays. Consequently, water droplets tend to spread out on the glass after heating steps of this sort.

Choice of oil phase: The oil used consists of a mixture of 40% hexadecane and 60% U.S.P. light mineral oil (specific gravity, 0.83–0.86). This represents a compromise in viscosity. If the viscosity is too low, droplets of fluid may not be adequately protected against access of CO_2 from the air. If the viscosity is too great, fluid droplets do not fall back rapidly to the bottom of the well when stripped off the pipette by the oil surface. Also, very viscous oil tends to adhere to an unpleasant degree to the pipette tip. When low temperatures are needed for experimental purposes, light mineral oil alone is used because the above mixture solidifies when the rack is kept on ice.

Heating and cooling of Teflon racks: As a cooling device for the Teflon rack a Petri dish containing a water-tight plastic bag packed with crushed ice is used as a base plate. Heating is accomplished in an oven or water bath of the desired temperature. To ensure uniform heating when a water bath is used, the Teflon racks are placed on a thin layer of sand in a metal pan. The pan is covered with aluminum foil and allowed to float on the water. The aluminum foil protects the Teflon rack against any water dripping down from the cover of the water bath. Because the Teflon rack is a good thermal insulator, it requires as long as 10 min for temperature equilibration.

Cleaning: The oil is removed by inverting and tapping out the Teflon block and washing thoroughly with acetone followed by boiling for 15 min in 0.5 N ethanolic KOH or NaOH. The racks are carefully rinsed with tap and distilled water and dried in an oven at 100°C. Glass tubes are similarly treated with acetone to remove all of the oil. Subsequently, the tubes are cleaned as described

for other microtubes by heating with concentrated nitric acid and thorough washings with water (8) and then resiliconized.

Special constriction pipettes: Quartz pipettes of special design were made for use with oil wells. Quartz rather than glass was used, since its higher melting point allows the construction of a more sharply defined constriction and a very sharp and sturdy tip. The tip of the pipette is drawn out with a slender taper to a fine point and is bent at a 45° angle 5–6 mm above the orifice (Fig. 1). Pipettes with a capacity as small as 0.001 μl can be constructed and handled accurately. The pipettes were calibrated colorimetrically, as previously described (8), except that stronger *p*-nitrophenol (4%) is used with dilutions of 10,000–50,000-fold.

Biochemicals: The necessary enzymes have been obtained from Boehringer and Sons, except for heart lactic dehydrogenase (Worthington Biochemical Corporation) and UDP-glucose dehydrogenase (Sigma Chemical Company). The other nonenzymic biochemicals were purchased usually from Sigma.

GENERAL PROCEDURE

Dry samples of about 0.1 μg are placed at the bottom of the Teflon wells. The samples are handled with a hair point modified by having a quartz fiber of 3–5-μ diameter fixed to the tip of hair with epoxy resin (5). All operations are carried out at low magnification of a stereo microscope with illumination from above or below. A variable magnification (Zoom) microscope with long focal distance is especially useful. Visibility can be improved if needed by restricting illumination from below to a single oil well by a hole drilled in an opaque plate. To exclude interference by static electricity the rack is briefly irradiated for 1 or 2 min with radium painted on a metal strip (U.S. Radium Corporation, Morristown, New Jersey, type T-200, active dimension ¼ inch x 1 inch). Otherwise, the aqueous droplets may migrate capriciously or even disintegrate into smaller droplets. As first step (see Table I) acid or alkali (0.05–0.25 μl) is added and the droplet is immediately covered with oil from an eye dropper. The initial heat treatment with acid or alkali is necessary in order to destroy tissue enzymes. Analytical considerations dictate whether acid or alkali is to be used. Thus acid is used if TPNH or DPNH has to be destroyed; conversely, alkali is used if TPN^+ or DPN^+ would interfere with the subsequent procedure. The choice of acid or alkali may also be governed by the stability of the particular metabolite to be measured. At this point appropriate standards dissolved in acid or alkali and blanks are introduced directly into the oil. A standard curve covering the range of sensitivity needed is prepared routinely. Blanks are usually provided at the beginning and at the end of the set of standards and at the beginning and at the end of a set of samples. If more than 40 samples are to be analyzed, extra blanks are also placed in the middle of the series. The acid or alkali and other reagents are kept under oil in the larger wells of the special Teflon rack described above. This rack is positioned so that both reagent and sample oil wells are visible simultaneously. As an alternative loading method, it is also possible, as suggested to us by Dr. J.-E. Edström, to reverse the order and to push the sample through the oil phase into the droplet of acid or alkali. The samples become translucent because of impregnation with oil and are somewhat difficult to visualize with the latter technique. This loading procedure is necessary, however, when the initial aqueous addition is less than 0.05 μl, because otherwise serious evaporation would occur before the sample could be covered with oil. Comparable results for glucose-6-P analysis in retinal samples were obtained with both loading techniques. After all samples have been loaded the rack is heated and, after cooling, reagent is added which contains enzymes and cofactors appropriate to the analysis (second step, Table I).

The presence of an oil-water interface creates some problems. (1) Inactivation of analytical enzymes may occur during incubation; this can be prevented with high concentrations of bovine serum albumin (1 mg/ml). (2) A precipitate forms during heating the protein-rich cycling mixture which may make it difficult to transfer. This problem can be circumvented by suitable dilution with water and/or use of a pipette with a large opening at the tip.

Incubations during the second step (Table I) are made at 22–26°C in a floating metal pan for the appropriate length of time. After completing the assay excess DPN^+ or TPN^+ is destroyed with alkali at 75°C or 100°C; excess DPNH or TPNH is destroyed by addition of HCl. The remaining amounts of reduced or oxidized pyridine nucleotides are equivalent to the substrate measured and are assayed by enzymatic cycling (Table II) (7). The cycling step (usually 1 hr) is carried out in the oil well itself as long as the volume does not exceed 10 μl. Otherwise this step is carried out in glass tubes of appropriate size, usually in 3-ml fluorometer tubes. The cycling reaction is terminated by heating, as described above, for 10 min at 100°C. The concentration of the enzymes used for the cycling systems is adjusted as described in the original paper (7) to give appropriate cycling rates. The final analysis of 6-P-gluconate or pyru-

TABLE I

Analytical Conditions Prior to the Cycling Step[a]

Metabolite	First Step	Second Step Reagent				
		Enzymes	Cofactors	Other additions	Buffer	Volume of second step
						μl
Glycogen	0.1 μl 0.015 *N* HCl, 10 min at 100°C	Glucose-6-P DH,[b] 5 μg/ml;[c] P-glucomutase, 2 μg/ml; Phosphorylase a, 30 μg/ml	TPN+, 75 μ*M*; 5′-AMP, 0.3 *mM*; P_i, 10 *mM*	$MgCl_2$, 7.5 *mM*; EDTA, 1.5 *mM*; mercaptoethanol, 3 *mM*; BSA[d], 0.1%	Imidazole-HCl, 0.04 *M*, pH 7.4	3[e]
Glucose	0.1 μl 0.015 *N* HCl, 10 min at 100°C	Hexokinase, 3 μg/ml; glucose-6-P DH, 1 μg/ml	TPN+, 100 μ*M*; ATP, 1 *mM*	$MgCl_2$, 7.5 *mM*; BSA, 0.1%	Tris-HCl, 0.1 *M*, pH 7.6	0.3
Glucose-6-P	0.15 μl 0.015 *N* HCl, 10 min at 100°C	Glucose-6-P DH, 1 μg/ml	TPN+, 20 μ*M*	BSA, 0.1%	Tris-HCl, 0.2 *M*, pH 8.0	0.15
UDP-glucose	0.1 μl 0.025 *N* HCl, 20 min at 60°C	UDP-glucose DH, 300 μg/ml	DPN+, 10 μ*M*	BSA, 0.1%	Glycine-HCl, 0.4 *M*, pH 9.0	0.1
Fructose diphosphate	0.05 μl 0.025 *N* HCl, 30 min at 60°C	Aldolase, 1 μg/ml; glyceraldehyde-P DH, 10 μg/ml; triose-P isomerase, 1 μg/ml	DPN+, 10 μ*M*; NaH_2AsO_4, 1 *mM*	Mercaptoethanol, 2 *mM*; EDTA, 1 *mM*; BSA, 0.1%	Imidazole-HCl, 0.2 *M*, pH 7.4	0.05
Lactate	0.2 μl 0.025 *N* HCl, 10 min at 100°C	Lactic DH (beef heart), 60 μg/ml	DPN+, 600 μ*M*	Hydrazine hydrate, 100 *mM*	AMP_2, 0.2 *M*, pH 9.5	0.2
ATP	0.1 μl 0.1 *N* NaOH, 15 min at 60°C	Hexokinase 2 μg/ml; glucose-6-P DH, 2 μg/ml	TPN+, 60 μ*M*; glucose, 2 *mM*	$MgCl_2$, 5 *mM*; BSA, 0.1%	Tris-HCl, 0.3 *M*, pH 8.0	0.3
P-creatine	0.2 μl from ATP, step 2, to 0.1 μl 0.3 *N* HCl, 10 min, 0°C	As for ATP, plus creatine-P-kinase, 2 mg/ml	As for ATP, plus 10 μ*M* ADP	As for ATP	Tris-HCl, 0.2 *M*, pH 8.0	0.6
P_i	0.25 μl 0.025 *N* NaOH, 20 min at 60°C	Phosphorylase *a*, 30 μg/ml; P-glucomutase, 5 μg/ml; glucose-6-P DH, 2 μg/ml	TPN+, 60 μ*M*; 5′-AMP, 20 μ*M*; glycogen, 10 *mM*	$MgCl_2$, 1 *mM*; EDTA, 2 *mM*; BSA, 0.1%	Imidazole-HCl, 0.1 *M*, pH 7.0	0.25

[a] All steps in this table are carried out under oil. Further details are given in text. The incubation period for most assays except for UDP-glucose (60 min) and for lactate (90 min) was 30–40 min at 22–26°C.

[b] Dehydrogenase.

[c] All concentrations refer to reagent volume.

[d] Bovine serum albumin.

[e] For layers 1, 2a and 2b, because of lower glycogen, only 0.25 μl of reagent is used.

TABLE II

Conditions for the Cycling Step[a]

Metabolite	Preparation for Cycling	Cycling Conditions	Incubation Conditions	Range of Sensitivity
				10^{-14} moles
Glycogen	3 µl[b] 0.2 *N* NaOH, 20 min at 75°C	G6PDH, 30 µg/ml; GDH, 150 µg/ml; 5,000 cycles/hr	1 hr, 38°C in 50 µl	200–4000[c]
Glucose	0.3 µl 0.2 *N* NaOH, 20 min at 75°C	G6PDH, 10 µg/ml; GDH, 100 µg/ml; 2,000 cycles/hr	1 hr, 38°C in 10 µl	50–500
Glucose-6-P	0.25 µl 0.2 *N* NaOH, 20 min at 75°C	G6PDH, 50 µg/ml; GDH, 250 µg/ml; 10,000 cycles/hr	1.5 hr at 38°C in 5 µl	10–100
UDP-glucose	1 µl 0.1 *N* NaOH, 20 min at 75°C	LDH, 15 µg/ml; GDH, 200 µg/ml; 1,000 cycles/hr	1 hr, 22–26°C in 30 µl	10–100
Fructose diphosphate	0.1 µl 0.2 *N* NaOH, 20 min at 75°C	LDH, 30 µg/ml; GDH, 400 µg/ml; 2,000 cycles/hr	1.5 hr, 22–26°C in 3 µl	0.2–4
Lactate	2 µl 0.05 *N* NaOH, 20 min at 75°C	LDH, 15 µg/ml; GDH, 200 µg/ml; 1,000 cycles/hr	1 hr, 22–26°C in 50 µl	100–600
ATP	0.6 µl 0.14 *N* NaOH to 0.2 µl aliquot, 20 min at 75°C	G6PDH, 10 µg/ml; GDH, 100 µg/ml; 2,000 cycles/hr	1 hr, 38°C in 10 µl	50–500
P-creatine	0.1 µl 1 *N* NaOH, 20 min at 75°C	As for ATP	As for ATP	50–500
P_i	2 µl 0.08 *N* NaOH, 20 min at 75°C	G6PDH, 30 µg/ml; GDH, 150 µg/ml; 5,000 cycles/hr	1 hr, 38°C in 50 µl	50–500

[a] Incubations in volumes of 10 µl or less were carried out in oil wells; the rest were carried out in test tubes. The cycling rates are only approximate.

[b] For retinal layers 1, 2a and 2b, because of lower glycogen, only 0.25 µl is used.

[c] Sensitivity, 20–200 × 10^{-14} moles under conditions given for layers 1–2b.

vate is performed by fluorometric enzymatic assays (6). 6-Phosphogluconate is measured with the aid of the TPN-dependent 6-P-gluconate dehydrogenase. For this purpose the cycling reagent is transferred quantitatively from the oil wells to a 3-ml fluorometer tube containing 1 ml of the indicator step reagent (0.02 *M* Tris buffer, pH 8.0, with 0.04 *mM* TPN^+, 0.1 *mM* EDTA and 6-P-gluconate DH from sheep liver or yeast. The enzyme concentration has to be sufficient to oxidize 50% of 5 µ*M* 6-P-gluconate in approximately 3 min). If the cycling step was carried out in 3-ml fluorometer tubes, the indicator step reagent is added to the cycling reagent. After incubation for 30 min at 22–26°C the fluorescence inherent in the TPNH formed is measured. Pyruvate is analyzed by reduction with lactic dehydrogenase and DPNH. To measure UDP-glucose and lactate to the cycling reagent are added 30 and 50 µl, respectively, of the indicator step reagent (0.65 *M* NaH_2PO_4, 0.15 *M* K_2HPO_4, 1.5 µg/ml crystalline rabbit skeletal muscle lactic dehydrogenase and DPNH at a concentration approximately 10 times that of the expected pyruvate). The tubes are incubated for 15 min at 22–26°C and then cooled in an ice bath, and 15 µl 5 *N* HCl are added to destroy excess DPNH. Fluorescence of DPN^+ is induced by pipetting to the tube 1 ml 6 *N* NaOH and heating at 60°C for 15 min. Fluorescence is recorded after cooling the tubes to room temperature again. In the case of fructose diphosphate the enzymatic indicator step is carried out under oil by adding 3 µl of the above reagent to cycling reagent in the oil well. The reaction is over within 20 min at 22–26°C. Excess DPNH is destroyed with 1 µl 5 *N* HCl and the reagent droplet is then transferred quantitatively to a 3-ml fluorometer tube containing 1 ml 6 *N* NaOH. Induction and meas-

urement of fluorescence are carried out as described for the other two DPN-dependent assays.

The native fluorescence of TPNH- and alkali-induced fluorescence of DPN^+ is read in the Farrand model A-3 fluorometer. As light sources both the line-operated high pressure mercury arc lamp and a GE no. 2331 lamp with tungsten filament run at 8 volts from batteries were employed. Galvanometer and microammeter were used interchangeably to record the photomultiplier output. In connection with the tungsten-filament lamp the primary filter was Corning no. 5840, and the secondary was a filter combination of Corning nos. 4303 and 3387, with the latter facing the phototube. When the mercury arc light was employed the primary filter was Corning no. 5860, and the secondary a combination of Corning nos. 3387, 4308 and 5562, the latter facing the phototube.

Methodologic problems could arise at any of the many steps. To spot a possible point of trouble in an analytical sequence appropriate standards may be introduced at every step of the procedure. For example, if difficulty arises in the glycogen assay the trouble may be found by checking the recovery of the following standards: glycogen, glucose-1-P, glucose-6-P, at the second step (Table I), TPNH at the cycling step and 6-P-gluconate at the final indicator reaction. Even in routine assays it has been found worthwhile to provide three kinds of standards: the metabolite to be measured, the pyridine nucleotide to be generated and the final indicator, *i.e.*, 6-P-gluconate or pyruvate. This allows calculation of the cycling rate as well as control of the over-all process.

Following the general scheme outlined the specific microanalytical systems have been developed. Most of the assay systems are adaptations of published enzymatic spectrophotometric or fluorometric methods for glycogen (12), glucose (6), glucose-6-P (6), UDP-glucose (4), fructose diphosphate,[4] lactate (1), ATP (2), creatine-P (2) and P_i (13). It is recommended that the original publications be consulted for further information about the basic methods. Specific assay conditions are given in Tables I and II. The enzymatic sequence of each assay and analytical details and special precautions not given in the tables are covered in the following comments regarding specific procedures.

NOTES ON SPECIFIC PROCEDURES

Glycogen:

$$\text{Branched glycogen} \xrightarrow{\text{debrancher complex}} \text{unbranched glycogen} \quad (1)$$

$$\text{Unbranched glycogen} + P_i \xrightarrow{\text{phosphorylase a}} \text{glucose-1-P} \quad (2)$$

$$\text{Glucose-1-P} \xrightarrow{\text{phosphoglucomutase}} \text{glucose-6-P} \quad (3)$$

$$\text{Glucose-6-P} + TPN^+ \xrightarrow{\text{glucose-6-P-DH}} \text{6-P-gluconate} + TPNH \quad (4)$$

$$\text{Branched glycogen} + P_i + TPN^+ \rightarrow \text{6-P-gluconate} + TPNH \quad (1\text{-}4)$$

To allow complete solution of the glycogen, samples are heated 10 min at 100°C in 0.015 *N* HCl. Glycogen analysis in retina is complicated by the nearly 400-fold range in concentrations (1.8–670 $\times 10^{-13}$ moles/0.1-μg sample). Accordingly, two different sets of standards and slight modifications of the procedure are necessary to cover this range. For samples from layers 1, 2a and 2b the specific enzyme step is carried out in 0.35 μl and the entire sample is carried forward, whereas with all other layers the specific enzyme step is performed in 3 μl and only a small aliquot is used for the subsequent cycling step.

Glucose:

$$\text{Glucose} + ATP \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + ATP \quad (1)$$

$$\text{Glucose-6-P} + TPN^+ \xrightarrow{\text{glucose-6-P-DH}} \text{6-P-gluconate} + TPNH \quad (2)$$

$$\text{Glucose} + ATP + TPN^+ \rightarrow \text{6-P-gluconate} + ADP + TPNH \quad (1\text{-}2)$$

Care has to be taken that the acid heat treatment is mild enough to prevent breakdown of glycogen to glucose. With 0.01 or 0.015 *N* HCl no detectable glucose is liberated at 100°C in 10 min (less than 0.1% of the glycogen present). The cycling amplification recommended is 2,000-fold. When very low levels of glucose occur, as, for example, in retina after short periods of ischemia, amplification can be increased to 10,000-fold by increasing the enzyme levels to those shown in Table II for glucose-6-P.

When absolute amounts of glucose are very small (about 10^{-14} moles), contamination becomes a problem and may result in high blanks and erratic measurements.

Another potential source of difficulty is the possible presence of significant amounts of pre-

[4] T. Bücher, personal communication.

formed glucose-6-P or of substances convertible to glucose or glucose-6-P with mild acid treatment. These include UDP-glucose which yields glucose, and glucose 1,6-diphosphate which yields glucose-6-P. It has been found, for example, that in some retinal layers both glucose-6-P and UDP-glucose may be present in amounts that are comparable to glucose levels (Table IV). Moreover, in brain, at least, it has been recently found that glucose 1,6-diphosphate is present in very substantial amounts.[5] For these reasons some caution must be exercised in interpreting glucose values, and another method of preparing samples for analysis may be desirable.

Glucose-6-P:

$$\text{Glucose-6-P} + \text{TPN}^+ \xrightarrow{\text{glucose-6-P-DH}} \text{6-P-gluconate} + \text{TPNH} \quad (1)$$

Certain batches of glucose-6-P dehydrogenase may be contaminated with glutathione reductase. Since in preparing the tissue for analysis significant amounts of reduced glutathione (GSH) may be converted to oxidized glutathione (GSSG), it is possible that this impurity leads to oxidation of a substantial fraction of the TPNH by GSSG. Accordingly the TPNH measured may not be stoichiometric with the G-6-P present. The loss can be prevented by incorporating 0.1 *mM* dithiothreitol in the second step reagent (Table I).[6] This is true for any of the reactions in which glucose-6-P dehydrogenase is the indicator enzyme but the danger is greatest when, as with glucose-6-P, the substance to be measured is present in relatively small quantity.

As in the case of glucose, the heating of tissue in dilute HCl converts any glucose 1,6-diphosphate (glucose-1,6-P_2) present quantitatively to glucose-6-P and P_i. Accordingly the sum of glucose-6-P and glucose-1,6-P_2 is measured with the described technique. From results of stability measurements of authentic glucose-6-P and glucose-1,6-P_2 in weak alkali and acid the following procedure is recommended to analyze the diphosphate after destruction of the monophosphate. Samples are heated in 0.1 μl 0.03 *N* NaOH for 10 min at 100°C to destroy glucose-6-P. After cooling to 0°C, the suspension is acidified with 0.1 μl 0.1 *N* HCl and heated once more for 10 min at 100°C in order to convert glucose-1,6-P_2 to glucose-6-P. After cooling the resulting glucose-6-P is measured by the usual method (second step, Table I). The difference between the routine procedure and the modification outlined would represent the true glucose-6-P.

Fructose diphosphate:

$$\text{Fructose diphosphate} \xrightarrow{\text{aldolase}} \text{3-P-glyceraldehyde} + \text{dihydroxyacetone-P} \quad (1)$$

$$\text{Dihydroxyacetone-P} \xrightarrow{\text{isomerase}} \text{3-P-glyceraldehyde} \quad (2)$$

$$\text{3-P-glyceraldehyde} + 2\ \text{DPN}^+ \xrightarrow[\text{arsenate}]{\text{3-P-glyceraldehyde-DH}} \text{3-P-glycerate} + 2\ \text{DPNH} \quad (3)$$

$$\text{Fructose diphosphate} + 2\ \text{DPN}^+ \rightarrow \text{3-P-glycerate} + 2\ \text{DPNH} \quad (1\text{-}3)$$

The usual enzymatic assay for fructose diphosphate is based on enzymatic conversion to α-glycero-P with the conversion of 2 moles DPNH to DPN^+ (6). This method is not satisfactory for the low amounts of fructose diphosphate found in some retinal layers. The reason is that when the excess DPNH is subsequently destroyed with acid an amount of DPN^+ is produced which, although ordinarily negligible, would be very disturbing at the low levels concerned. Therefore, an alternative method was developed based on the production of DPNH *via* aldolase, triose-P isomerase and glyceraldehyde-P dehydrogenase. In the presence of arsenate, glyceraldehyde-P is converted rapidly and irreversibly to 3-phosphoglyceric acid, and the destruction of excess DPN^+ presents no problem. All steps of this assay, except the final induction of fluorescence of DPN^+ by strong NaOH, are carried out under oil as described under "General Procedure." With this procedure the sum of fructose diphosphate and dihydroxyacetone-P is measured. Whether or not a correction is needed depends upon the relative concentrations of the two compounds. Dihydroxyacetone-P may be destroyed before carrying out the actual analysis by treatment with alkali (0.04 *N* NaOH for 20 min at 60°C).

Uridine diphosphoglucose:

$$\text{UDP-glucose} + 2\ \text{DPN}^+ \xrightarrow{\text{UDP-glucose-DH}} \text{UDP-glucuronate} + 2\ \text{DPNH} \quad (1)$$

UDP-glucose was converted to uridine diphosphoglucuronic acid with aid of UDP-glucose dehydrogenase and DPN^+. Two molecules of DPNH are formed per molecule of the intermediate. Complete inactivation of tissue enzymes without

[5] J. V. Passonneau, D. W. Schulz and O. H. Lowry, unpublished data.

[6] P. Needleman, personal communication.

destruction of this metabolite is difficult to achieve, since this sugar derivative is both acid- and alkali-labile. Under the conditions given (Table I) there was 15% loss in the initial heating step. The over-all recovery compared to DPNH standards was 60%. The reasons for this low recovery have not been completely determined; nevertheless, the assay is reproducible and linear over the indicated range.

Lactate:

$$\text{Lactate} + \text{DPN}^+ + \text{hydrazine} \xrightarrow{\text{lactic DH}} \text{hydrazone of pyruvate} + \text{DPNH} \quad (1)$$

The measurement of lactate was troublesome for several reasons. It is difficult to reduce lactate contamination in the reagent to less than 10^{-6} *M*. In addition, it is difficult to destroy completely the high levels of DPN^+ needed to ensure stoichiometric conversion of lactate to pyruvate; any remaining DPN^+ contributes to the cycling blank. Furthermore, hydrazine, commonly employed to trap pyruvate, enhances DPNH oxidation during treatment with hot alkali and causes low recovery. This destruction can be minimized if 2-amino-2-methyl-1-propanol-HCl buffer is substituted for carbonate which is recommended in the original procedure (1). Even with this change, recovery of lactate has been only 60% compared to DPNH standards.

ATP:

$$\text{ATP} + \text{glucose} \xrightarrow{\text{hexokinase}} \text{glucose-6-P} + \text{ADP} \quad (1)$$

$$\text{Glucose-6-P} + \text{TPN}^+ \xrightarrow{\text{glucose-6-P-DH}} \text{6-P-gluconate} + \text{TPNH} \quad (2)$$

$$\text{ATP} + \text{glucose} + \text{TPN}^+ \rightarrow \text{6-P-gluconate} + \text{ADP} + \text{TPNH} \quad (1\text{-}2)$$

P-creatine:

$$\text{P-creatine} + \text{ADP} \xrightarrow{\text{creatine-P-kinase}} \text{ATP} + \text{creatine} \quad (1)$$

2 and 3 as for ATP 1 and 2

$$\text{P-creatine} + \text{ADP} + \text{glucose} + \text{TPN}^+ \rightarrow \text{creatine} + \text{6-P-gluconate} + \text{TPNH} \quad (1\text{-}3)$$

ATP and P-creatine were measured separately in the same sample (2). Treatment of the tissue with 0.1 *N* NaOH for 15 min at 60°C destroys interfering enzymes, as well as glucose-6-P, without cleavage of ATP or P-creatine. The TPNH content of retinal samples is very low compared to ATP and can be ignored. After the specific enzyme reaction for ATP is complete the sample is split. One-half of the sample (0.2 μl) is carried forward according to Table II for the measurement of ATP. The other half of the sample is pipetted into 0.1 μl 0.3 *N* HCl in another oil well at 0°C to destroy the TPNH formed in the ATP reaction. After neutralization of this acid solution with 0.1 μl 0.3 *M* Tris base the reagent for P-creatine is added and the procedure is continued as indicated in Table I.

Inorganic phosphate:

$$\text{Glycogen} + \text{P}_i \xrightarrow{\text{phosphorylase a}} \text{glucose-1-P} \quad (1)$$

$$\text{Glucose-1-P} \xrightarrow{\text{P-glucomutase}} \text{glucose-6-P} \quad (2)$$

$$\text{Glucose-6-P} + \text{TPN}^+ \xrightarrow{\text{glucose-6-P-DH}} \text{6-P-gluconate} + \text{TPNH} \quad (3)$$

$$\text{Glycogen} + \text{P}_i + \text{TPN}^+ \rightarrow \text{6-P-gluconate} + \text{TPNH} \quad (1\text{-}3)$$

In the determination of P_i there are two common sources of error. Endogenous ATP may be split by ATPase, which is at present a contaminant of commercial phosphorylase a. This can be

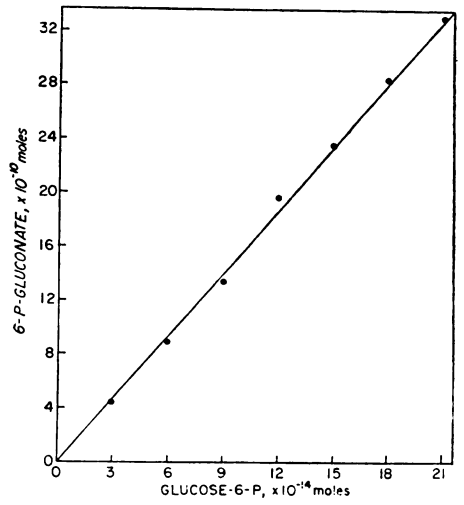

FIG. 2. Yield of product in the assay for glucose-6-P. The ordinate records the amounts of 6-P-gluconate produced by the amounts of glucose-6-P shown on the abscissa. It is seen that amplification at the cycling step was approximately 15,000-fold. Each point is the mean of duplicate measurements.

TABLE III

Standard Deviations and Over-All Blanks of Assays[a]

Metabolite	S.D.	Over-All Blank
	%	*moles* × 10^{-14}
Glycogen	5 (8)	20
Glucose	10 (8)	70
Glucose-6-P	5 (8)	6
Fructose diphosphate	8 (18)	1
UDP-glucose	7 (9)	20
Lactate	12 (8)	200
ATP	8 (14)	40
P-creatine	11 (8)	150
P_i	2 (12)	100

The number of analyses carried out in each instance is given in parentheses. The over-all blank is the fluorescence resulting without addition of the intermediate or tissue. Depending upon the procedure all reagents added and chemical reactions occurring contribute variably to the over-all blank. It gives an indication of the useful sensitivity of the method and is expressed by comparison with the fluorescence delta due to the intermediates concerned.

prevented by using phosphorylase a that has been recrystallized several more times to remove ATPase or, as prescribed in Table I, by using ethylene diaminetetraacetate (EDTA) in excess of Mg^{++} ions to prevent ATPase activity (13). The danger of contamination with exogenous P_i is also great. Special care has to be taken during the preparation of reagents and tools to avoid this contamination.

VALIDATION OF THE TECHNIQUE

Reproducibility and linearity of the various assays are illustrated by analyses of standard solutions and samples from individual layers of the retina.

Standard curves with authentic compounds: Results with glucose-6-P are representative of several TPN-dependent assays (Fig. 2). Reproducible measurements were obtained with samples ranging from 3–20 × 10^{-14} moles. The over-all blank of the assay system could be kept as low as 6 × 10^{-14} moles (Table III). The assay was linear and the standard deviation was about 5%.

Fructose diphosphate is chosen to demonstrate the potential of a DPN-dependent system. Between 0.5 and 4 × 10^{-14} moles could be measured accurately (Fig. 3). The over-all blank of this system was 1 × 10^{-14} moles. The assay was linear over the full range and the standard deviation was approximately 8% (Table III). In the useful analytical range (see Table II), the standard deviation of the other assays was in most cases below 10% (Table III), regardless of whether high or low levels are measured.

Retina assays: The efficacy of the oil well techniques for analysis of glycolytic metabolites and cofactors in tissues is demonstrated by measurements made in layers of rabbit and monkey retina (Table IV). This is a test of biologic variation as well as analytical precision. The over-all standard deviations in most cases were less than 20%, permitting estimation of mean values to within 10% with four or five samples.

The tissue levels of all intermediates exhibit complicated patterns which it is not the present purpose to discuss in detail. However, a short comment is indicated at this point. Glucose levels when corrected for glucose-6-P and UDP-glucose are high in the outer layers of the retina and decline in the vitreal direction. All of the other intermediates analyzed show gradients, increasing with varying degree of steepness from the choroidal to the vitreal side of the retina. The most dramatic differences between outer and inner layers are seen in the glycogen profile. Fructose diphosphate is exceptional in being relatively high in the layer of outer segments.

The high rate of aerobic glycolysis of the retina was described 40 years ago by Otto Warburg (14). These data supplement at the cellular level our

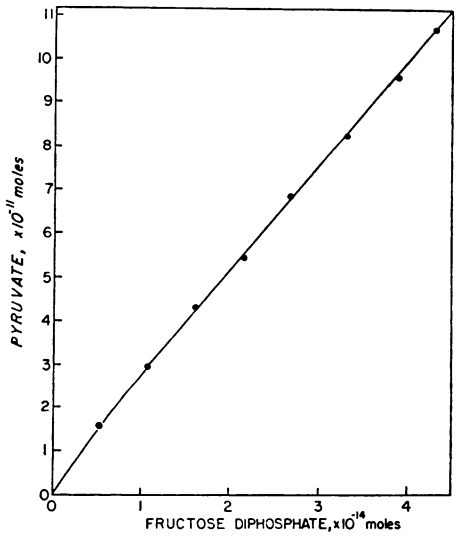

FIG. 3. Yield of product in the assay for fructose diphosphate. The ordinate records the amounts of pyruvate produced by the amounts of fructose diphosphate shown on the abscissa. It is seen that amplification was approximately 3000-fold. Each point is the average of duplicate measurements.

TABLE IV

Distribution of Nine Intermediates in the Layered Structure of Retina[a]

Layer	Glycogen	Glucose[b]	Glucose-6-P[c]	Fructose Diphosphate[d]	UDP-Glucose	Lactate	P_i	ATP	P-Creatine
1. Epithelium	3.8 ± 0.8	11.8 ± 0.7	0.32 ± 0.03	0.14 ± 0.01	1.4 ± 0.1	86 ± 5	7.3 ± 0.3	11.5 ± 0.9	3.1 ± 0.3
2a. Outer segments	1.8 ± 0.1	9.3 ± 0.7	0.28 ± 0.01	0.43 ± 0.02	0.3 ± 0.1	77 ± 8	4.7 ± 0.5	2.1 ± 0.1	1.4 ± 0.1
$2b_1$. Inner segments, outer part	4.5 ± 1.6	7.8 ± 0.3	0.64 ± 0.05	0.30 ± 0.02	1.1 ± 0.1	90 ± 8	6.5 ± 0.2	6.3 ± 0.3	5.9 ± 0.3
$2b_2$. Inner segments, inner part	53.7 ± 10.3	12.2 ± 0.6							
4[e]. Outer nuclei	110 ± 7.8	11.2 ± 1.0	1.44 ± 0.03	0.19 ± 0.01	2.1 ± 0.1	63 ± 16	10.5 ± 0.3	7.7 ± 0.3	8.3 ± 0.3
5. Outer plexiform layer	275 ± 52.0	11.5 ± 0.7		0.63 ± 0.02		150 ± 26		11.0 ± 0.8	8.5 ± 0.1
6. Inner nuclei	422 ± 19.8	12.7 ± 0.3	3.04 ± 0.17	0.42 ± 0.02	3.9 ± 0.1	168 ± 7	21.8 ± 0.6	11.3 ± 0.8	9.5 ± 0.7
7. Inner plexiform layer	356 ± 19.5	11.8 ± 0.7	2.15 ± 0.07	0.72 ± 0.04	3.1 ± 0.3	157 ± 18	25 ± 0.9	11.3 ± 0.4	8.8 ± 0.4
8. Ganglion cells	530 ± 44.0	14.6 ± 1.4	3.75 ± 0.07	0.38 ± 0.06	4.8 ± 0.3	189 ± 28	30 ± 0.9	12.6 ± 0.8	7.8 ± 0.4
9. Nerve fibers	670 ± 31.6		3.93 ± 0.11		1.3 ± 0.3	204 ± 20	31 ± 1.0	12.4 ± 0.5	6.5 ± 0.3

[a] These results were obtained from frozen-dried specimens (about 0.1 μg) of retinal layers sampled as previously described (9). ATP and P-creatine data are from monkey retina; all of the rest are from the rabbit. To preserve the *in vivo* situation as truly as possible, eyes were taken out under local anesthesia (rabbit) or general anesthesia (monkey) and frozen immediately in Freon-12 at −150°C (6). Levels of intermediates are recorded as millimoles per kilogram of dry tissue. The means of usually five determinations and the standard errors are given.

[b] Not corrected for glucose-6-P and UDP-glucose.

[c] Not corrected for glucose 1,6-diphosphate.

[d] Not corrected for dihydroxyacetone-P.

[e] Layer 3 (outer limiting membrane) is not discernible in the frozen-dried section.

knowledge about the function of glycolysis in the retina. Emergency stores of glycogen are highest in areas remote from the blood supply and active aerobic glycolysis sustains a steep gradient of lactate from inside to outside. The distribution patterns of glucose-6-P and UDP-glucose are correlated with the glycogen profile. Further discussion of retinal metabolites will be deferred until there is opportunity to present more detailed results with monkey and rabbit retina under normal and ischemic conditions.[7]

DISCUSSION

The oil well techniques for glycolytic intermediates and cofactors have been essential for other recent studies regarding the energy metabolism of microscopic structures of the inner ear and in experiments concerning penetration and phosphorylation of glucose in islets of Langerhans (10, 11). This further illustrates the validity and the potential of the methodology. In fact, the combination of enzymatic cycling and oil well technique provides almost unlimited sensitivity for quantitative analysis of any metabolic intermediate or enzyme which can be led to a pyridine nucleotide-coupled system. With a single cycling step as little as 10^{-15} moles of a compound can be measured. This degree of sensitivity is sufficient to measure a number of glycolytic intermediates in single anterior horn cells. Moreover, sensitivity can be increased by working in volumes as small as 1 nl (10^{-6} ml) and by introducing a second cycling step (7).

With these techniques in hand, enzymes and metabolic intermediates can be measured quantitatively in the smallest existing cells. Even cellular organelles such as a nucleus or mitochondrion could be analyzed provided that the resolution power of the sampling procedures is refined to a comparable degree.

[7] F. M. Matschinsky, in preparation.

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