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A note on the assay of the contribution of the pentose phosphate pathway to glucose metabolism in human red cells

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When human red cell suspensions were incubated with [$^{14}C_1$] glucose a substantial fraction of the incorporated radioactivity was found in phosphate compounds. If some of these phosphates are precursors of $^{14}CO_2$ then an error is introduced into the conventional calculation of the proportion of glucose metabolised by the pentose phosphate pathway. The existence of this error was demonstrated and its magnitude was assessed by studying the effects of the subsequent addition of unlabelled glucose to the reaction mixtures in either the absence or the presence of methylene blue.

THE human red cell metabolises glucose by the glycolytic and pentose phosphate pathways. Mature erythrocytes lack certain enzymes of the tricarboxylic acid cycle (Prankerd, 1955; Murphy, 1960) and the oxidative reactions of the pentose phosphate pathway appear to be the sole mechanism for the conversion of glucose carbons to CO_2. The relative contribution of this pathway to glucose metabolism has been assessed by measuring the evolution of $^{14}CO_2$ from red cells incubated with [$^{14}C_1$] glucose (De Loecker & Prankerd, 1961). The use of radioactive glucose, labelled in the 1 position only, avoids difficulties arising from the possible recycling of hexose phosphates (Murphy, 1960). The ratio:

$$\frac{\text{Radioactivity in evolved } ^{14}CO_2}{\text{Radioactivity lost from } [^{14}C_1] \text{ glucose}}$$

is the basis of calculations performed to derive the proportion of glucose metabolised by the pentose phosphate pathway (De Loecker, 1964). It is assumed that the radioactivity lost from the [$^{14}C_1$] glucose and metabolised by the pentose phosphate pathway has all been recovered as $^{14}CO_2$. The validity of this assumption is questionable since under the experimental conditions used by many investigators (De Loecker & Prankerd, 1961; Bonsignore, Fornaini, Segni, Leoncini & Chieffi, 1963) variable amounts of radioactivity may remain in certain intermediary compounds such as 6-phosphogluconate and glucose-6-phosphate, which are precursors of $^{14}CO_2$. Retention of radiocarbon in these intermediates at the end of the incubation would be expected to yield low values for the percentages of glucose metabolised by the pentose phosphate pathway. The following experiments were performed to assess the magnitude of this error under defined conditions.

Experimental

White cells were removed from defibrinated human blood by filtration through cotton wool and washing three times with a solution consisting of seven parts of 0·15 M sodium chloride and three parts of 0·1 M phosphate

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buffer, pH 7·4 (Buchanan, 1960). The remaining red cells were resuspended in an equal volume of the above medium, which was also used to prepare all other solutions. The incubations were at 37° in stoppered Warburg flasks with two side arms, one of which contained 0·5 ml of 50% w/v trichloroacetic acid and the other contained quantities of unlabelled glucose, ranging from 0–70 μmoles, dissolved in 0·5 ml of medium; the centre well contained 0·2 ml of 20% w/v potassium hydroxide. Red cell suspension (2 ml) was added to the main compartment of each flask followed by 1 ml of medium containing [$^{14}C_1$] glucose (1 μc, 0·03 μmole) and sufficient methylene blue, when present, to give a final concentration of 1 mM. After an initial incubation period of 2 hr the unlabelled glucose was added from one side arm and the incubation continued for a further 2 hr. The trichloroacetic acid was then added from the other side arm and the incubation continued for an additional hour to ensure that all the liberated $^{14}CO_2$ was absorbed in the centre well. Glucose, lactic acid and phosphate compounds were separated by chromatography on Whatman No. 4 paper using n-butanol: propionic acid: water (3:2:2) and radioactive compounds were located by a Nuclear-Chicago Actigraph scanner. The amounts of [$^{14}C_1$] glucose initially present were measured in corresponding mixtures to which the trichloracetic acid was added at zero time. No radioactive glucose was detected in the incubation mixtures at the end of the experiments. The $^{14}CO_2$ liberated from the cell suspensions was absorbed in potassium hydroxide and portions of the resulting solution dried on Whatman No. 4 paper. All radioactive counting was performed directly on paper with a Packard Tri-Carb liquid scintillation counter, using as phosphor 15 ml of 0·4% 2,5-diphenyloxazole and 0·01% 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene in toluene.

Results and discussion

The results (Table 1) show that when the red cells were incubated with [$^{14}C_1$] glucose for 4 hr, the incorporated radioactivity was distributed

TABLE 1. EFFECT OF THE SUBSEQUENT ADDITION OF UNLABELLED GLUCOSE ON THE DISTRIBUTION OF ^{14}C FROM [$^{14}C_1$] GLUCOSE INTO PHOSPHATES, LACTIC ACID AND CARBON DIOXIDE OF HUMAN RED CELL SUSPENSIONS
The results represent the means of four experiments ± standard deviations and are expressed as the radioactivity in each fraction calculated as a percentage of that lost from the [$^{14}C_1$] glucose.

Unlabelled glucose added (μmoles)	Phosphates	Lactic acid	Carbon dioxide
0	61·6 ± 0·9	13·1 ± 0·7	14·3 ± 0·2
0·7	36·8 ± 0·8	27·4 ± 0·6	14·9 ± 0·3
7	24·3 ± 1·0	40·2 ± 0·9	16·6 ± 0·3
35	16·6 ± 0·9	49·5 ± 0·5	17·8 ± 0·2
70	16·4 ± 0·9	50·7 ± 0·6	18·1 ± 0·3
In presence of methylene blue			
0	16·5 ± 0·8	3·3 ± 0·1	63·4 ± 0·3
0·7	10·2 ± 0·5	4·8 ± 0·1	65·9 ± 0·4
7	3·1 ± 0·2	5·5 ± 0·4	73·3 ± 0·7
35	2·5 ± 0·2	6·8 ± 0·3	76·8 ± 0·7
70	2·3 ± 0·2	6·8 ± 0·3	76·6 ± 0·3

between intermediate phosphate compounds and metabolic end products, lactic acid and carbon bioxide. If some of the labelled phosphates were precursors of $^{14}CO_2$ then the conventional method (cf. Murphy, 1960) for the calculation of the proportion of glucose metabolised by the pentose phosphate pathway must be in error. This hypothesis was tested by adding increasing amounts of unlabelled glucose to the incubation mixtures after 2 hr and continuing the incubation for a further 2 hr. Marked changes in the distribution of the radiocarbon from the [$^{14}C_1$] glucose between the various fractions were observed. More isotope appeared in lactic acid and carbon dioxide and less in the phosphates. Thus the proportion of [$^{14}C_1$] glucose converted to $^{14}CO_2$ was increased up to 25% by the addition of the unlabelled glucose. The amount of $^{14}CO_2$ liberated from red cells incubated with concentrations of [$^{14}C_1$] glucose such that all the labelled glucose is utilised during the experiments (Bonsignore & others, 1963) is, therefore, not a reliable measure of the quantity of sugar metabolised by the pentose phosphate pathway. The present results show that the magnitude of this error may be diminished by adding unlabelled glucose to the incubation mixtures, but it will be affected by variation in experimental conditions such as the time of incubation and the glucose concentrations present at the beginning of the experiments.

In the presence of methylene blue, added as an electron acceptor to facilitate the oxidation of $NADPH_2$ by molecular oxygen (Brin & Yonemoto, 1958), the distribution of radioactivity from the [$^{14}C_1$] glucose was found to alter in a manner consistent with a higher proportion of the radioactive glucose being metabolised by the pentose phosphate pathway (Table 1). Nevertheless, the addition of unlabelled glucose again increased the evolution of $^{14}CO_2$ and decreased the retention of radiocarbon in phosphates. The magnitude of this effect was similar to that found in the absence of the dye.

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