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OF ERYTHROCYTIC HEXOSE  
MONOPHOSPHATE SHUNT ACTIVITY

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## AN *IN VITRO* MICRO-VOLUME PROCEDURE FOR RAPID MEASUREMENT OF ERYTHROCYTIC HEXOSE MONOPHOSPHATE SHUNT ACTIVITY

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**Abstract**—1. A radiometric micro-volume procedure for measurement of erythrocytic hexose monophosphate shunt (HMS) activity in intact cells *in vitro* is described.

2. The procedure is rapid, allowing 200 individual HMS determinations in a single experiment of 5 hr duration.

3. The procedure is reproducible, yielding HMS activity means insignificantly different ( $P > 0.05$ ) between replicate experiments.

4. A profile of sodium nitrite-induced HMS stimulation is reported: HMS was elevated 2-fold ( $P < 0.001$ ) between zero and 2.5 mM  $\text{NaNO}_2$ ; HMS elevation was more distinct (7-fold) between 2.5 and 5.0 mM  $\text{NaNO}_2$ ; maximum activity (22-fold) was observed between 10 and 20 mM  $\text{NaNO}_2$ ;  $>20$  mM  $\text{NaNO}_2$  caused significant ( $P < 0.001$ ) diminution of HMS; glucose carbon recycling through the HMS occurred only with  $>2.5$  mM  $\text{NaNO}_2$  where this process contributed  $\leq 16\%$  to total HMS activity.

### INTRODUCTION

In persons genetically deficient in erythrocytic glucose-6-phosphate dehydrogenase (G6PD), a hemolytic anemia is generally believed to result from the inability of the deficient erythrocyte to generate a reduction potential in response to oxidative challenge presented by certain drugs (Carson and Frischer, 1966; Beutler, 1978; Gordon-Smith, 1980); however, the mechanism by which oxidation may be related to hemolysis is unknown (Keller, 1971; Carson *et al.*, 1981). In this regard, the HMS is a likely subject of investigation because HMS-generated NADPH is required for reduction of glutathione, and reduced glutathione affords the primary protection against oxidative challenge in the erythrocyte (Keller, 1971; Srivastava *et al.*, 1980). The determination of HMS activity levels has recently been used by Kirkman *et al.* (1980) for the investigation of possible modes of metabolic regulation operating on the shunt, and by Carson *et al.* (1981) to differentiate at least two mechanisms through which HMS stimulation is achieved.

The methods used in these and similar investigations (Rose and O'Connell, 1963; Welt *et al.*, 1971; Gaetani *et al.*, 1974; Harvey and Kaneko, 1977) are modified versions of the procedure described by Brin and Yonemoto (1958). In these procedures, 2-4 ml of drug-erythrocyte-glucose isotope mixture is incubated in a sealed vessel containing a hydroxide trap from which HMS-liberated  $^{14}\text{CO}_2$  is quantitated.

The procedure we have developed also uses retrieval of HMS-generated  $^{14}\text{CO}_2$  but has been de-

signed to allow simultaneous measurement of several treatment groups with a high number of replicates. This design is illustrated in Fig. 1. The final incubation volume is  $50 \mu\text{l}$  and the  $^{14}\text{CO}_2$  trap seals the microtiter well which serves as the incubation vessel.

This report presents the results obtained using the micro-volume technique. The procedure appears to be somewhat inefficient in recovery of HMS-liberated  $^{14}\text{CO}_2$ , but it offers replicable, quantitative data which may be statistically analyzed with a high degree of confidence.

### MATERIALS AND METHODS

#### Buffers

A physiological balanced salts solution (PBSS) buffer was prepared by addition of the following chemicals (Sigma

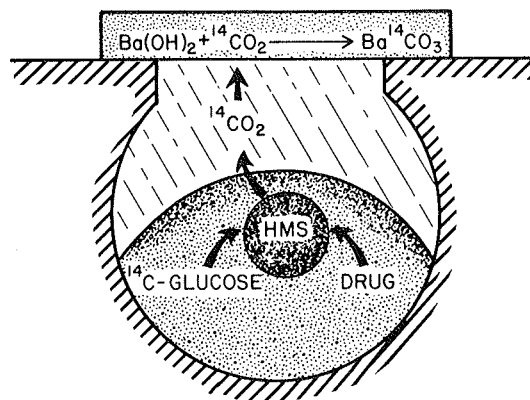


Fig. 1. Schematic representation of hexose monophosphate shunt (HMS) activity measurement by the micro-volume procedure. The incubation mixture is  $50 \mu\text{l}$  and the  $\text{Ba}(\text{OH})_2$  soaked  $\text{CO}_2$  trap is  $20 \text{ mm}^2$ . The  $\text{Ba}^{14}\text{CO}_3$  product precipitates in the filter pad and its emission is quantitated by gas flow spectrometry.

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Chemical Co., St Louis, Missouri, U.S.A.) to sterile distilled and deionized water (Travenol Laboratories, Deerfield, Illinois, U.S.A.) to the indicated final concentrations: NaCl, 112 mM; KCl, 5.4 mM; CaCl<sub>2</sub>, 1.26 mM; KH<sub>2</sub>PO<sub>4</sub>, 0.44 mM; MgSO<sub>4</sub>, 0.42 mM; Na<sub>2</sub>HPO<sub>4</sub>, 10.0 mM. The pH of this solution was adjusted to 7.4 with another solution prepared as above except that Na<sub>2</sub>HPO<sub>4</sub> was substituted with 10.0 mM NaH<sub>2</sub>PO<sub>4</sub>. The PBSS solution (500 ml) was sterilized by filtration through a Nalgene type LS sterilization filter unit (Sybron Corp., Rochester, New York, U.S.A.) and stored at 4°C.

A phosphate buffered salts (PBS) buffer (pH 7.4) was prepared as described above except for deletion of CaCl<sub>2</sub>, KCl and MgSO<sub>4</sub>.

#### Isotopes

D[1-<sup>14</sup>C]glucose and D[2-<sup>14</sup>C]glucose were obtained at 50 μCi/250 μl 2% ethanol in water (Amersham Corp., Arlington Heights, Illinois, U.S.A.). D[1-<sup>14</sup>C]glucose and D[2-<sup>14</sup>C]glucose had specific activities of 59.8 and 55 μCi/μmol, respectively. Isotope solutions were prepared in PBSS to yield 100,000 dpm per 25 μl and a final glucose concentration of 3.0 mM by addition of reagent grade D-glucose (Sigma Chemical Co., St Louis, Missouri, U.S.A.). Final specific activities were 0.60 and 0.55 μCi/μmol for D[1-<sup>14</sup>C]glucose and D[2-<sup>14</sup>C]glucose, respectively. Solutions were sterilized by membrane filtration (Gelman Acrodisc, disposable filter unit, 0.2 μm pore size, Gelman Sciences Inc., Ann Arbor, Michigan, U.S.A.), dispensed into sterile screw cap vials and stored at -25°C.

#### Reagents

Reagent grade sodium nitrite (Aldrich Chemical Co., Inc., Milwaukee, Wisconsin, U.S.A.) stock solution (250 mM) was prepared in PBSS. Solutions for tests were prepared by dilution of the stock in PBSS. Nitrite solutions older than 1 hr were not used.

Saturated barium hydroxide was prepared by addition of 250 g reagent grade anhydrous barium hydroxide (Fisher Scientific Co., Fair Lawn, New Jersey, U.S.A.) to approx. 300 ml autoclaved distilled and deionized water, cooled under vacuum. This mixture was gently shaken under vacuum for 10 min and then held under vacuum overnight before use. The saturated stock Ba(OH)<sub>2</sub> solution was maintained under vacuum to minimize exposure to atmospheric CO<sub>2</sub>.

#### Erythrocytes

Ten ml whole blood were withdrawn by venipuncture from a 24-year-old Caucasian male (with no family history of hemolytic sensitivity), added to 40 ml PBS (25°C) and centrifuged immediately at 4°C, 500 g for 10 min. The buffy coat was removed by aspiration prior to decanting the supernatant. The red cell pellet was resuspended in 40 ml PBSS (4°C) and again centrifuged as before. This centrifugal wash was repeated once more and the pellet plus PBSS was adjusted to a total volume of 10 ml. Without disturbing the red cell pellet, 500 mg human serum albumin (essentially globulin free, Fraction V, Sigma Chemical Co.) was allowed to dissolve into the supernatant. The preparation was mixed gently and kept at 0°C until used. Red cell preparations older than two hours were not used. Albumin was added to the incubation mixture as a component to aid in solubilization of hydrophobic HMS-stimulating agents used in related experiments.

#### Incubation

Twenty-five μl of <sup>14</sup>C-glucose solution were added to each well of a microtiter tray (Unipac Inc., Rockville, Maryland, U.S.A.). Sodium nitrite solution (12.5 μl) was added to these wells immediately before addition of red blood cells. For measurement of nonstimulated HMS activity, PBSS was substituted for the nitrite solution. A dropper bottle of

saturated Ba(OH)<sub>2</sub> solution and filter pads (circular, 20 mm<sup>2</sup>, Scheicher and Schull Inc., Keene, New Hampshire, U.S.A.) were prepared prior to addition of erythrocytes, to ensure rapid sealing of microtiter wells and to minimize air-exposure of the stock bottle of hydroxide reagent.

Red cell suspension (12.5 μl, 0°C) was added to each well of a replicate set of ten wells. One drop of Ba(OH)<sub>2</sub> was quickly added to each filter pad for the set and the soaked pads were immediately placed over the wells. Trays were sealed with friction-fit lids, shaken gently for about 10 sec and placed at 37°C under ambient atmosphere for 30 min.

#### HMS activity measure

Following incubation, filter pads were placed on aluminum planchettes (20 mm<sup>2</sup>) held in plastic sample storage trays and rapidly dried under infrared light. The emission rate from Ba<sup>14</sup>CO<sub>3</sub> on each pad was determined using an argon-methane (9:1) gas flow, α, β proportional spectrometer (LB 5110, Tennelec Inc., Oak Ridge, Tennessee, U.S.A.). The following equation was used for HMS activity calculation:

$$\begin{aligned} \text{dpm} \times \frac{1.0 \mu\text{Ci}}{2.22 \times 10^6 \text{ dpm}} \times \frac{1.0 \mu\text{mol glucose}}{n \mu\text{Ci}} \\ \times \frac{1000 \text{ nmol CO}_2}{\mu\text{mol glucose}} \times \frac{1}{0.0125 \text{ ml RBC}} \times \frac{1}{0.5 \text{ hr}} \\ = \text{nmol CO}_2 \text{ trapped} \cdot \text{ml RBC}^{-1} \cdot \text{hr}^{-1} \end{aligned}$$

Because 1 mol <sup>14</sup>CO<sub>2</sub> is released per mol <sup>14</sup>C-glucose oxidized, it is valid to express HMS activity in terms of glucose oxidized on the basis of recovered <sup>14</sup>CO<sub>2</sub> quantitation, but only if close to 100% recovery of <sup>14</sup>CO<sub>2</sub> has been demonstrated. As described herein, the micro-volume procedure does not permit complete recovery of <sup>14</sup>CO<sub>2</sub>; incubation was not terminated by addition of acid which would drive CO<sub>2</sub> from solution. It was found that the micro-volume procedure was 32% efficient in recovery of <sup>14</sup>CO<sub>2</sub> when acid treatment was omitted. While it is possible to include acid treatment in this procedure, doing so was found to decrease the rapidity of the procedure. We elected to omit acid treatment and to express HMS activity in terms of <sup>14</sup>CO<sub>2</sub> trapped rather than glucose oxidized.

The *n* μCi term in the equation depends on the specific activity of the isotope and its treatment with non-labeled substrate. In the experiments reported, *n* = 0.60 (D[1-<sup>14</sup>C]glucose) or 0.55 (D[2-<sup>14</sup>C]glucose).

#### Statistical analysis

Tests of significance of differences between two means were conducted using the analysis of variance (ANOVA) F-test as described by Sokal and Rohlf (1981). Tests of significance of differences among more than two means were conducted using the Student-Newman-Keuls (SNK) test. All ANOVA tests were carried out using a 95% degree of certainty and the statistical tables employed were those compiled by Rohlf and Sokal (1981).

## RESULTS

Stimulation of HMS activity from 0 to 80 mM NaNO<sub>2</sub> is shown in Fig. 2 where two activities are plotted. These plots represent activity measured from (1) D[1-<sup>14</sup>C]glucose incubation, and (2) D[2-<sup>14</sup>C]glucose incubation. Activity measurement using the 1-<sup>14</sup>C glucose isotope accounts for glucose molecules having made a single pass through the HMS and the 2-<sup>14</sup>C glucose isotope provides a measure of glucose carbon recycling through the HMS (Brin and Yonemoto, 1958). The sum of the two activity means was considered to represent total HMS activity.

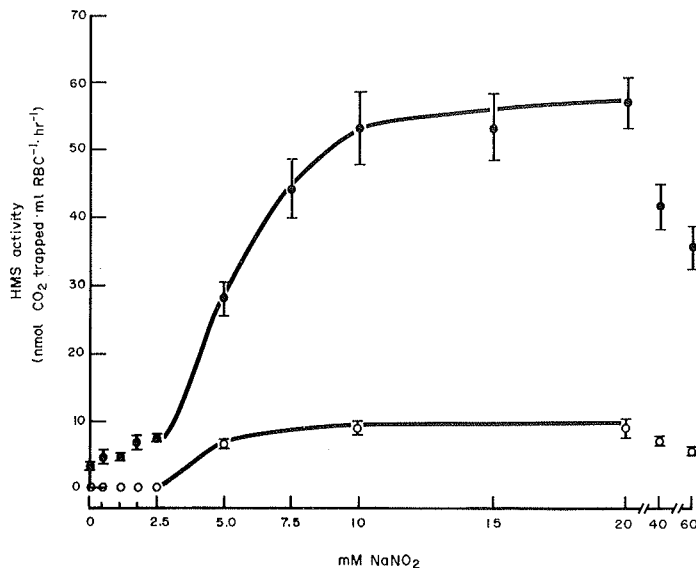


Fig. 2. Activation of red cell hexose monophosphate shunt by increasing concentrations of sodium nitrite. The two plots are those obtained from  $d[1-^{14}\text{C}]$ glucose incubation (●) or  $d[2-^{14}\text{C}]$ glucose incubation (○). Each data point is the mean of ten simultaneously obtained measurements which are bracketed with limits of the 95% confidence interval.

Total HMS activity was significantly greater ( $P < 0.001$ ) than activity measured from  $1-^{14}\text{C}$  glucose incubation alone at sodium nitrite concentrations greater than 5.0 mM. Below this concentration the two activities were insignificantly ( $P > 0.05$ ) different. At maximum HMS activity (20 mM  $\text{NaNO}_2$ ), glucose recycling accounted for 16% of total HMS activity.

Saturation of activity was observed between 10 and 20 mM  $\text{NaNO}_2$ , as evidenced by an insignificant ( $P > 0.10$ ) difference between the means. The 37% decrease in HMS activity between 20 and 80 mM  $\text{NaNO}_2$  was significant ( $P < 0.001$ ).

At the lowest nitrite concentration (0.625 mM), HMS activity was significantly greater ( $P < 0.001$ ) than control activity. Each activity increase between 0, 0.625, and 2.5 mM  $\text{NaNO}_2$  was significant ( $P < 0.001$ ).

This experiment was repeated on a separate occasion and HMS activities at several nitrite concentrations were compared by SNK and found to be uniformly similar ( $P > 0.05$ ). In both experiments mean coefficients of variation were calculated for each treatment group and found to range between 2 and 8%.

Statistical analysis of the means used to construct the HMS stimulation plot illustrated in Fig. 2 has provided a profile of red cell response to sodium nitrite-induced oxidative challenge. The following conclusions were drawn from these measurements:

(1) HMS activation induced by nitrite was sigmoid, i.e. acceleration of HMS activity was bimodal; between 0 and 2.5 mM  $\text{NaNO}_2$  the increase was linear at a rate of  $4.2 \times 10^{-3} \text{ mol CO}_2 \cdot \text{mol NaNO}_2^{-1} \cdot \text{hr}^{-1}$ . Between 2.5 and 5.0 mM  $\text{NaNO}_2$  the increase was linear at a rate of  $11.7 \times 10^{-3} \text{ mol CO}_2 \cdot \text{mole NaNO}_2^{-1} \cdot \text{hr}^{-1}$ .

(2) Nitrite treatment saturated HMS activity between 10 and 20 mM  $\text{NaNO}_2$  with an activity max-

imum of 57 units ( $\text{nmol CO}_2 \text{ trapped} \cdot \text{ml RBC}^{-1} \cdot \text{hr}^{-1}$ ). HMS activities at 10 and 20 mM  $\text{NaNO}_2$  were not significantly different.

(3) HMS activity means at concentrations greater than 20 mM  $\text{NaNO}_2$  were significantly diminished by as much as 37%.

(4) Nitrite-induced glucose carbon recycling through the HMS made a significant contribution to total HMS activity at nitrite concentrations greater than 2.5 mM  $\text{NaNO}_2$ . Below 5.0 mM  $\text{NaNO}_2$  recycling activity was not detectable.

Nitrite-induced glucose carbon recycling accounted for  $\leq 16\%$  of total HMS activity. Though this contribution was significant ( $P < 0.001$ ), it did not alter the shape of the HMS response curve. Activation was sigmoidal, saturated between 10 and 20 mM  $\text{NaNO}_2$ , and was diminished between 20 and 80 mM  $\text{NaNO}_2$  whether or not correction for recycling was made.

## DISCUSSION

The micro-volume procedure is a method in which a reproducible, highly resolved profile of oxidant-induced elevation of HMS activity can be obtained. The method also offers the advantages of requiring relatively minute quantities of blood and HMS stimulant samples.

The units of HMS activity employed herein are not directly comparable with unit values reported elsewhere (usually as  $\mu\text{mol glucose oxidized} \cdot \text{ml RBC}^{-1} \cdot \text{hr}^{-1}$ ). However, comparison of relative HMS activities reveals published values in good agreement with those obtained using the micro-volume procedure. For example, Methylene Blue-induced elevation of HMS activity has been reported as 51-fold (Gaetani *et al.*, 1974), 48-fold (Metz *et al.*, 1977), and 52-fold (Kirkman *et al.*, 1980), whereas the micro-volume procedure measured a 45-fold

elevation of HMS above baseline activity (Baird, 1984). Further agreement is evident among estimates of the Methylene Blue-induced glucose carbon recycling contribution to total HMS activity; 34% (Brin and Yonemoto, 1958), 27% (Harvey and Kaneko, 1977), 39% (Kirkman *et al.*, 1980), and 28% using the micro-volume procedure (data not given). These comparisons indicate that the indirect measure of HMS activity provided by this procedure is reliable.

The micro-volume procedure provides reproducible estimates of HMS activity with rapidity, allowing simultaneous measurement of a large number of experimental treatments with replication sufficient for statistical analysis of the data with a high degree of confidence. In this laboratory the micro-volume procedure has been employed to examine the relationship between HMS activation and the recently described proteolytic system in erythrocytes which is activated by oxidative stress (Goldberg and Boches, 1982). It has been found that sodium nitrite-induced proteolysis is sensitive to the level of HMS activity, whereas Methylene Blue may stimulate the HMS independently of proteolytic activity (Baird, 1984). These observations may be related to the fact that nitrite-induced activation of the HMS was distinctly sigmoidal (Fig. 2) and that induced by Methylene Blue was strongly hyperbolic (Kirkman *et al.*, 1980; Baird, 1984). The micro-volume procedure is well-suited to detecting such differences in HMS response.

This procedure also offers the advantage of requiring relatively minute quantities of blood and test oxidant. We have studied the oxidative activity of various putative primaquine metabolites in human erythrocytes *in vitro* (Baird *et al.*, 1983). These compounds are very limited in supply; reproducible estimates of their effects on the HMS were obtained at several concentrations with an expenditure of < 5 mg per compound.

#### SUMMARY

A procedure which permits simultaneous measurement of HMS activity in intact erythrocytes *in vitro* from 200 individual incubation mixtures in a single experiment is described. The incubation mixture (washed RBC, oxidant,  $^{14}\text{C}$ -glucose; 1:1:2) is 50  $\mu\text{l}$  contained in a microtiter well sealed with a  $\text{Ba}(\text{OH})_2$ -soaked paper filter pad which traps evolved  $^{14}\text{CO}_2$  with 32% efficiency. The emission rate from  $\text{Ba}^{14}\text{CO}_3$  precipitate is measured directly from the filter pad by a gas flow,  $\alpha$ ,  $\beta$  detector. The procedure has been used to define a highly resolved, reproducible profile of elevation of HMS activity with increasing  $\text{NaNO}_2$  concentration. The data given illustrate the information-gathering potential this method offers. The large number of replicates possible allow one to detect relatively small departures from normal HMS activity; e.g. elevation of erythrocytic HMS activity *in vivo* by the hemolytic antimalarial, primaquine, has been estimated to be  $\leq 0.20$ -fold above baseline (Welt *et al.*, 1971). The procedure may also be particularly useful when one is experimentally limited by the amounts of blood and/or test compound available.

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