Estimation of Pathways of Glucose Metabolism Employing [^{14}C]Glucose

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A method of quantitative estimation of the contribution of the pentose cycle and the Embden-Meyerhof pathway to erythrocyte carbohydrate metabolism employing [1-^{14}C]glucose and [6-^{14}C]glucose, is described. It has been found that the ratio of both pathways changes with time of incubation. The contribution of the pentose cycle to glucose metabolism was about 50% and below 10% after 1 hour and 3—5 hour incubation, respectively. Methylene blue (1.84×10^{-4} M) increased the contribution of the pentose cycle to glucose metabolism from 1.5 times to 7 times after 20 min und 3 hour incubation, respectively. In view of the inhibitory effect of ATP on glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49) the dependence of the contribution of the pentose cycle to glucose metabolism on the length of incubation may be related to the possible changes of erythrocyte ATP content during the separation and washing of these cells.

Introduction

Several aspects, *e. g.* the activity of glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: $NADP^+$ oxidoreductase EC 1.1.1.49) [25], the ratio $\frac{NADP^+}{NADPH}$ [10, 11], and the accumulation of ribose [8, 23 24], have been used to appreciate the contribution of the pentose cycle (PC) and the Embden-Meyerhof pathway (EMP) to glucose metabolism. Many investigators have attempted to evaluate PC using differently labelled types of [^{14}C] glucose but most of them have not attempted to evaluate PC in terms of the total metabolism of glucose * and have estimated only the relative roles of PC and EMP; they also failed to consider the effect of recycling in PC.

KATZ and WOOD [13, 15, 27] have proposed several methods for the evaluation of PC employing differently labelled types of [^{14}C] glucose. The methods can be divided into two groups: a) methods for estimation of the participation of PC in terms of total glucose metabolism and b) a method for estimation of the relative roles of PC and EMP. The method b) when used per se is valuable only in such a system involving only PC and EMP and has little or no value when used in a system involving NTP.**

All the methods have been derived on the basis of an assumed model system of glucose metabolism. The system involves the effect of recycling but has many theoretical limitations and the whole assumption may not be valid for every living system. Erythrocytes seem to fufil most of the assumptions made in this model [14]. It appears that PC and EMP are major metabolic pathways in erythrocytes. In this case the method b can be used. It is based on the assumption that EMP yields a mole of labelled triose phosphate (Tr-P) both from [1-^{14}C] glucose ([1-^{14}C] G) and from [6-^{14}C] glucose ([6-^{14}C] G) and that PC yields a mole of labelled Tr-P solely from [6-^{14}C] G. Expression (1) for calculation of PC has been derived:

$$PC = \frac{1 - [\frac{1}{6}]}{2 \cdot [\frac{1}{6}] + 1} \qquad (1)$$

where

$$[\tfrac{1}{6}] = \frac{^{14}\text{C yield in Tr-P from [1–}^{14}\text{C]G}}{^{14}\text{C yield in Tr-P from [6–}^{14}\text{C]G}}$$

It must be stressed again that the method is valid only when no glucose is metabolized via NTP. If NTP

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* Total metabolism of glucose: The sum of the nontriose-P pathways, the Embden-Meyerhof pathway, and the pentose cycle. It is assumed that triose phosphates are formed only by the Embden-Meyerhof pathway and the pentose cycle.

** Nontriose-P pathways (NTP): Conversion of glucose-6-phosphate into compounds without formation of triose phosphate or rearrangement of the 6-carbon skeleton. (Conversion to glycogen, lactose, glucosamine, pentoses, uronic acid, etc.).

contributes to glucose metabolism, the values of PC obtained by means of this method will be overestimated.

In practice, the ^{14}C yields are not measured in Tr-P but are determined in a suitable derivative of Tr-P. Lactate is such a suitable derivative for erythrocytes. It is the most important metabolic product of glucose metabolism since mature erythrocytes posses only some enzymes of the KREBS cycle and pyruvate cannot be oxidized to CO_2 [20]. If the relative specific activities of lactate formed from [1-^{14}C] G and [6-^{14}C] G are determined, their ratio and the contribution of PC to glucose metabolism can be calculated.

Methods

Separation of Erythrocytes

Blood was obtained from healthy donors. After having been collected in heparin, blood was centrifuged at 3000 *g* for 10 min in a cooled centrifuge at about 4 °C. The supernatant plasma and the layer of leucocytes were withdrawn and erythrocytes were washed twice by the same volume of modified Krebs buffer[5]. The standard erythrocyte suspension was obtained by diluting the mass of washed erythrocytes with buffer (v/v).

Estimation of Lactate Specific Activity

The specific activity of lactate in deproteinized samples of incubation medium was determined as the specific activity of ethylidene dimedon according to BRIN and OLSON [4]. Ethylidene dimedon was transferred into preweighed glass vials for ^{14}C assay and 10 ml of scintillation liquid (SLD-31, Spolana Neratovice; primary solute PBD, secondary solute POPOP, solvent dioxane) were added. Every sample was counted twice in an automatic liquid scintillation counter (MARK I, Nuclear Chigaco) and the mean value used for further calculation. Corrections for quenching were made by the External Standard Method. The specific activities were related to a milligram of formed ethylidene dimedon.

Results and Discussion

Estimation of Lactate Specific Activity

It is evident from Expression (1) that an error of the lactate specific activity estimation will influence the ratio of the specific activities and, thus, will also influence the reliability of PC estimation. Two experiments were carried out to test the reproducibility of the method.

Incubation medium in each 50 ml test tube contained 16 ml of standard erythrocyte suspension, 3 ml of modified Krebs buffer (pH 7.4) and 1 ml of standard solution of labelled glucose (0.4 μc; Amersham England). Final glucose concentration was 100 mg per 100 ml. Nine test tubes contained [1-^{14}C] G, and eight test tubes contained [6-^{14}C] G. Incubation was performed at 37 °C on the Dubnoff metabolic apparatus in the air for 5 hours; the pH did not change during the incubation period. All test tubes were shaken vigorously. At the end of the incubation, 8 test tubes with [1-^{14}C] G and 7 tubes with [6-^{14}C] G were deproteinized by adding 20 ml of 10 % trichloroacetic acid (TCA) to each test tube and the specific activities of lactate were estimated from the supernatants. Aliquot samples from the two remaining test tubes containing [1-^{14}C] G and [6-^{14}C] G were dropped into 5 ml of 10 % TCA containing carrier lactic acid (5 g/1 l) and the specific activities of lactate were estimated.

The values gained from both the experiments are similar. When the estimations without carrier were performed, the values of the coefficient of variation were 5.4 % (n=8) and 0.84 % (n=4) for [1-^{14}C] G, and 1.5 % (n=7) and 0.63 % (n=4) for [6-^{14}C] G as the substrate. When carrier lactate was used, the values were 0.96 % (n=9) and 0.64 % (n=9) for [1-^{14}C] G and 1.04 % (n=9) and 1.42 % (n=9) for [6-^{14}C] G as the substrate. The values obtained from these experiments suggest that the reproducibility of the method is quite acceptable and that the method can be taken as a rapid and reliable one for the estimation of PC. The modified method is, perhaps, better than the original one and has a great advantage requiring a smaller quantity of blood for analysis.

Estimation of PC

Five series of experiments were carried out to examine the reproducibility of this method. Methylene blue (MB), which has been reported to stimulate PC not only by rapid reoxidation of NADPH [5, 14] but also by increased reutilization of pentoses via transaldolase and transketolase reactions [7, 12, 18, 22], was present in several cases. The reaction mixture contained 4 ml of standard erythrocyte suspension, 0.5 ml of buffer, 0.25 ml of H_2O and 0.25 ml of standard solution

of [^{14}C] glucose (2 μc; final concentration of 100 mg per 100 ml). When MB was present 0.25 ml of its solution (final concentration of $9.2 \cdot 10^{-5}$ M) were added. Incubation was performed for 2 hours, under the conditions described above. Aliquots (2 ml) were deproteinized by 5 ml of 10 % TCA (with carrier lactate) and lactate specific activities were measured.

The results presented in Table I suggest that the reproducibility of PC estimation is satisfactory. Triplicates are in good agreement. Experiments No. 2 and No. 3 were performed with blood taken from the same person, within an interval of two months and do not differ substantially. Experiments No. 4 and No. 5 confirm earlier observations on the individual differences of erythrocyte PC values.

Table I. Estimation of the pentose cycle (two hour incubation).

No. of experiment	Person	Methylene blue 9.2×10^{-5} M	No. of parallel estimations	PC [%] mean value ± S.D.
1	A	no	3	9.9 ± 0.6
		yes	1	41
2	B	no	3	10.4 ± 1.1
		yes	1	53.6
* 3	B	no	3	10.0 ± 0.9
		yes	1	50.1
4	C	no	3	22.9 ± 2.2
5	D	no	3	5.9 ± 1.3

* Experiment No. 2 was repeated with the same person after two months period.

Effect of Length of Time of Incubation on PC

In several experiments the effect of the length of incubation time was tested. The incubation mixture and conditions were the same as described above. Aliquots were taken according to a time schedule up to 7 hours. Mean values of the lactate specific activities obtained from three parallel incubations were used for further calculations. The contributions of PC to glucose metabolism for a given length of incubation are presented in Fig. 1. These experiments were repeated with MB (final concentration of $1.84 \cdot 10^{-4}$ M) under the same conditions (Fig. 2).

The calculated contribution of PC to glucose metabolism obtained from long time incubation experiments, is conformable to the values obtained by other investigators. BRIN and YONEMOTO [5] studied the metabolism of [1-^{14}C] G, [2-^{14}C] G, and [6-^{14}C] G by human erythrocytes and the incorporation of labelling into lactate and CO_2 in the presence or absence of MB. Using their results KATZ [14] has calculated the contribution of PC to glucose metabolism – from 37

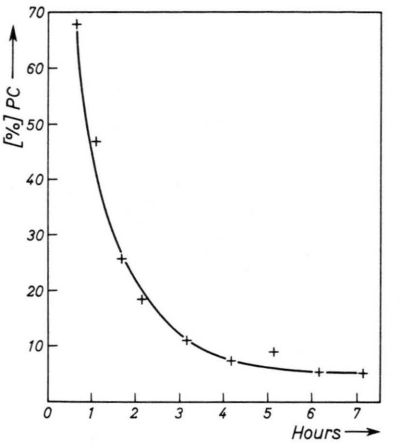

Fig. 1. Effect of length of incubation time on the pentose cycle (seven hour incubation).

% to 41 % when the dye was present and 7 % when absent. The influence of MB on $^{14}CO_2$ production from [1-^{14}C] G and [2-^{14}C] G by erythrocytes was also studied by SAGONE *et. al.*[21]. From their results the contribution of PC to glucose metabolism can be calculated – 5 % (MB absent) and 35 % (MB present). These values are in close agreement with those obtain-

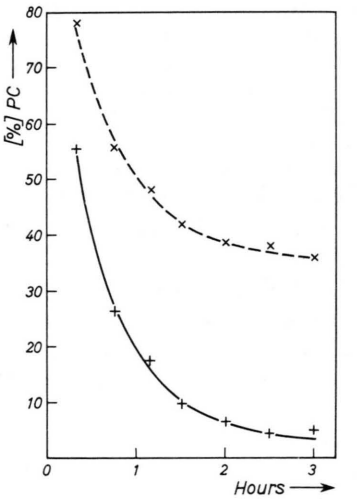

Fig. 2. Effect of length of incubation time and methylene blue on the pentose cycle (three hour incubation). —— + —— Control, – – – x – – – methylene blue 1.84 x 10^{-4} M.

ed from our experiments made under similar conditions – the contributions of PC to glucose metabolism were 36 % and 5 % when MB was present or absent, respectively.

The role of the time of incubation to the $\frac{PC}{EMP}$ ratio, independent of the presence of MB, is however surprising. The real decrease is, in fact, greater than it is possible to determine experimentally. As has been said, this method is based on the assumption that only EMP yields a mole of [^{14}C] lactate from [1-^{14}C] G but the ^{14}C yield in this lactate is lowered by recycling in PC. The higher the contribution of PC to glucose metabolism is, the lower the ^{14}C yield in lactate from [1-^{14}C] G will be. At the beginning of incubation, the contribution of PC to glucose metabolism is high, and low specific activity [^{14}C] lactate is formed via EMP. This low specific activity [^{14}C] lactate will *dilute* [^{14}C] lactate arising via EMP during the late incubation period, the specific activity of which is much greater since the contribution of PC to glucose metabolism, is low in the late incubation period and does not influence the specific activity of ^{14}C lactate arising via EMP from [^{14}C] G to such a great extent. This *dilution* of [^{14}C] lactate reduces the ratio [$\frac{1}{6}$] substituted into Expression (1) and the contribution of PC to glucose metabolism is overestimated.

The sharp decrease of PC participation in glucose metabolism during incubation may be due to enhanced oxidation of NADPH during the separation and washing of erythrocytes. NADP$^+$ serves as a hydrogen acceptor for glucose-6-phosphate dehydrogenase (G6PD) [2, 17, 20] and, hence, for the whole cycle. It may be assumed that at the beginning of incubation the concentration of NADP$^+$ is high and enables an enhanced activity of PC. NADP$^+$ concentration decreases with growing incubation time since NADP$^+$ is reduced to NADPH. This mechanism, however, cannot explain the action of MB. MB should maintain a high activity of G6PD [5, 14] and keep PC [7, 12, 18, 22] on a constant level during the whole incubation period. But it is evident from experiments with MB that the ratio $\frac{PC}{EMP}$ also varies with time of incubation.

On the other hand, ATP has been shown to inhibit G6PD [1, 3, 9, 16, 19]. It may be assumed that ATP is degraded during the separation and washing of erythrocytes, ATP depletion occurs and ATP dependent G6PD inhibition is abolished. Incubation with glucose leads to a restoration of erythrocyte ATP content and the depressed activity of G6PD reduces the PC contribution to normal values. This explanation is in accord with the view of BRAND *et. al.*[2] that ATP is the main factor controlling erythrocyte glucose metabolism. The problem of decreasing activity of PC during incubation will be further investigated from this point of view.

Estimation of PC in other tissues

Preliminary experiments were made with rat liver and heart slices using [1-^{14}C] G, [2-^{14}C] G and [6-^{14}C] G. Adult male albino rats (220–240 g; food and water ad lib.) were killed by decapitation. The incubation mixture contained 2.5 ml of buffer, 0.25 ml of glucose (final concentration of 360 mg per 100 ml) and 300–500 mg of pooled liver or heart slices. Incubations were performed for 3 hours under the conditions described above. From the values of [$\frac{2}{6}$] ratio the relative contributions of PC and EMP were determined and from the values of [$\frac{2}{1}$] ratio the contributions of PC to total glucose metabolism were determined using the curves derived from the values published by KATZ and WOOD [13]. The contributions of EMP to total glucose metabolism were also calculated. Results are presented in Table II. The values of PC in liver are comparable with the values calculated by KATZ [14] from the results published by other investigators.

Table II. Contribution of the pentose cycle to glucose metabolism by rat liver and heart slices (three hour incubation).

Tissue	PC/EMP + PC [$\frac{1}{6}$] [a]	[%] [$\frac{2}{6}$] [b]	Contribution to total glucose metabolism [%]		
			PC	EMP	NTP [c]
Liver	41	49	23	20	57
Heart	16	21	10	34	56

[a] Determined from [$\frac{1}{6}$] ratio. [b] Determined from [$\frac{2}{6}$] ratio. [c] Calculated by the difference.

The method described is not more laborious than methods employing $^{14}CO_2$ measured as Ba CO_3. The methods using $^{14}CO_2$ yields are valid only in tissues with no metabolism via NTP but even if this condition is observed the information obtained on glucose metabolic patterns depends on the Krebs cycle activity and its value is therefore limited [13, 27]. In tissues with additional metabolism via NTP these methods provide limited qualitative and no useful quan-

titative information on the pathways of glucose metabolism [13, 15, 27]. The method described here is independent of the activity of the K r e b s cycle and employing [2-^{14}C] G provides quantitative information on glucose metabolic patterns even when NTP is present [13, 14].

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