The Biosynthesis of Trehalose in the Locust Fat Body

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The haemolymph of a number of insects has been shown to contain the non-reducing disaccharide trehalose as the principal carbohydrate (Wyatt & Kalf, 1956, 1957). This is so in the desert locust,

Yeast cells are also able to convert glucose into trehalose, and Cabib & Leloir (1958) have shown that yeast contains an enzyme which catalyses the reaction:

Schistocerca gregaria, the haemolymph of which may contain up to 2% of trehalose (Howden & Kilby, 1956). Treherne (1958*a, b*) demonstrated that the introduction of radioactive glucose into the alimentary canal or directly into the haemolymph of *S. gregaria* *in vivo* resulted in the appearance of radioactive trehalose in the haemolymph within a short time, but the site and mode of conversion of glucose into trehalose were not investigated. We have been able to show that the fat body is the most active tissue of the locust in this respect.

The insect fat body is a conspicuous organ which extends throughout the abdominal and thoracic cavities, and consists of a loose meshwork of anastomosing lobes formed of sheets of single or double layers of yellow cells. It occupies the space between the gut and the body wall and is everywhere in contact with the blood, so a ready interchange of metabolites between fat-body cells and the blood would be expected. One function of the fat body which has long been recognized is that of a storage organ, since the cells become loaded with globules of fat, protein and glycogen during the development of the insect. Recently it has been shown that fat-body tissue is active in carrying out a number of different metabolic reactions (Kilby & Neville, 1957; Bellamy, 1958; Zebe & McShan, 1959), and the organ may possibly be considered as an equivalent in some respects of the mammalian liver as a site of intermediary metabolism. For these reasons, it was thought that it might be involved in trehalose biosynthesis.

Fat body forms a very convenient tissue for biochemical investigation as it is readily dissected from the insect and can be obtained almost free from other tissues. It was found that the fat body of *S. gregaria* would convert radioactive glucose into trehalose *in vitro*, and the subsequent preparation of active cell-free extracts from it facilitated the study of the pathway of trehalose biosynthesis.

Their yeast preparation also contained a specific phosphatase for trehalose phosphate.

In the present paper we describe the identification of similar enzymes in the fat body of *S. gregaria*, and also of other enzymes for the regeneration of uridine diphosphate glucose and the formation of glucose 6-phosphate. Parts of this work have been briefly reported elsewhere (Candy & Kilby, 1959, 1960).

MATERIALS AND METHODS

Locusts. Fifth-instar hoppers of the desert locust, *S. gregaria* Forskål, phase *gregaria*, were obtained from the Anti-Locust Research Centre, London, S.W. 7, and maintained in cages at approx. 34° during a daytime period of 16 hr. Heat and light were switched off for a night-time period of 8 hr. During the early stages of the work, the locusts were fed on a mixture of fresh grass and an artificial diet. Later, however, they were fed solely on the artificial diet described by Howden & Hunter-Jones (1958).

Preparation of fat-body extract. Adult locusts were used, and 7–10 days after the final moult. The fat bodies were removed and homogenized with ice-cold water in a Potter-Elvehjem blender. The homogenate was then centrifuged for 5 min. at 1000*g*, forming an upper fatty layer, an aqueous layer and a sediment. The aqueous layer was withdrawn and a similar volume of ice-cold water was added to the residue. After mixing, this was again centrifuged at 1000*g* for 5 min. and the aqueous layer was withdrawn and mixed with the aqueous fraction from the previous centrifuging. The final volume was in the proportion of about 0.5 ml./original fat body. This preparation was then freeze-dried and stored. When sufficient of this material had accumulated, it was dissolved in 0.1 ml. of water/original fat body and centrifuged at 12 000*g* for 30 min. (stage 1). The clear aqueous layer was then removed and dialysed for 8 hr. against three changes of distilled water at 5°. The dialysate was then freeze-dried and stored at –20° until used.

Paper chromatography. Solvents used for the chromatography of sugars were: solvent 1, propan-1-ol-ethylacetate-water (7:1:2, by vol.) (Baar & Bull, 1953); solvent 2,

ethyl acetate–water–pyridine (4:4:1, by vol.); solvent 3, ethyl acetate–water–acetic acid (3:3:1, by vol.); solvent 4, propan-1-ol–aq. NH_3 soln. (sp.gr. 0.88)–water (6:3:1, by vol.) (Hanes & Isherwood, 1949). Sugars were detected by the method of Trevelyan, Procter & Harrison (1950). Solvents used for the chromatography of sugar phosphates were: solvent 5, propan-2-ol–isobutanol–aq. NH_3 soln. (sp.gr. 0.88)–water (40:20:1:39, by vol.) (Sato & Tsumura, 1954); solvent 6, propan-2-ol–aq. NH_3 soln. (sp.gr. 0.88)–water–trichloroacetic acid (75 ml.:0.3 ml.:25 ml.:5 g.) (Sato & Tsumura, 1954); solvent 7, acetone–aq. 35% (w/v) monochloroacetic acid (6:4, v/v) (Sato & Tsumura, 1954); solvent 8, methyl Cellosolve–butan-2-one–aq. 3N- NH_3 soln. (7:2:3, by vol. plus boric acid to saturation) (Harrop, 1958) was used for the separation of glucose 1-phosphate and glucose 6-phosphate. Sugar phosphates were detected by the method of Hanes & Isherwood (1949). Nucleotides were separated with two solvents described by Paladini & Leloir (1952): solvent 9, 95% ethanol–M-ammonium acetate (pH 7.5) (15:6, v/v) and solvent 10, 95% ethanol–M-ammonium acetate buffer (pH 3.8) (15:6, v/v). The nucleotides were detected by contact printing of the chromatograms in ultraviolet light as described by Markham & Smith (1949, 1951). All chromatograms were developed on Whatman no. 4 paper, which was washed in 2N-acetic acid followed by water when sugar phosphates or nucleotides were to be separated. In all cases one-dimensional chromatograms were developed, with the descending technique. Radioactive spots on chromatograms were detected by radioautography with Kodirex X-ray film. For measurement of radioactivity a spot was eluted into a known volume of water and a portion of the eluate was spotted on to a standard 3 cm. diameter circle of filter paper for which fitting planchets were available. The radioactivity was then measured with a thin-walled Geiger–Müller tube. A statistical analysis of 16 results obtained by this method gave a standard deviation of 7.6%, which was acceptable for our purposes.

Enzyme reactions were stopped by heating at 100° for 3 min., and the precipitated protein was removed by centrifuging before chromatography.

Other analytical methods. Phosphate was estimated by the method of Fiske & Subbarow (1925) and acid-labile and total phosphate content of compounds were assayed as described by Leloir & Cardini (1957). Pentose was estimated by the method of Mejbaum (1939).

Enzyme preparations. A crude trehalase preparation was made from locusts by the technique employed by Kalf & Rieder (1958), who used wax-moth larvae as starting material. Samples of alkaline phosphatase and hexokinase were purchased from L. Light and Co. Ltd.

Special chemicals. Generally labelled D- ^{14}C glucose was obtained from The Radiochemical Centre, Amersham, Bucks. Trehalose phosphate was isolated from dried brewer's yeast (Robison & Morgan, 1928), and a sample was also received from Professor W. T. J. Morgan. DL- α -Phosphoglycerol was prepared from DL-acetone glycerol according to the method of Baer (1952). Adenosine triphosphate (ATP), fructose 6-phosphate, glucose 1-phosphate and glucose 6-phosphate were purchased from L. Light and Co. Ltd., and uridine triphosphate (UTP), uridine diphosphate (UDP) and uridine diphosphate glucose (UDPG) from the Sigma Chemical Co., Mo., U.S.A.

RESULTS

Formation of trehalose from glucose by whole-tissue preparation of fat body. Fat body and other tissues were removed by dissection from decapitated locusts and used immediately. Our preliminary experiments were carried out on whole tissues. Fat-body tissue (17 mg.) was incubated with 0.01 ml. of a solution of generally labelled ^{14}C glucose (0.2 μC) for 60 min. at 37°, and the products were separated by paper chromatography with solvent 1. The most conspicuous spot on the radioautograph of the chromatogram was that which corresponded in position with trehalose. Similar experiments were carried out on tissues of the leg muscle, haemolymph, crop, midgut and hindgut, but in no case was the spot corresponding to trehalose comparable in intensity with that obtained in the fat-body experiment.

Formation of trehalose from glucose by a fat-body extract. A fat-body extract (prepared as described in the Materials and Methods section) was virtually inactive in catalysing the conversion of glucose into trehalose unless fortified with both ATP and UDPG. The enzyme preparation was incubated with ^{14}C glucose, ATP and UDPG, and control reactions were also carried out (see Table 1). UTP was partially effective in replacing UDPG.

The reaction products were separated by paper chromatography with solvent 1, and the radioactive spots corresponding to glucose and trehalose were then counted. When ATP alone was added to the system, most of the glucose gave rise to radioactive products other than trehalose. The radioactive trehalose was eluted and identified by co-chromatography with authentic trehalose in solvents 1, 2, 3 and 4. In all cases the radioactive

Table 1. *Activation of fat-body extract by adenosine triphosphate and uridine diphosphate glucose*

The reaction mixture consisted of 3 mg. of enzyme, 0.1 μmole of magnesium sulphate, 0.1 μmole of ^{14}C glucose (0.2 μC) and additions as shown, in tris (2-amino-2-hydroxymethylpropane-1,3-diol)–maleate buffer, pH 6.6, in a final volume of 0.06 ml. This was incubated at 37° for 60 min. The figures given below refer to the radioactivity of the spots expressed as a percentage of the total radioactivity spotted on the chromatogram.

Additions	Radioactivity in trehalose (%)	Radioactivity in glucose (%)
None	1	76
ATP, 0.5 μmole	14	5
UDPG, 0.2 μmole	2	75
UDPG, 0.2 μmole + ATP, 0.5 μmole	42	3
UTP, 0.2 μmole + ATP, 0.5 μmole	37	6

spot was found to be identical with the spot reacting with silver nitrate in the detection method of Trevelyan *et al.* (1950). Also the rate of hydrolysis of the radioactive compound by a crude preparation of trehalase was the same as that of the authentic material, and the product of such a hydrolysis was a compound chromatographically identical with glucose.

Intermediates in trehalose formation. A complete reaction mixture (i.e. [^{14}C]glucose plus fat-body extract fortified with ATP and UDPG) was incubated at 37° and samples were withdrawn at various time intervals (see Table 2).

The reaction products were separated by paper chromatography with solvent 1. When a chromatogram was run for 16 hr., then the compounds with the highest R_f values (glycerol, glucose and trehalose) were separated. When a similar chromatogram was developed in the same solvent for 72 hr., the compounds with high R_f values ran off the end of the paper, and those with lower R_f values were separated. In this experiment a total of nine different compounds were resolved in easily detectable amounts.

The identity of the glycerol and of the glucose was confirmed by co-chromatography with the authentic materials with solvents 1, 2 and 3. In all cases the radioactive spot was identical with the spot reacting with silver nitrate in the sugar-detection method. The identity of the trehalose had already been shown in a previous experiment (see above), but was confirmed by co-chromatography in solvents 1, 2 and 3. The identity of α -glycerophosphate, fructose 6-phosphate, glucose 6-phosphate and trehalose phosphate was confirmed by co-chromatography with the authentic materials in solvents 5 and 6. After hydrolysis by alkaline phosphatase, fructose 6-phosphate gave

fructose, glucose 6-phosphate gave glucose, and trehalose phosphate gave trehalose. The identity of these sugars was confirmed by co-chromatography with authentic sugars in solvents 1, 2 and 3. The remaining two reaction products remain unidentified.

By measuring the percentage of radioactivity present in each of these compounds after different reaction times, the progress of formation and disappearance of the compounds was followed. Fig. 1 shows the changes during incubation in the percentage of the total radioactivity of the reaction mixture found in glucose, glucose 6-phosphate, trehalose phosphate and trehalose. The radioactivity in fructose 6-phosphate and the two unknown compounds showed an initial rise followed by a fall, whereas that in α -glycerophosphate and glycerol increased during incubation; these changes are omitted from Fig. 1 to preserve clarity, and there is no reason at present for supposing that they are on the direct pathway of trehalose biosynthesis.

Formation of trehalose from glucose 6-phosphate and uridine diphosphate glucose. Glucose 6-phosphate was found to replace glucose and ATP in the system. 1 μmole of glucose 6-phosphate, 0.8 μmole of UDPG, 0.25 μmole of magnesium sulphate, 1 mg. of enzyme in tris-maleate buffer, pH 7.0, in a total volume of 0.09 ml., were incubated at 37° for 60 min. Paper chromatography of the reaction products with solvents 1 and 9 revealed that substances corresponding to trehalose and UDP had been formed. (A new batch of enzyme was used in

Table 2. Products formed by the incubation of [^{14}C]glucose with fortified fat-body extract

Enzyme (19 mg.), 0.6 μmole of [^{14}C]glucose (0.015 mc), 7.5 μmoles of ATP, 0.75 μmole of UDPG and 0.4 μmole of magnesium sulphate in tris-maleate buffer, pH 6.6, and a total volume of 0.6 ml., were incubated at 37° and samples were removed at different times between 1 and 120 min. The compounds shown below were separated and identified as described in the text. The R_g values refer to the mobility of the compounds in solvent 1 relative to that of glucose.

Compound	R_g
Glycerol	1.71
Glucose	1.00
Trehalose	0.58
α -Glycerophosphate	0.33
Fructose 6-phosphate	0.24
Unknown 1	0.21
Glucose 6-phosphate	0.17
Trehalose phosphate	0.12
Unknown 2	0.07

Fig. 1. Progress of appearance and disappearance of radioactivity in glucose and some reaction products. The reaction was carried out as described in Table 2. O, Glucose; ●, glucose 6-phosphate; ■, trehalose phosphate; △, trehalose. Radioactivity is expressed as a percentage of the total counts spotted on the chromatograms.

this experiment, and a trehalose-phosphatase activity higher than before may account for trehalose predominating as the end product.) If glucose 6-phosphate, UDPG or the enzyme was omitted from the reaction mixture this reaction did not take place. Glucose 1-phosphate would replace glucose 6-phosphate.

Hydrolysis of trehalose phosphate. Substances chromatographically identical with trehalose and inorganic phosphate were formed when the fat-body preparation was incubated with trehalose phosphate. The rate of this hydrolysis was maximal at pH 6.25 and in the presence of mM-magnesium sulphate. As shown in Table 3, the rate of hydrolysis of trehalose phosphate is greater than that of the other sugar phosphates tested.

Partial fractionation of enzyme with calcium phosphate gel. The fat bodies of 100 locusts were treated as far as the stage 1 (see Material and Methods section). The resultant 10 ml. supernatant fraction was dialysed for 4 hr. against distilled water and added very slowly, with stirring, to a mixture of 70 ml. of ethanol and 30 ml. of ether at a temperature of -20° .

After 2 hr. at -20° , the precipitate was washed in 200 ml. of ether and dried. This process was carried out in order to remove fat and phospholipid from the preparation (Hartley, 1925). The precipitate was dissolved in 10 ml. of water, and the solution was adjusted to pH 5.5 by the addition of 0.01M-acetic acid. The resultant precipitate was collected by centrifuging (fraction 1). To the supernatant was added 15 mg. dry wt. of 6-months-old calcium phosphate gel (Keilin & Hartree, 1938). The mixture was centrifuged, and the precipitate (containing fraction 2) was collected. This process was repeated on the supernatant fraction with 15 mg. dry wt. of gel (which absorbed fraction 3) and then 45 mg. dry wt. (fraction 4). The supernatant fraction then contained fraction 5. The protein fractions were eluted from the gel by a 5% solution of ammonium sulphate in phosphate

buffer at pH 7.5. The fractions were then dialysed, to remove ammonium sulphate, and freeze-dried.

Phosphoglucumutase activity of fractionated fat-body extract. Preliminary experiments showed that fraction 5 contained the highest phosphoglucumutase activity. In further experiments 1 μ mole of glucose 1-phosphate, 1 μ mole of cysteine and 0.3 μ mole of magnesium sulphate were incubated with 1 mg. of enzyme (fraction 5) in tris buffer, pH 7.5, in a volume of 0.05 ml. at 37° for 60 min. Paper chromatograms of the reaction products (with solvent 8) showed that most of the glucose 1-phosphate had disappeared and that a large spot corresponding to glucose 6-phosphate had been formed. In the absence of cysteine, however, the glucose 1-phosphate remained largely unchanged. The identity of the glucose 6-phosphate formed was confirmed by chromatography. Chromatograms were developed with solvents 6, 7, 8 and 9, and in each case the reaction product behaved in the same way as the authentic glucose 6-phosphate. After hydrolysis by alkaline phosphatase, a substance chromatographically identical with glucose in three different solvents was obtained.

Nucleoside diphosphate-kinase activity in fractionated fat body. Fraction 1 obtained as above was found to have nucleoside diphosphate-kinase activity; 0.2 μ mole of UTP, 0.2 μ mole of ADP, 0.3 μ mole of magnesium sulphate, 1 mg. of enzyme in tris buffer, pH 8.2, in a total volume of 0.06 ml., were incubated at 37° for 60 min. The reaction products were separated on chromatograms developed for 72 hr. in solvent 9. A substance chromatographically identical with ATP was formed only if the reaction mixture was complete. If UTP, ADP or the enzyme was left out then no reaction took place. The reaction was carried out on a larger scale, and the substance suspected to be ATP was separated on paper chromatograms and eluted. An absorption spectrum of this eluate corresponded to that of ATP, and analyses of total and labile phosphate and of ribose all corresponded to the values given for ATP. The product was also active in the phosphorylation of glucose to glucose 6-phosphate by a partially purified hexokinase preparation. The reverse reaction (the formation of UTP and ADP from UDP and ATP) was also shown qualitatively on paper chromatograms.

Uridine diphosphoglucose pyrophosphorylase in the fat body. The presence of this enzyme was demonstrated in a different fat-body preparation. Fat body was treated as far as the stage 1. The supernatant fraction was then made 100% saturated with ammonium sulphate at pH 7.0 and the precipitate was collected by centrifuging and dissolved in water. This was then dialysed to remove ammonium sulphate and freeze-dried. This preparation catalysed the formation of UDPG from

Table 3. *Hydrolysis of sugar phosphates by fat-body extract*

The reaction mixture consisted of 5 μ moles of substrate and 2 mg. of enzyme in 0.025M-maleate buffer, pH 6.25, containing mM-magnesium sulphate, in a total volume of 0.8 ml. The figures represent the percentage of substrate hydrolysed after 60 min. at 38° as measured by the increase in inorganic phosphate.

Substrate	Hydrolysis (%)
Trehalose phosphate	74
Glucose 6-phosphate	7
Glucose 1-phosphate	3
Fructose 6-phosphate	7
Fructose 1:6-diphosphate	44
Ribose 5-phosphate	12

UTP and glucose 1-phosphate. UTP (0.2 μ mole), 1 μ mole of glucose 1-phosphate, 0.1 μ mole of magnesium sulphate, 1 mg. of enzyme in tris buffer, pH 8.2, in a total volume of 0.08 ml., were incubated at 37° for 60 min. The reaction products were separated on chromatograms developed for 20 hr. in solvent 9. A substance corresponding to UDPG was present in the reaction products. The reaction did not take place in the absence of either UTP or glucose 1-phosphate. The reaction was carried out on a larger scale, and the reaction product corresponding to UDPG was separated and eluted from a paper chromatogram. The absorption spectrum of the eluted compound, the total and labile phosphate content and the amount of reducing sugar after acid hydrolysis all corresponded to those for UDPG. Hydrolysis in 0.1N-hydrochloric acid at 100° for 10 min. gave a sugar chromatographically identical with glucose in solvents 1, 9 and 10.

DISCUSSION

The experimental results are consistent with the scheme shown in Fig. 2 for the biosynthesis of trehalose in *Schistocerca* fat body. The necessity for ATP and UDPG was shown by the activation produced on their simultaneous addition to a dialysed fat-body-enzyme preparation of low activity. Carey & Wyatt (1960) have recently shown the presence of UDPG in the fat body of *Cecropia* pupae, and there is no reason to doubt its occurrence in locust fat body. The presence of hexokinase in various insects has been shown by previous workers, and Kerly & Leaback (1957), for example, have studied the properties of the enzyme in thoracic muscle and salivary gland of *Locusta migratoria*. The rapid disappearance of [¹⁴C]glucose on incubation with the fat-body enzyme fortified with ATP, and the concomitant production of sugar phosphates, shows that it is likewise present in

Schistocerca fat body. The transfer of a glucose unit from UDPG to glucose 6-phosphate then leads to the formation of trehalose phosphate. This is supported by the curves in Fig. 1, which show that glucose 6-phosphate is formed at a rapid rate and then decreases as trehalose phosphate builds up. It was also shown that glucose and ATP could be replaced by glucose 6-phosphate. It is not possible to say unequivocally that glucose 6-phosphate rather than glucose 1-phosphate is involved in the reaction, since phosphoglucumutase could not be removed from the extract without inactivating phosphotrehalose-UDP transglucosylase. A similar difficulty arose in the early work of Leloir & Cabib (1953) when they demonstrated trehalose phosphate formation from hexose phosphate and UDPG by an enzyme preparation from yeast. In subsequent work, however, they were able to show that glucose 6-phosphate was involved (Cabib & Leloir, 1958). The final stage in the formation of trehalose by the fat body is the splitting of the trehalose phosphate to trehalose and inorganic phosphate. Fig. 1 shows that trehalose builds up throughout the incubation, whereas trehalose phosphate, which is formed more rapidly during the early stages, reaches a maximum and then decreases. The crude fat-body-enzyme preparation was also shown to break down trehalose phosphate more rapidly than the other sugar phosphates tested. The postulation of the presence of a specific trehalose phosphate phosphatase would be consistent with the known presence of such an enzyme in the trehalose-synthesizing system of yeast (Cabib & Leloir, 1958). Friedman (1960), using a diethylaminoethylcellulose column, has recently fractionated a crude trehalase preparation from *Phormia regina*, and obtained a peak in the eluate which, according to the preliminary report, is said to be a specific trehalose phosphate phosphatase.

The presence in the fat body of nucleoside diphosphate kinase and UDPG pyrophosphorylase is consistent with a mechanism for the regeneration of UDPG:

Fig. 2. Scheme for trehalose biosynthesis. The enzymes involved are: a, hexokinase; b, phosphotrehalose uridine diphosphate transglucosylase; c, trehalose phosphate phosphatase; d, nucleoside diphosphate kinase; e, UDPG pyrophosphorylase; f, phosphoglucumutase.

The presence of an enzyme system which will convert glucose into trehalose, and the presence also of trehalase (Howden & Kilby, 1956; Kalf & Rieder, 1958), which splits trehalose into glucose, suggests that trehalose formation is a device used by insects to store dietary carbohydrate in a readily mobilizable reserve. Howden & Kilby (1960) have shown that the trehalose concentration in the haemolymph of fifth-instar nymphs of *Schistocerca* increases throughout the 10 days of the stadium, but falls sharply during the moulting

period. Trehalose can serve as an energy source, since Bücher & Klingenburg (1958) found a pronounced fall in concentration after 2 hr. flight in *Locusta*, and a similar result was found in *Phormia* (Evans & Dethier, 1957). Before the time of moulting, chitin is required for the formation of the new cuticle, and it seems possible that the N-acetylglucosamine units in it may be ultimately derived from the trehalose carbohydrate store.

SUMMARY

1. An extract prepared from the fat body of *Schistocerca gregaria* catalyses the formation of trehalose from glucose in the presence of adenosine triphosphate and uridine diphosphate glucose.

2. Glucose 6-phosphate and trehalose phosphate were indicated as intermediates in this reaction.

3. The extract hydrolyses trehalose phosphate at a greater rate than any other sugar phosphate tested.

4. The presence was shown of phosphoglucosyltransferase, nucleoside diphosphate-kinase and uridine diphosphoglucose-pyrophosphorylase activities in the fat body.

5. A scheme for the biosynthesis of trehalose from glucose in the fat body of *Schistocerca* is presented.

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Pathways of Glucose Metabolism in Ox Retina

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In retina, metabolism of glucose is much faster in a medium buffered with bicarbonate than in one buffered with phosphate (Laser, 1937; Craig & Beecher, 1943), but the mechanism of this effect has never been satisfactorily explained. It has been suggested (Hopkinson & Kerly, 1959) that it

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may be concerned with oxidation of triphosphopyridine nucleotide during assimilation of carbon dioxide by pyruvate and the maintenance of a high ratio of oxidized to reduced triphosphopyridine nucleotide. If this is so, and if the availability of oxidized triphosphopyridine nucleotide limits oxidation of glucose 6-phosphate, then the ratio of glucose metabolized by the hexose monophosphate