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Catalysis of Pentose Phosphate Pathway Reactions by Cytoplasmic Fractions from Muscle, Uterus and Liver of the Rat, and the Presence of a Reduced Nicotinamide–Adenine Dinucleotide Phosphate–Triose Phosphate Oxidoreductase in Rat Muscle

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1. The enzymes of the pentose phosphate pathway were assayed in supernatant fractions from rat muscle, liver and uterus. 2. On incubation of ribose 5-phosphate with uterus and liver supernatants, triose phosphate, sedoheptulose 7-phosphate and hexose monophosphate accumulated. 3. When a muscle supernatant was used, glycerol 3-phosphate instead of triose phosphate appeared and there was a negligible accumulation of hexose monophosphate. 4. Hexose monophosphate production from ribose 5-phosphate was also followed by measuring $NADP^+$ reduction in the presence of an excess of phosphoglucose isomerase, glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. 5. With a muscle supernatant, NADPH was reoxidized as rapidly as it was formed owing to the presence of a NADPH–triose phosphate oxidoreductase. 6. A modification of the pentose phosphate pathway in skeletal muscle incorporating this enzyme is proposed.

As part of an investigation of the pentose phosphate pathway in smooth and striated muscle, the activities of the enzymes of the pathway in a cytoplasmic fraction from rat skeletal muscle, liver and uterus were measured and compared with the abilities of fractions from these tissues to convert ribose 5-phosphate into hexose monophosphate. Discrepancies between the nature and amounts of the intermediates that accumulated and those expected on the basis of the pathway as usually formulated, indicated the presence of a NADPH–triose phosphate oxidoreductase in rat skeletal muscle and suggested that a modified pathway may be operative in this tissue.

Materials and Methods

Materials

Sugar and triose phosphates, coenzymes and enzymes were obtained either from Sigma Chemical Co., St. Louis, Mo., U.S.A., or from Boehringer Mannheim Corp., New York, N.Y., U.S.A. Diaflo PM-30 membranes were from Amicon Corp., Cambridge, Mass., U.S.A. D-Erythrose 4-phosphate was prepared by oxidation of D-glucose 6-phosphate and precipitated as the barium salt of the hydrazone (Sieben *et al.*, 1966). D-Xylulose 5-phosphate and a mixture of D-ribose 5-phosphate and D-ribulose 5-phosphate were prepared as described previously (Kiely *et al.*, 1973).

Preparation of tissue extracts

Adult Wistar rats of body weight 250–360g were used. Water and Purina rat chow (Ralston Purina of Canada, Woodstock, Ont., Canada) were allowed *ad lib.* Skeletal muscle was taken from male rats, and uterus and liver from virgin female rats. Muscle and liver were homogenized in 4ml of ice-cold 50mM-triethanolamine–HCl buffer, (pH7.4)/g in the 50ml stainless-steel container of a Lourdes Multi-Mixer (Lourdes Instrument Corp., Old Bethpage, N.Y., U.S.A.) cooled in ice. Uterus was homogenized in the same buffer at 5°C by using the Eberbach micro-container attachment to a Waring Blendor (Eberbach Corp., Ann Arbor, Mich., U.S.A.). Homogenates were subsequently centrifuged for 2h at 100000*g* at 4°C in a Spinco model L preparative ultracentrifuge and the clear supernatants were collected.

Enzyme assays

All assays were performed at 37°C, pH7.4, in a final volume of 2.00ml with a 1cm-light-path cell. Absorbance changes were measured with a Beckman DB spectrophotometer connected to a Varicord 43 linear–log recorder (Photovolt Corp., New York, N.Y., U.S.A.). Enzyme activities were calculated in

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units/g of tissue as the mean ±S.E.M. for six animals. D-Glucose 6-phosphate dehydrogenase (EC 1.1.1.49) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) were measured as described by Glock & McLean (1953) and Shonk & Boxer (1964) respectively. D-Ribose 5-phosphate ketol-isomerase (EC 5.3.1.6) and D-ribulose 5-phosphate 3-epimerase (EC 5.1.3.1) were assayed at 290nm by previously published procedures (Wood, 1970; Kiely *et al.*, 1973). Transketolase (EC 2.2.1.1) and transaldolase (EC 2.2.1.2) were assayed as described previously (Tan & Wood, 1969).

NADPH–triose phosphate oxidoreductase activity. This was measured in 50mM-triethanolamine–HCl buffer, pH7.4, containing 1mM-dithiothreitol and 0.1mM-NADPH. The rate of NADPH oxidation in the absence of triose phosphate was first recorded from the E_{340}, then DL-glyceraldehyde 3-phosphate was added to 0.4mM and the rate measured again. The activity was calculated from the difference between the two rates.

Assay of metabolic intermediates

Triose phosphate was measured as described by Bucher & Hohorst (1965) and glycerol 3-phosphate as described by Hohorst (1965). Sedoheptulose 7-phosphate was determined by using ATP and fructose 6-phosphate kinase as described by Wood & Poon (1970). A correction was made for fructose 6-phosphate in the mixture by assuming that 30% of the hexose monophosphate present was the fructose derivative. The reaction, repeated in the absence of fructose 6-phosphate kinase and ATP, gave the sum of fructose diphosphate and sedoheptulose diphosphate and a correction was made for the contribution of these compounds in the sedoheptulose 7-phosphate assay. Hexose monophosphate was measured fluorimetrically as described previously (Wood, 1972). Erythrose 4-phosphate was determined with transaldolase (Racker, 1965), and ribulose 5-phosphate and xylulose 5-phosphate were measured with transketolase and ribulose 5-phosphate 3-epimerase (Wood, 1974).

Incubation experiments

These were performed at 37°C and pH7.4 in 50mM-triethanolamine–HCl buffer containing 1mM-dithiothreitol and 0.1mM-thiamin pyrophosphate. The concentration of D-ribose 5-phosphate was 10mM and the mixtures contained, in 15ml, 0.1vol. of a fresh muscle or uterus supernatant fraction, or 0.01vol. of a liver supernatant. Controls contained all components except ribose 5-phosphate. Samples (5ml) were removed at 20, 40 and 60min and ultrafiltered at room temperature through a Diaflo membrane to halt the reaction. Ultrafiltration took between 5 and 8min. Triose phosphates and diphosphates were determined immediately. Other intermediates were measured after storage of the ultrafiltrates at −20°C.

Spectrophotometric experiments

The reduction of $NADP^+$ was followed directly at 340nm and 37°C, over a 60min period, in a Beckman spectrophotometer connected to a recorder. The cuvette contained, in a final volume of 2.00ml, 50mM-triethanolamine–HCl buffer, pH7.4, 1mM-dithiothreitol, 0.1mM-thiamin pyrophosphate, 1mM-$NADP^+$, 10mM-D-ribose 5-phosphate, 0.3unit of glucose 6-phosphate dehydrogenase, 0.1unit of 6-phosphogluconate dehydrogenase, 2.1units of phosphoglucose isomerase, and the same proportions of the tissue supernatants as were used in the incubation experiments. The observed rates were corrected for any reaction occurring in the absence of ribose 5-phosphate.

Results

Enzyme assays

The activities of the enzymes in rat liver and uterus were in accord with the values reported by Novello & McLean (1968) and Baquer & McLean (1972), except for uterus transaldolase, which was higher at 1.70±0.38 units/g of tissue. The activities in muscle of ribose 5-phosphate ketol-isomerase (6.4±0.5units/g) and ribulose 5-phosphate 3-epimerase (16.0±0.5 units/g) were higher than reported previously (Tan & Wood, 1969), owing to the use of saturating concentrations of substrate and improved assay procedures.

Spectrophotometric experiments

The formation of hexose monophosphate was measured directly in a spectrophotometer cuvette by observing $NADP^+$ reduction in the presence of auxiliary hexose monophosphate dehydrogenases and phosphoglucose isomerase. Ribose 5-phosphate was the substrate. With supernatants from liver and uterus, in the absence of substrate, the rates were relatively low, but in the presence of 10mM-ribose 5-phosphate the rates of reduction gradually accelerated as intermediates built up, until, between 50 and 60min later, they had attained constant values comparable with the rates of hexose monophosphate accumulation in the incubation experiments discussed below.

When the same experiment was carried out with a muscle supernatant, there was a steady rate of increase in E_{340} in the absence of ribose 5-phosphate,

but addition of this compound to a 10mM concentration did not increase this rate, and on occasion even decreased it to zero. A model system containing purified shunt enzymes in the amounts present in 0.2ml of muscle supernatant responded with a ready reduction of $NADP^+$ when ribose 5-phosphate was added at concentrations between 0.5 and 5mM, and it was concluded that the failure of a muscle supernatant to behave similarly was due to interfering enzyme reactions. When pure NADPH was added to the system it was not oxidized in the absence of ribose 5-phosphate, but became oxidized when this substance was added. The oxidation was not inhibited by 0.1mM-NaCN, a concentration that inhibited the direct oxidation of NADH by the muscle supernatant, so a transfer of reducing equivalents to NAD^+ with a subsequent oxidation of NADH could not explain the observed behaviour.

Further experiments revealed that when NADPH was added, or accumulated in the system, to a concentration greater than 0.03mM, it was oxidized when ribose 5-phosphate, fructose diphosphate, dihydroxyacetone phosphate, or 0.2mM-ATP in the presence of ribose 5-phosphate, were added. Apparently the NADPH underwent a substrate-level oxidation in the presence of triose phosphate, or any compound that could give rise to triose phosphate. ATP fell into this caregory, since, by the action of fructose 6-phosphate kinase and aldolase present in the muscle supernatant, the sedoheptulose 7-phosphate that had accumulated would be phosphorylated to sedoheptulose 1,7-diphosphate and then split into erythrose 4-phosphate and dihydroxyacetone phosphate.

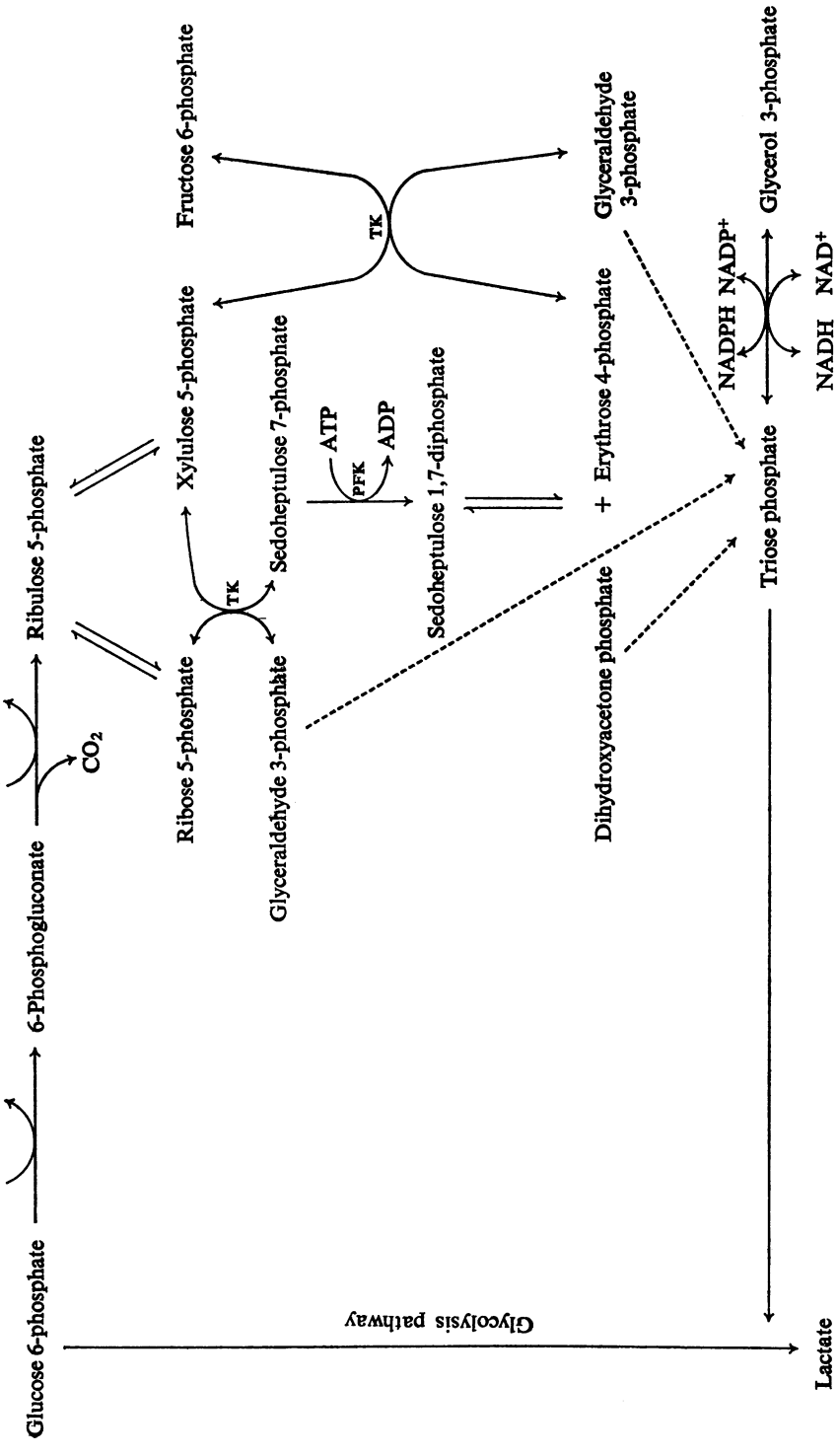
Finally, it was demonstrated that muscle extracts could catalyse the direct oxidation of NADPH by glyceraldehyde 3-phosphate to form glycerol 3-phosphate. This reaction was not catalysed by a mixture of rabbit muscle glycerol 3-phosphate dehydrogenase and triose phosphate isomerase in the amounts present in the rat muscle supernatant, and it appeared to be due to an activity distinct from that of glycerol 3-phosphate dehydrogenase described by Borrebaek *et al.* (1965). The product of the reduction of 0.18μmol of D-glyceraldehyde 3-phosphate gave 0.21μmol of glycerol 3-phosphate when assayed with glycerol 3-phosphate dehydrogenase by the method of Hohorst (1965). Supernatants from liver and uterus had NADPH–triose phosphate oxidoreductase activities of 0.16 ± 0.02 and less than 0.02unit/g of tissue respectively, compared with 0.90 ± 0.03unit/g for a muscle supernatant (means ± S.E.M. for four animals). This was additional evidence for the non-identity of the activity with glycerol 3-phosphate dehydrogenase, as rat liver contains larger amounts of the latter enzyme than does muscle (von Fellenberg *et al.*, 1962; Shonk & Boxer, 1964).

Table 1. *Formation of intermediates by tissue supernatants incubated with ribose 5-phosphate*

Values are the means ± S.E.M. for supernatants from four animals. Controls contained all components except ribose 5-phosphate. Further details of the conditions are given in the text. Initial concentrations of xylulose 5-phosphate and ribulose 5-phosphate in the tubes containing the 10mM-ribose 5-phosphate substrate were 10μM and 50μM respectively.

	Concentration (μM)							
	Muscle			Uterus		Liver		
Incubation time (min) ...	0 (control)	60 (control)	60	0 (control)	60 (control)	60	0 (control)	60 (control)	60
Triose phosphate	5±1	2±1	10±2	11±5	9±3	476±24	2±1	2±1	233±18
Sedoheptulose 7-phosphate	5±1	6±1	37±6	1±1	4±3	463±29	4±2	4±2	300±25
Glycerol 3-phosphate	13±1	20±6	59±10	6±1	6±1	8±3	0	0	2±2
Hexose monophosphate	0.8±0.1	0.8±0.2	1.2±0.3	26±1	25±1	319±46	1.7±0.2	1.5±0.2	40±5
Fructose diphosphate and sedoheptulose diphosphate*	0	0	1	0	0	70±3	0	0	25±5
Xylulose 5-phosphate	0	0	1440±60	0	0	1890±95	0	0	725±45
Ribulose 5-phosphate	0	0	1180±30	0	0	1340±180	0	0	550±35

* Calculated as fructose diphosphate equivalents from the reactions with aldolase, triose phosphate isomerase and glycerol 3-phosphate dehydrogenase.



Scheme 1. *Alternative formulation of the pentose phosphate pathway in muscle*

Abbreviations are: TK, transketolase; PFK, fructose 6-phosphate kinase.

Incubation experiments

Ribose 5-phosphate was incubated with the supernatant fractions from muscle, uterus and liver, and the intermediates that accumulated were characterized by paper chromatography (Wood, 1961, 1968) and determined by enzymic assay. The concentrations after 60min incubation and, for the control tubes, the concentrations initially and after 60min, are presented in Table 1. With the supernatants from all three tissues adequate amounts of xylulose 5-phosphate for the operation of transketolase had accumulated after 20min. In supernatants from uterus and liver, triose phosphate and sedoheptulose 7-phosphate accumulated in parallel, hexose monophosphate was synthesized, and there was no accumulation of glycerol 3-phosphate. With a muscle supernatant, however, there was no significant accumulation of hexose monophosphate, apparently owing to the absence of triose phosphate to serve as a substrate for transaldolase. It appeared that the triose phosphate had been reduced to glycerol 3-phosphate, which accumulated markedly when ribose 5-phosphate was present but only increased slightly in the 60-min incubated control. When 1mM-triose phosphate was incubated with a 1:10 dilution of a muscle supernatant and 1mM-dithiothreitol for 60min, 0.52mM-glycerol 3-phosphate was produced, showing that, in the absence of added coenzymes, sufficient endogenous NAD^+ was present to catalyse the coupled oxidoreduction of triose phosphate to glycerol 3-phosphate.

Although all samples were assayed for erythrose 4-phosphate, concentrations greater than 8μM did not accumulate, probably owing to the high affinity of erythrose 4-phosphate for transketolase and its ready conversion into fructose 6-phosphate and glyceraldehyde 3-phosphate in the presence of xylulose 5-phosphate. Thus Novello & McLean (1968) have shown that the transketolase-catalysed reaction with xylulose 5-phosphate as a donor and erythrose 4-phosphate as an acceptor ($K_m = 0.04$mM) is much faster than with ribose 5-phosphate as the acceptor ($K_m = 0.4$mM).

Discussion

Supernatants from liver and uterus when incubated with ribose 5-phosphate accumulated the intermediates expected from their content of the shunt enzymes and the usual formulation of the pentose phosphate pathway. The hexose monophosphate produced NADPH in the spectrophotometric experiments. Muscle supernatants did not accumulate hexose monophosphate in the incubation experiments and the concentration of triose phosphate remained low, owing to a NAD^+-linked oxidoreduction to glycerol 3-phosphate as soon as it was formed. In the spectrophotometric experiments, no net $NADP^+$ reduction could be obtained owing to the relatively high activity in muscle of a NADPH–triose phosphate oxidoreductase that kept the coenzyme in the oxidized state and diminished hexose monophosphate formation by converting the intermediate triose phosphate into glycerol 3-phosphate.

These results lead one to ask whether the oxidation of triose phosphate by NADPH in muscle has biological significance. The activity of the NADPH–triose phosphate oxidoreductase in muscle is greater than the sum of the glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities and could provide a new type of 'hexose monophosphate shunt' in which NADPH formed by these two enzymes is reoxidized with triose phosphate formed by transketolase, giving glycerol 3-phosphate (Scheme 1). Such a system would tend to accumulate sedoheptulose 7-phosphate, which would, however, be removed in the presence of the high ATP concentration and fructose 6-phosphate kinase activity of this tissue to form sedoheptulose 1,7-diphosphate, which, in turn, would be split by aldolase to form dihydroxyacetone phosphate and erythrose 4-phosphate (the sequence of reactions used here to assay sedoheptulose 7-phosphate). The erythrose 4-phosphate would react readily in the presence of transketolase and xylulose 5-phosphate to form fructose 6-phosphate and a further molecule of glyceraldehyde 3-phosphate. The overall reaction, as formulated in Scheme 1, would result in the conversion of 2 molecules of hexose monophosphate into 3 molecules of glycerol 3-phosphate and the production of 3 molecules of CO_2 and 3 molecules of NADPH without the participation of transaldolase.

Whether such a cycle operates in muscle is a matter for further experiment; however, that such a pathway is possible is demonstrated by the observation described above that NADPH oxidation by a muscle supernatant incubated with ribose 5-phosphate was increased by the addition of ATP. The pentose phosphate pathway as usually formulated leads to the production of 2 molecules of triose phosphate, 6 molecules of CO_2 and 12 molecules of NADPH for each 2 hexose monophosphate molecules broken down. Hence, the advantage to the organism of possessing a NADPH-specific triose phosphate oxidoreductase may be that the pentose phosphate pathway of muscle can be kept turning over with a lesser requirement for concomitant biosynthesis to remove the NADPH formed. The accumulated glycerol 3-phosphate could be removed by the usual mechanisms involving NAD^+-linked enzymes and mitochondrial oxidation (Sols, 1968). In liver and uterus such a mechanism would be required less urgently in the presence of active biosynthesis.

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