EVIDENCE FOR THE EXISTENCE AND FUNCTIONAL ACTIVITY OF THE PENTOSE PHOSPHATE PATHWAY IN THE LARGE PARTICLE FRACTION ISOLATED FROM RAT TISSUES

Najma Zaheer Baquer and Patricia McLean

Courtauld Institute of Biochemistry, The Middlesex Hospital Medical School, London, W1P 5PR, Great Britain.

Received October 21, 1971

SUMMARY: Evidence is presented to show that all the enzymes of the pentose phosphate pathway are present in the 12,000*g* particulate fraction in a wide range of tissues. That these are constituent and functional is suggested by the findings that: (1) significant activity of the entire sequence of enzymes could be revealed after treatment of the washed particulate fraction by freezing and thawing and Triton X-100 or by mechanical disruption in phosphate buffer although no such activity could be detected in untreated particles; (2) the relative rates of $^{14}CO_2$ production from glucose labelled on C-1, C-2 or C-6 were consistent with the operation of the cycle; (3) accumulation of intermediates of the pathway occurred when the liver large particle fraction was incubated in a fortified medium. The tissues studied were rat liver, ethionine-induced hepatoma, brain, adrenal gland, ovary and uterus.

Early studies with animal tissues led to the view that the pentose phosphate pathway was largely confined to the cytosol [1,2]. More recently evidence has accumulated pointing to an association of the oxidative enzymes of this cycle with particulate fractions from brain [3,4], liver [5-7] and muscle [8]. Zaheer *et al.* [5] showed that, under appropriate treatment e.g. with Triton X-100, the activity of G6P dehydrogenase and 6PG dehydrogenase exposed in these particles constituted a significant proportion of the activity in the supernatant fraction. The question arises as to whether the entire sequence of enzymes of the pentose phosphate pathway are to be found in association with the particulate fraction of the cell and the extent of the functional activity in different tissues.

The finding that freezing and thawing and treatment with Triton X-100 could reveal activity of all the enzymes of the pentose phosphate pathway in the LPF, as much as 24% of the cytosolic activity, could have significance in two respects. Firstly, in relation to NADPH-requiring reactions, e.g. steroid synthesis, which occur in mitochondria of liver, adrenal gland and placenta [9,10] and of chain elongation of fatty acids which occur in liver mitochondria [11]; secondly, in relation to the formation of mitochondrial RNA [12].

Abbreviations: G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; R5P, ribose 5-phosphate; Ru5P, ribulose-5-phosphate; LPF, large particle fraction sedimenting at 120,000*g*.mins.

METHODS

Preparation of cell fractions and exposure and solubilisation of enzymes

Adult female virgin rats of the albino strain were used. The tissues were homogenised and fractionated by previously established methods [5,13,14]. The large particulate fraction sedimenting at 120,000*g*.mins. (LPF) was washed four times to remove, as far as possible, contaminating cytosolic enzymes. In the freshly-prepared, washed LPF the enzymes of the pentose phosphate pathway were too low to be detected and were certainly less than 1% of the soluble fraction activity. The characteristics of this liver fraction, the biochemical parameters and electron microscopy, have already been reported [15]. Exposure and solubilisation of the 'latent' enzymes was effected by freezing and thawing and subsequent treatment with Triton X-100 (Sigma) used in a final concentration of 0.5—1.0% [5].

In order to compare the extractability of the dehydrogenases of the pentose phosphate pathway with that of other mitochondrial enzymes such as glutamate dehydrogenase and the transaminases, the fractional extraction procedures of Klingenberg [16] were used with the LPF prepared from rat liver and brain. Three fractions were obtained: (I) that extracted on incubation with 0.1M phosphate buffer, pH 7.2, the readily soluble fraction; (II) that extracted after mechanical breakage with an Ultraturrax homogeniser (2 mins), the soluble fraction and (III) the residue from the Ultraturrax extraction treated with Triton X-100 (1% final concentration for 1—2 hours), the difficultly soluble fraction. The dehydrogenases of the pentose phosphate pathway appeared in all three fractions.

Estimation of the enzymes and intermediates of the pentose phosphate pathway

The enzymes of the pentose phosphate pathway were measured as previously described [1,17]. Some difficulties were experienced in measuring the low activities of the non-oxidative enzymes of the pathway in fresh and in frozen and thawed particles because of the high rate of oxidation of NADH; corrections for this were made with appropriate blank values. When particles were frozen and thawed and then treated with Triton X-100, the supernatant obtained after these procedures was virtually devoid of NADH-oxidase activity. Metabolites were estimated as previously described [18].

RESULTS

The relative specific activities of enzymes of the pentose phosphate pathway in the cytosol and the LPF treated with Triton X-100 are shown in Table 1. Freshly-prepared, untreated LPF show very low, or negligible, activities; some activity is revealed by freezing and thawing and this is further increased by exposure to Triton X-100. Three effects of Triton X-100

Table 1. The specific activities of enzymes of the pentose phosphate pathway in the cytosol and Triton X-100-treated particulate fraction from liver, ethionine-induced hepatoma, brain, adrenal glands, ovary and uterus.

Tissue	G6P dehydrogenase	6PG dehydrogenase	R5P isomerase	Ru5P epimerase	Trans-ketolase	Trans-aldolase
			milliunits/mg. protein			
Cytosolic fraction						
Liver	19± 2	40± 2	32± 3	100±12	18±1	16± 2
Hepatoma	96±24	90±12	63±10	332±14	34±5	56±10
Brain	13± 2	10± 2	11± 1	28± 3	8±2	11± 3
Adrenal	89±16	80±13	60±10	528±74	10±2	21± 5
Ovary	24± 1	10± 0.3	30± 1	146± 2	4±0.4	6± 0.1
Uterus	20± 3	11± 3	38± 4	136±15	3±0.1	7± 0.5
LPF, treated with Triton X-100						
Liver	5.2±0.6	4.7±0.2	5.7±0.7	8.3±1.4	5.3±0.4	6.0±0.3
Hepatoma	32.0±5.0	18.4±3.5	21.4±3.9	23.9±4.8	17.1±2.2	24.0±3.5
Brain	8.9±1.3	5.5±1.5	5.8±1.2	7.9±0.7	5.6±1.5	5.2±1.5
Adrenal	12.3±0.8	2.9±0.1	5.3±0.2	21.7±0.7	4.2±0.2	4.7±0.2
Ovary	15.2±0.3	2.4±0.3	6.6±0.2	26.2±1.3	4.6±0.2	6.8±0.3
Uterus	2.5±0.5	1.7±0.3	4.8±0.4	10.5±0.8	2.2±0.4	2.8±0.8

The cell fractions were prepared as described in Methods. The protein contents of liver, hepatoma, brain, adrenal, ovary and uterus in the cytosol from 1g of tissue were: 102±8; 50±1.4; 84±5; 74±5; 56±3; 42±5 mg respectively. The corresponding figures for the LPF treated with Triton X-100 were 28±6; 7±1; 22±4; 16±0.5; 7.3±0.2; 14±1 mg in the fraction derived from 1 g tissue. Protein was estimated by the method of Lowry *et al.* [25]. The enzyme content of fresh mitochondria was in most cases too low to measure. Each value is the mean ± SEM of six determinations.

must be considered: (i) the effect of facilitating access of enzymes and substrate; (ii) the effect of decreasing NADH-oxidase activity, which causes marked interference with the assay of the non-oxidative enzymes; (iii) the possible inhibitory effects of Triton X-100 on these enzymes, particularly at low protein concentration.

While no absolute quantitative significance may be given to the activities of the enzymes revealed in the LPF by Triton X-100, nevertheless it may be noted that there is a marked uniformity in the specific activities of the 'latent' enzymes released in a wide variety of tissues despite considerable variations in the corresponding cytosolic values. The LPF from the hepatomas was somewhat exceptional in this respect and contained significantly more of

all six enzymes measured than did the same fraction from the other tissues examined.

The extractability of the oxidative enzymes of the pentose phosphate pathway (studied by the procedure of Klingenberg [16]), was compared with that of the marker enzymes glutamate dehydrogenase (GLDH), glutamate-oxaloacetate transaminase (GOT) and isocitrate dehydrogenase (ICDH). G6P and 6PG dehydrogenases appeared in all three fractions (*ca.* 20% in the readily soluble fraction and the remainder distributed in fractions II and III). ICDH showed a parallel distribution while GLDH, GOT and MDH were largely located (80—90%) in fraction II. While it is possible that the readily-extractable G6P dehydrogenase and 6PG dehydrogenase (fraction I) represents enzyme loosely bound to the particles which could be derived from the soluble fraction by an artefact of the isolation and extraction procedures, it seems less likely that the fraction of enzyme activity found only after mechanical disruption falls into this category and it is therefore possible that this fraction of the activity represents constituent enzymes of the LPF.

Table 2. The oxidation of specifically labelled glucose by rat liver 12,000*g* particulate fraction and the activities of oxidative enzymes of the pentose phosphate pathway.

	12,000*g* large particle fraction	
	Fresh	Treated with Triton X-100
	mμmoles product/min at 37°/LPF from 1 g liver	
Conversion of ^{14}C-glucose into $^{14}CO_2$		
[1-^{14}C]glucose	17 ± 2.2	80 ± 22
[6-^{14}C]glucose	<1	<1
Activity of oxidative enzymes		
G6P dehydrogenase	<20	290 ± 30
6PG dehydrogenase	<20	133 ± 4.0

Mitochondria were incubated with 4.5 ml Krebs—Ringer bicarbonate medium containing: ATP, 25 μmoles; sodium succinate, 25 μmoles; $NADP^+$, 2 μmoles; glucose, 50 μmoles; NaF, 90 μmoles; phenazine methosulphate, 0.5 μmole; 1 unit of yeast hexokinase (Boehringer) and 1 ml of LPF suspension or extract equivalent to the amount derived from 1 g liver. The results of six values are given as means ± SEM.

The functional activity of the pentose phosphate pathway in the LPF was tested by two further procedures. Firstly, the rate of formation of $^{14}CO_2$ from [1-^{14}C]glucose in the presence of phenazine methosulphate was used to assess the activity of the two oxidative reactions of the pentose phosphate pathway, correction being made for the very low rate of oxidation of carbon-6 of glucose (Table 2). This rate approximated to that of 6PG dehydrogenase activity in the Triton X-100-treated LPF. Evidence for the functioning of the non-oxidative reactions of the pathway was obtained from studies on the rate of formation of $^{14}CO_2$ from [2-^{14}]glucose on the basis that $^{14}CO_2$ is liberated from the second carbon of glucose only after complete recycling in the pentose phosphate cycle had occurred. The observation that the rate of $^{14}CO_2$ formation from carbon-2 of glucose was approximately half that from carbon-1 and significantly greater than from carbon-6 is in accord with a functional pentose phosphate cycle.

Secondly, the accumulation of intermediates of the pentose phosphate pathway in the LPF from mouse liver, when incubated in a supplemented medium,

Table 3. The accumulation of metabolites of the pentose phosphate pathway during incubation of mouse liver 12,000*g* particulate fraction with glucose.

	Metabolite content		Fisher's *P*
	Glucose absent	Glucose present	
	mμmoles/LPF from 1 g liver		
Glucose 6-phosphate	1.59 ± 0.61	4.76 ± 0.34	0.03
6-Phosphogluconate	1.01 ± 0.22	1.97 ± 0.32	0.01
Pentose phosphate	16.3 ± 5.9	44.7 ± 1.2	<0.01
Sedoheptulose 7-phosphate	1.23 ± 0.24	3.23 ± 0.39	0.01
Triose phosphate	16.2 ± 4.2	25.8 ± 4.4	0.04

The incubation medium containing 4.0 ml Krebs–Ringer bicarbonate medium, 90 μmoles NaF, 25 μmoles sodium succinate, 100 μmoles of glucose and LPF fraction from mouse liver (the yield from 2g liver was used in each flask), final volume 5.0 ml, gas phase O_2:CO_2 95/5; incubation time 30 min at 37°. The reaction was stopped and protein removed by addition of cold 0.5 ml 2*N* $HClO_4$. The metabolites were estimated as previously described [18]. The results of 6 values are given as the means ± SEM. Fisher's *P* values are given for corresponding pairs of results obtained on the same LPF with and without glucose.

including glucose, provided additional supporting evidence (Table 3). Mouse liver particles were used for this experiment because, in contrast to rat liver, they have an appreciable particulate-bound form of hexokinase [19]. The LPF incubated in the supplemented medium, without added glucose, accumulated measureable amounts of all the intermediate phosphorylated derivatives of the pathway with the exception of erythrose-4-phosphate. When glucose was added to the medium, there was a significant increase in content of all the intermediates, although erythrose-4-phosphate still remained below the limits of the sensitivity of the assay system. Significance appeared to attach to the increase in the presence of glucose rather than to the absolute values of the sugar phosphates. An extension of this approach was the demonstration that, when Triton X-100-treated particulate fraction was incubated with R5P there was a rise in Ru5P, sedoheptulose-7-phosphate and hexose monophosphate, a sequence of change similar to that found by Horecker *et al.* [20].

DISCUSSION

Compelling evidence exists for the occurrence of 'latent mitochondrial' forms of G6P dehydrogenase and 6PG dehydrogenase in liver [5] and rabbit muscle [8]. It has been shown that the cytosolic and particulate forms of the two enzymes in liver differ from each other in kinetic properties and in reaction to antisera prepared using the cytosolic enzymes [5]. Differences between cytosolic and mitochondrial forms of G6P dehydrogenase have also been demonstrated by gel electrophoresis [6,7]. Mandula *et al.* [21] have described a microsomal-linked hexose 6-phosphate dehydrogenase which reacts with both NAD and NADP. It seems unlikely that this enzyme accounts for the activity in the LPF in the present experiments, firstly because of the differences in centrifugal speeds used; secondly because the G6P dehydrogenase of the LPF reacts only with NADP and not with NAD [5] and thirdly, because the microsomal enzyme was absent from brain and mammary gland, two tissues having a significant content of 'latent' G6P dehydrogenase in the LPF.

The present experiments extend the work of Zaheer *et al.* [5] to the non-oxidative enzymes of the pentose phosphate pathway and present preliminary evidence which suggests that the entire sequence of enzymes of the pathway may be present in the LPF of a wide range of tissues. The association of enzymes of the pentose phosphate pathway with membrane fractions is illustrated by the work of Lionetti & Fortier [22] with erythrocyte stroma.

Mitochondrial RNA synthesis is now well-established [12] and in this context it may be noted that the ethionine-induced hepatoma has an exceptionally high specific activity of enzymes of the pentose phosphate pathway associated with the LPF, moreover the LPF from mammary glands of pregnant rats

also shows a high specific activity of the enzymes of this pathway [23]. Both these observations are consistent with the high rates of nucleic acid synthesis found in these tissues.

A second possible role for the pentose phosphate pathway in the LPF may reside in the formation of NADPH. Simpson and Estabrook [24] have suggested that 'malic' enzyme may be of importance in certain of the reductive steps of steroid synthesis. G6P and 6PG dehydrogenases have activities comparable to the 'malic' enzyme of the LPF but both are markedly less active than the NADP-linked isocitrate dehydrogenase.

While it is difficult to assign functional significance to the enzymes of the pentose phosphate pathway in the LPF until a wider range of tissues under different physiological conditions have been studied, the high levels found in adrenals, ovaries and hepatoma may suggest a rôle in both steroid and nucleic acid synthesis.

This work was supported by a grant from the Wellcome Trust; we also wish to acknowledge the Medical Research Council for provision of the SP800 recording spectrophotometer.

REFERENCES

1. Glock, G.E., and McLean, P., *Biochem.J.* 55, 400 (1953).
2. Newburgh, R.W., and Cheldelin, V.H., *J.Biol.Chem.* 206, 625 (1954).
3. Yamada, K., and Schimazono, N., *J.Biochem.* 51, 242 (1962).
4. Bagdasarian, G., and Hulanicka, D., *Biochim.Biophys.Acta* 99, 367 (1965).
5. Zaheer, N., Tewari, K.K., and Krishnan, P.S., *Arch.Biochem.Biophys.* 120, 22 (1967).
6. Hori, S.H., Matsui, S.-I., *J.Histochem.Cytochem.* 16, 62 (1968).
7. Shatton, J.B., Halver, J.E., and Weinhouse, S., *J.Biol.Chem.* 246, 4878 (1971).
8. Razumovskaya, N.I., Pleskov, V.M., and Perova, T.L., *Biochemistry (USSR) (English Transl.)* 35, 162 (1970).
9. Billiar, R.B., and Little, B., *Biochim.Biophys.Acta* 187, 243 (1969).
10. Simpson, E., and Estabrook, R.W., *Advances in Enzyme Regulation* 7, 259 (1969)
11. Quagliariello, E., Landriscina, C., and Coratelli, P., *Biochim.Biophys. Acta* 164, 12 (1968).
12. Saccone, C., Gadaleta, M.N., and Gallerani, R., in *Atti del Seminario di Studi Biologici* (Ed. E.Quagliariello) Vol.IV p.43 (Adriatica Editrice, Bari, 1969).
13. Gorski, J., and Nicolette, J.A., *Arch.Biochem.Biophys.* 103, 418 (1963).
14. McKerns, K.W., in *Functions of the Adrenal Cortex* (Ed. K.W.McKerns) p.948 (North-Holland Publishing Company, 1968).
15. Zaheer, N., Tewari, K.K., and Krishnan, P.S., *Arch.Biochem.Biophys.* 109, 646 (1965).
16. Klingenberg, M., in *Methods in Enzymology* (Ed. R.W.Estabrook and M.E. Pullman) Vol.X p.94 (Academic Press, New York, 1967).
17. Novello, F., and McLean, P., *Biochem.J.* 107, 775 (1968).
18. Gumaa, K.A., and McLean, P., *Biochem.J.* 115, 1009 (1969).
19. Rose, I.A., and Warms, J.V.B., *J.Biol.Chem.* 242, 1635 (1967).
20. Horecker, B.L., Gibbs, M., Klenow, H., and Smyrniotis, P.Z., *J.Biol.Chem.* 207, 393 (1954).

21. Mandula, B., Srivastava, S.K., and Beutler, E., *Arch.Biochem.Biophys.* 141, 155 (1970).
22. Lionetti, F.J., and Fortier, N.L., *Arch.Biochem.Biophys.* 103, 15 (1963).
23. Baquer, N.Z., and McLean, P., unpublished observations.
24. Simpson, E.R., and Estabrook, R.W., *Arch.Biochem.Biophys.* 129, 384 (1969).
25. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., *J.Biol.Chem.* 193, 265 (1951).