

A Theoretical Analysis of NADPH Production and Consumption in Yeasts

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Theoretical calculations of the NADPH requirement for yeast biomass formation reveal that this parameter is strongly dependent on the carbon and nitrogen source. The data obtained have been used to estimate the carbon flow over the NADPH-producing pathways in these organisms, namely the hexose monophosphate pathway and the NADP⁺-linked isocitrate dehydrogenase reaction. It was calculated that during growth of yeasts on glucose with ammonium as the nitrogen source at least 2% of the glucose metabolized has to be completely oxidized via the hexose monophosphate pathway for the purpose of NADPH synthesis. This figure increases to approximately 20% in the presence of nitrate as the nitrogen source. Not only during growth on glucose but also on other substrates such as xylose, methanol, or acetate the operation of the hexose monophosphate pathway as a source of NADPH is essential, since the NADP⁺-isocitrate dehydrogenase reaction alone cannot meet the NADPH demand for anabolism. NADPH production via these pathways requires an expenditure of ATP. Therefore, the general assumption made in calculations of the ATP demand for biomass formation that generation of NADPH does not require energy is, at least in yeasts, not valid.

INTRODUCTION

Despite its well-known function as a reductant in anabolic metabolism, little is known about the quantitative aspects of NADPH production and consumption in micro-organisms. In contrast, the ATP balance in micro-organisms has been analysed in detail (Stouthamer, 1973, 1977). As for ATP, the amount of NADPH required for biosynthesis of cell constituents from central metabolic intermediates and ammonia is a constant. This requirement only depends on the relative amounts of monomers, i.e. amino acids, fatty acids, nucleotides and hexose phosphates. However, due to differences in NADPH production and consumption in the conversion of various carbon sources to, for example, triose phosphates, and of various nitrogen sources to ammonia, the NADPH requirement for biosynthesis will be strongly dependent on the carbon and nitrogen source used for growth. It is therefore evident that the NADPH balance of the cell must be carefully controlled, and that the NADPH-producing systems must be tuned to the NADPH-consuming processes in relation to environmental conditions.

In this paper an attempt has been made to quantify the NADPH requirement for biomass synthesis from different carbon and nitrogen sources. Since in yeasts NADPH must be generated via intermediary pathways of carbon metabolism (see below) attention has been focused on the metabolic consequences of the NADPH requirement in terms of carbon flow over NADPH-producing pathways.

Before a quantitative estimation is made of the NADPH consumption in anabolism and NADPH production in catabolism, some relevant information on the nature of these processes in yeasts will be discussed.

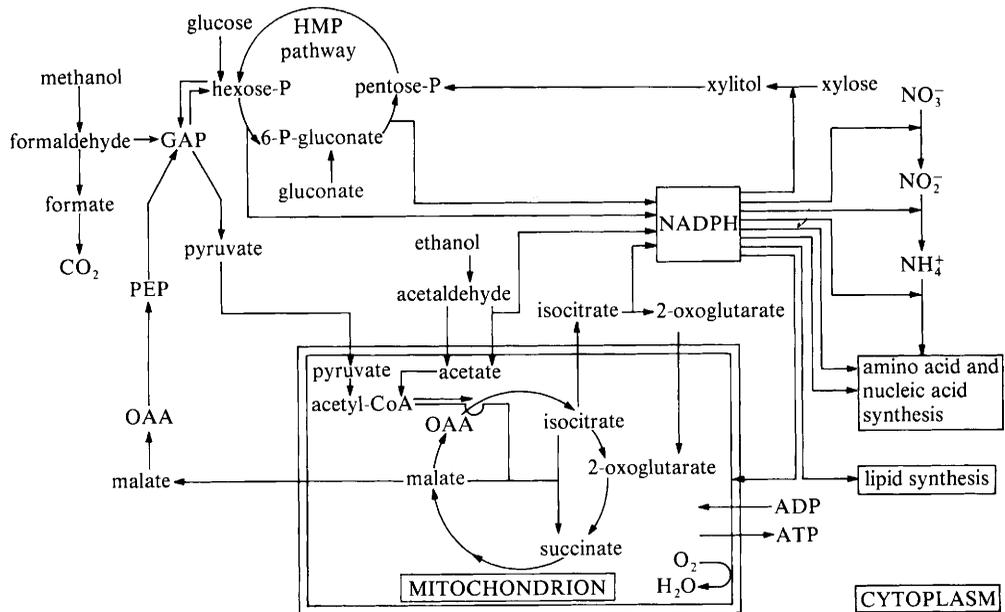
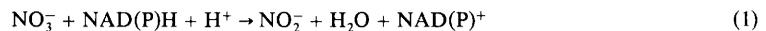


Fig. 1. Schematic representation of NADPH-producing and NADPH-consuming processes and their localization in the yeast cell. Note that, for simplicity, the enzymes of the glyoxylate cycle have been presented as mitochondrial. This may not reflect the real situation since they may occur in cytoplasm or peroxisomes. OAA, oxaloacetic acid; PEP, phosphoenolpyruvic acid; GAP, glyceraldehyde-3-phosphate.

NADPH-consuming processes in yeasts

A simplified scheme of NADPH-consuming and NADPH-producing processes in the yeast cell is given in Fig. 1. Lipid synthesis, a major NADPH-consuming process, occurs in the cytoplasm (Botham & Ratledge, 1979). Except for some mitochondrial reduction steps in the biosynthesis of arginine, valine, leucine and isoleucine (Ryan *et al.*, 1973; Ryan & Kohlhaw, 1974; Wipf & Leisinger, 1977) also the NADPH-consuming reactions of amino acid synthesis are located in the cytoplasm.

When nitrate is used as the nitrogen source this compound is reduced via cytoplasmic nitrate and nitrite reductases with NADPH [equations (1) and (2)]:



The coenzyme specificities of nitrate and nitrite reductases may differ amongst yeasts and other fungi. In *Aspergillus nidulans* both are NADPH-dependent (Hankinson & Cove, 1974). In *Candida utilis*, depending on the strain, nitrate reductase may be either NADH- or NADPH-linked, whereas nitrite reductase is NADPH-dependent (Bruinenberg *et al.*, 1983).

The NADPH requirement for biosynthesis is strongly dependent on the carbon source as well. For example, the metabolism of xylose in the majority of yeasts proceeds via a reduction to xylitol (Fig. 1) which is strictly NADPH-dependent (Barnett, 1976). Therefore, in contrast to glucose, the conversion of xylose to a central intermediate, like triose phosphate, requires NADPH. On the other hand a net production of NADPH occurs in the conversion of gluconate to triose phosphate, since gluconate is oxidized via 6-phosphogluconate dehydrogenase (Bruinenberg *et al.*, 1983) which is an NADP⁺-dependent enzyme in yeasts.

It is not only assimilatory processes that may be involved in NADPH consumption. Schuurmans Stekhoven (1966) showed that yeast mitochondria can oxidize NADPH with respiratory control. Also submitochondrial particles can oxidize NADPH (Ragan & Garland,

1971; Djavadi *et al.*, 1980). Direct oxidation of exogenous NADPH seems a general property of plant and fungal mitochondria (Palmer & Møller, 1982). The quantitative importance of this process *in vivo* is unknown.

NADPH-producing processes in yeasts

The localization of NADPH-producing processes in the yeast cell is an important parameter since, as mentioned above, most NADPH-consuming processes are located in the cytoplasm. Since the inner mitochondrial membrane is impermeable to reduced pyridine nucleotides (Von Jagow & Klingenberg, 1970) it follows that NADPH production must occur in the same compartment of the cell, namely in the cytoplasm. A theoretical alternative would be mitochondrial production of NADPH and export of this reduction equivalent by a shuttle mechanism. So far, enzymes which could be involved in such a process have not been encountered.

Several pathways for NADPH production may exist in eukaryotic cells. An energy-independent or energy-dependent transhydrogenase, present in mitochondria of mammalian cells (Rydström *et al.*, 1970), has not been detected in *Candida utilis* (Bruinenberg *et al.*, 1983). Such a system would, however, not contribute significantly to the NADPH production for biosynthesis as a result of the permeability barrier of the inner mitochondrial membrane. This is in line with the observations that fat synthesis in mammalian cells is dependent on the activity of the hexose monophosphate (HMP) pathway as a source of NADPH (Gumaa *et al.*, 1973).

In certain moulds a mannitol cycle has been postulated as a source of cytoplasmic NADPH (Hult *et al.*, 1980). This transhydrogenating system, which requires the net input of one ATP per NADPH, has so far not been detected in yeasts. In *Saccharomyces cerevisiae* very low activities of a mitochondrial NAD(H) kinase have been detected (Griffiths & Bernofsky, 1970). This enzyme catalyses the reaction:



As with transhydrogenase, the localization of the enzyme makes it an unlikely source of cytoplasmic NADPH.

The information presented above shows that the generation of NADPH in oxidation reactions of intermediary carbon metabolism must be the major source of NADPH in yeasts. Undoubtedly the HMP pathway and possibly the NADP⁺-linked isocitrate dehydrogenase reaction are the most important in this respect. The enzymes of these pathways have been detected in all species investigated so far, regardless of the growth conditions. Indeed, on the basis of a theoretical calculation of the NADPH demand for biosynthesis as well as radiorespirometric studies, Lagunas & Gancedo (1973) concluded that the HMP pathway is the major route of NADPH formation in glucose-grown *Saccharomyces cerevisiae*, and that transhydrogenation does not play an important role in this organism when growing on glucose.

Apart from the HMP pathway and NADP⁺-linked isocitrate dehydrogenase, an NADP⁺-dependent acetaldehyde dehydrogenase may contribute to cytoplasmic NADPH synthesis during growth on, for example, ethanol (Fig. 1) (Llorente & Núñez de Castro, 1977). Another possible source of NADPH, the NADP⁺-linked malic enzyme, cannot be detected in a variety of yeasts (Hirai *et al.*, 1976a; Bruinenberg *et al.*, 1983). The enzyme is present in malate-grown *Rhodotorula glutinis* but its synthesis is repressed during growth on glucose and acetate (Fernández *et al.*, 1967).

In yeasts both dehydrogenases of the HMP pathway are strictly NADP⁺-dependent (Bonsignore & De Flora, 1972; Scott & Mahoney, 1976) and located in the cytoplasm (Hirai *et al.*, 1976b). The pentose phosphates produced via the action of these enzymes can be rearranged via transaldolase/transketolase reactions to hexose phosphates (Fig. 2a). The complete oxidation in the HMP pathway of one glucose-6-phosphate to six CO₂ yields 12 NADPH. However, the operation of the HMP pathway as a full cycle requires the presence of fructose-1,6-bisphosphatase (Fig. 2a). In certain yeasts the synthesis of this enzyme is repressed during growth on glucose (Gancedo & Gancedo, 1971). In this case oxidation of one glucose 6-phosphate in the HMP pathway can yield only six NADPH (Fig. 2b). When the NADP⁺-

dependent isocitrate dehydrogenase would be the only source of NADPH, maximally two NADPH can be generated from one glucose 6-phosphate (Fig. 2c). Thus, when fructose-1,6-bisphosphatase is not present, the maximum yield in the complete oxidation of one glucose 6-phosphate to CO₂ is seven NADPH via the combined action of the HMP pathway and the tricarboxylic acid cycle.

As already mentioned above, the localization of the NADPH-producing pathways in the cell is a very important parameter. Glucose 6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase are known to be cytoplasmic enzymes but the sub-cellular distribution of the NADP⁺-dependent isocitrate dehydrogenase in yeasts is less clear. The relative distribution of the enzyme between mitochondria and cytoplasm may depend on the carbon source for growth (Hirai *et al.*, 1976b). However, irrespective of the growth conditions most of the enzyme is present in the cytoplasm, which is a prerequisite for a possible function in the supply of cytoplasmic NADPH.

METHODS

Estimation of the NADPH requirement for biomass formation during growth of yeasts on different carbon and nitrogen sources can be performed by summation of all NADPH-requiring anabolic reactions. Similar calculations have been made by Payne (1970), Stouthamer (1973) and Harder & van Dijken (1976) but attention was focused on the ATP requirement of biomass formation. In this paper essentially the same calculations are performed but here only the oxidation-reduction processes in anabolism are considered, analogous to earlier contributions by Oura (1972) and Lagunas & Gancedo (1973).

For the present calculations the following macromolecular composition of yeast was used (g per 100 g cells): polysaccharide 42, protein 40, nucleic acid 7, lipid 7 and ash 4 (Oura, 1972; Alroy & Tannenbaum, 1973). Nucleic acids were taken as RNA since the RNA content of yeast cells is an order of magnitude higher than the DNA content (Oura, 1972). Large differences have been found in the amount of lipids in yeasts. In order to obtain a minimal figure for the NADPH requirement in biomass formation, a low lipid content (taken as neutral fat) is used in our calculations.

The monomer composition given by Oura (1972) is used. Thus, the amino acid composition is supposed to be (mmol per 100 g cells): alanine 45.9; arginine 16.1; asparagine 10.2; aspartate 29.7; glutamate 30.2; glutamine 10.5; cysteine 0.7; glycine 29; histidine 6.6; isoleucine 19.3; leucine 29.6; lysine 28.6; methionine 5.1; phenylalanine 13.4; proline 16.5; serine 18.5; threonine 19.1; tryptophan 2.8; tyrosine 10.2 and valine 26.5. For nucleotides the values (mmol per 100 g cells): AMP 5.1; GMP 5.1; UMP 6.7, and CMP 5.0 and for lipids (mmol per 100 g cells): palmitoleic acid 13.65; oleic acid 5.5; lauric acid 9.2 and glycerol 9.1 are introduced in the calculations.

The cell formula used in the equations was that of Dekkers *et al.* (1981): C₄H_{7.32}O_{2.24}N_{0.68}S_{0.006}. With this cell formula and the calculated amounts of NADPH, NADH and CO₂ produced or consumed during biomass synthesis, the amount of carbon source required in assimilation is calculated. From this amount and the cell yield on the carbon source, the amount of carbon source dissimilated was calculated.

For the calculation of the NADPH requirement the following assumptions and simplifications have been made.

- (1) The same macromolecular and monomer composition is taken for biomass formation from different carbon and nitrogen sources.
- (2) During growth on glucose, methanol, xylose and gluconate, oxaloacetate is produced via pyruvate carboxylase (EC 6.4.1.1), whereas during growth on ethanol or acetate this compound is formed via the glyoxylate cycle.
- (3) Pentoses are formed via transaldolase/transketolase rearrangement reactions. It appears that only a small amount of pentoses is needed, as a result of a net production of pentoses in the biosynthesis of aromatic amino acids.
- (4) In order to arrive at a minimal figure of the NADPH requirement it is assumed that the biosynthesis of 2-oxoglutarate proceeds exclusively via NADP⁺-linked isocitrate dehydrogenase.
- (5) Cytochrome and vitamin synthesis is not considered since the amount of NADPH required in these processes is negligible.
- (6) The reduction of nitrate to ammonia requires 4 NADPH.
- (7) Excess formate produced in biosynthetic reactions is oxidized to CO₂ with the production of 1 NADH.
- (8) Dissimilatory consumption of NADPH by mitochondria is not taken into consideration.
- (9) Some NADPH-requiring steps in amino acid biosynthesis are known to be mitochondrial in *Saccharomyces cerevisiae* (Ryan *et al.*, 1973; Ryan & Kohlhaw, 1974; Wipf & Leisinger, 1977). Although the amount of NADPH required in these reactions is 10% of the total NADPH requirement for biomass formation from glucose and ammonia, for simplicity all the NADPH required was taken to be cytoplasmic.

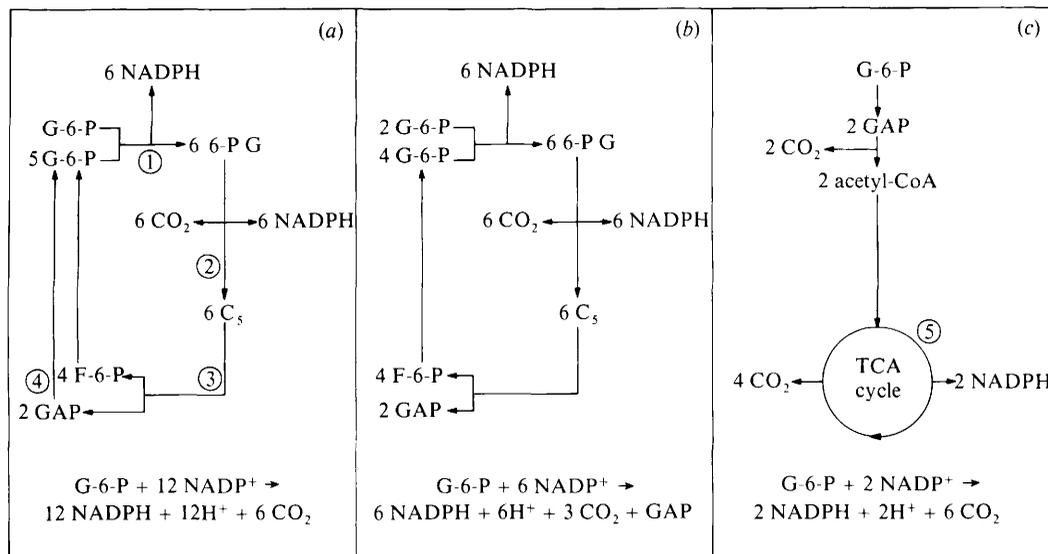


Fig. 2. Generation of NADPH by the HMP pathway and the tricarboxylic acid cycle in yeasts. G-6-P, glucose 6-phosphate; 6-P-G, 6-phosphogluconate; F-6-P, fructose 6-phosphate; C₅, pentose phosphate; GAP, glyceraldehyde-3-phosphate. The enzymes catalysing key reactions in these routes have been indicated by numbers (circled): 1, glucose-6-phosphate dehydrogenase; 2, 6-phosphogluconate dehydrogenase; 3, transaldolase and transketolase; 4, fructose-1,6-bisphosphate aldolase and fructose-1,6-bisphosphatase; 5, NADP⁺-linked isocitrate dehydrogenase.

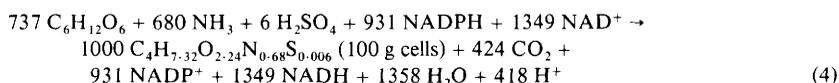
For the calculation of the carbon flow over NADPH-producing pathways the following assumptions will be made.

- (1) The maximum NADPH yield from the oxidation of one molecule of glucose 6-phosphate is taken as 7 or 12 (see Fig. 2a, b). The maximum yield of NADPH from glucose 6-phosphate in the HMP pathway during growth on substrates which require gluconeogenesis is taken as 12, since in this case the HMP pathway is more likely to operate as a full cycle.
- (2) The possibility of NADPH generation during growth on glucose via decarboxylation of pyruvate and subsequent oxidation of acetaldehyde via an NADP⁺-linked dehydrogenase is not taken into consideration. In *Candida utilis* the latter enzyme is not present during growth on glucose (Bruinenberg *et al.*, 1983).
- (3) Dissimilatory oxidation of NADPH by mitochondria is not considered. This and other assumptions (see above) have been made in order to obtain minimal figures for the NADPH demand of the cell.

RESULTS

Calculation of the NADPH requirement for biosynthesis

The amounts of NADPH required for the synthesis of the various cell components from glucose and ammonia are listed in Table 1. Although these processes require an input of NADPH, it is noteworthy that a net synthesis of reduction equivalents occurs. With the cell formula C₄H_{7.32}O_{2.24}N_{0.68}S_{0.006} an overall equation for the biosynthesis of 100 g cells from glucose and ammonia can be constructed from these data:



From this equation the amount of NADPH required for cell synthesis from glucose and nitrate can be derived since the only additional NADPH required is that for the reduction of NO₃⁻ to NH₄⁺ (931 + 4 × 680 = 3651 NADPH):

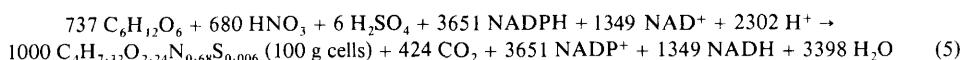


Table 1. *Production (+) or consumption (-) of NADH, NADPH and CO₂ during synthesis of the amount of monomers present in 100 g yeast*

For the monomer composition, see Methods. The values are expressed in mmol.

	NADH	NADPH	CO ₂
Amino acids	+878.5	-541.1	+237.9
Nucleic acids	+64.2	-47.4	-21.9
Lipids	+406.7	-342.0	+207.9
Polysaccharide	0	0	0
Total	+1349.4	-930.5	+423.9

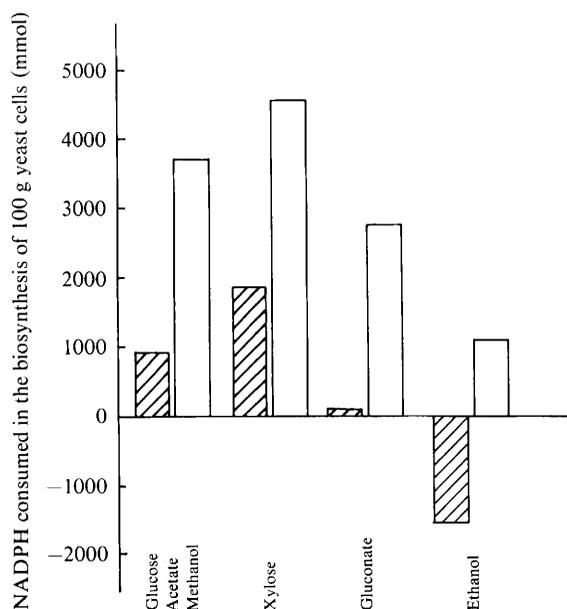


Fig. 3. NADPH requirement (mmol per 100 g yeast cells) for biosynthesis with various carbon sources with ammonia or nitrate as nitrogen sources. The data for ethanol were obtained by assuming that this compound is assimilated exclusively via an NADP⁺-linked acetaldehyde dehydrogenase. ▨, NH₄⁺; □, NO₃⁻.

Similar calculations can be made for xylose, gluconate, methanol, acetate or ethanol as carbon sources, with ammonia or nitrate as nitrogen sources. The NADPH requirement for biomass formation with these combinations of carbon and nitrogen sources is shown in Fig. 3. Different carbon sources such as glucose, methanol and acetate require the same amount of NADPH for biomass formation. This results from the fact that the conversion of these compounds to a common precursor for monomer synthesis, for example triose phosphate, does not involve NADP(H)-linked oxidation-reduction reactions. Biomass formation with nitrate results in an almost fourfold increase in the NADPH requirement with these carbon sources [Fig. 3; equations (5) and (6)]. With xylose as the carbon source a much higher NADPH requirement is calculated, resulting from the initial NADPH-linked reduction of xylose to xylitol. When gluconate and ammonia serve as carbon and nitrogen sources, the NADPH requirement for biomass formation is very low since the assimilation of each molecule of gluconate is obligatorily linked to the production of one molecule NADPH in the 6-phosphogluconate dehydrogenase reaction. The calculated NADPH requirement for growth on gluconate with nitrate is almost entirely due to the reduction of this nitrogen source to ammonia.

The NADPH requirement for growth with ethanol as the carbon source cannot exactly be estimated since the contribution of each of the two isoenzymes of acetaldehyde dehydrogenase

(Fig. 1) is not known. When all the ethanol assimilated is oxidized via the NADP⁺-linked acetaldehyde dehydrogenase, the formation of cell material results in a net production of NADPH when ammonia serves as the nitrogen source (Fig. 3).

The calculated differences in NADPH required per unit of biomass synthesized from the carbon and nitrogen sources considered here must have important consequences for the carbon flow over NADPH-producing pathways, namely the HMP pathway and the NADP⁺-linked isocitrate dehydrogenase reaction. The data presented above permit an estimation of the minimal carbon flow over these routes. This is outlined below.

Calculation of the carbon flow over NADPH-producing pathways

NADPH required for anabolic reactions must be produced in the catabolism of the carbon and energy source. It will be clear that when, for example, ammonia is replaced by nitrate as a nitrogen source, the flow over the NADPH-producing pathways has to increase. This can be evaluated quantitatively as follows:

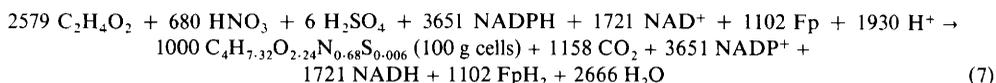
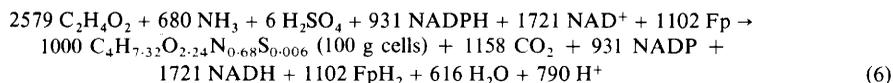
The yield (g cells per g substrate) of yeasts growing with glucose as a carbon source and ammonia as a nitrogen source is 0.5 (P. M. Bruinenberg, unpublished). It then follows that the amount of glucose catabolized for the formation of 100 g cells equals $100/(0.5 \times 180) = 1111$ mmol (total amount metabolized) – 737 mmol [amount assimilated, equation (4)] = 374 mmol. In the absence of the HMP pathway as an NADPH-generating system a maximum of $374 \times 2 = 748$ mmol NADPH can be generated via the NADP⁺-linked isocitrate dehydrogenase (Fig. 2c). This is not enough to meet the amount required for assimilation [931 mmol NADPH, equation (4)]. It can therefore be concluded that the HMP pathway must operate as a source of NADPH. In the case of an HMP pathway lacking fructose-1,6-bisphosphatase (Fig. 2b) the minimum amount of dissimilatory glucose which has to be oxidized via this pathway can be calculated with the formula:

$$6x + kx + 2k(374 - x) = 931$$

in which x represents the amount of glucose metabolized via the HMP pathway and k the relative amount of isocitrate oxidized by the NADP⁺-dependent isocitrate dehydrogenase. When all the isocitrate generated during glucose catabolism is oxidized by this enzyme ($k = 1$) it follows that 10% of the dissimilatory glucose (3% of the total amount metabolized) has to be oxidized via this pathway. In the case of a HMP pathway including a fructose-1,6-bisphosphatase activity (Fig. 2a) it follows via $12x + 2k(374 - x) = 931$ that at least 5% of the dissimilatory glucose (2% of the total glucose) will have to be oxidized by such a type of HMP pathway. It will be clear that the theoretical contribution of the HMP pathway has to increase when both the NADP⁺- and NAD⁺-linked isocitrate dehydrogenase play a role in glucose dissimilation ($0 \leq k < 1$).

With nitrate as a nitrogen source a much higher NADPH production is required in order to meet the NADPH requirement for biosynthesis. With a cell yield of 0.43 on glucose and nitrate (P.M. Bruinenberg, unpublished) it can be calculated that a minimum of 91% of the dissimilatory glucose (36% of the total amount of glucose) has to be metabolized via a HMP pathway lacking fructose-1,6-bisphosphatase. In the presence of this enzyme a minimum of 45% is required. This figure accounts for 19% of the total glucose metabolized.

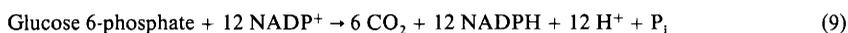
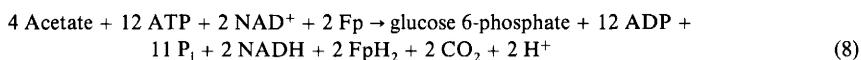
For other carbon sources similar calculations can be made. Biomass formation from acetate and ammonia or nitrate yields the following equations:



(Fp stands for flavin prosthetic group of succinate dehydrogenase).

Like the equations for glucose (4) and (5), these equations for the assimilation of acetate to cell material show a small deficit of 20–30 H on the right. This is due to the hydrogen : oxygen ratio

in the cell formula chosen for these calculations. More important, however, is the fact that the magnitude of this deficit is independent of the substrate chosen, indicating the correctness of the calculations as such. With equation (6) it can be calculated that during growth on acetate + NH_4^+ ($Y = 0.4$ g cells per g acetic acid) enough NADPH is generated in the tricarboxylic acid cycle provided that at least 60% of the isocitrate is oxidized via the NADP⁺-dependent enzyme. However, during growth on acetate with nitrate as the nitrogen source this is not the case even when all the isocitrate is oxidized via the NADP⁺-dependent enzyme. With a yield of 0.28 g cells per g acetic acid (P. M. Bruinenberg, unpublished) 3373 mmol acetate are dissimilated during the formation of 100 g cells. Since the NADPH yield in the tricarboxylic acid cycle is maximally 1 mol per mol acetate oxidized (Fig. 2c) only 3373 mmol NADPH can be generated which does not meet the requirement for assimilation [3651 mmol NADPH, equation (7)]. Since, as outlined above, the only alternative to the NADP⁺-linked isocitrate dehydrogenase for the synthesis of NADPH is the HMP pathway, acetate has to be converted to glucose-6-phosphate to meet this requirement [equations (8) and (9)]:



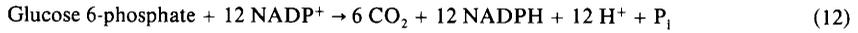
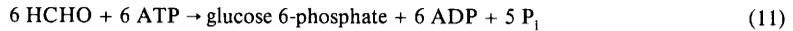
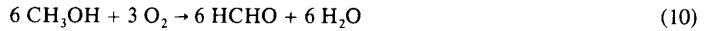
Only via this sequence of reactions can the required amount of NADPH be generated since in the oxidation of acetate via the HMP pathway the NADPH yield is three times as high as in the tricarboxylic acid cycle [3 NADPH/acetate, equations (8) and (9)]. The necessity of gluconeogenesis from acetate for NADPH production during growth with nitrate is in particular evident when, more realistically, it is assumed that both the NADP⁺- and NAD⁺-linked isocitrate dehydrogenase play a role in the dissimilation of acetate. When there is a fifty-fifty division in the carbon flow over these two enzymes, 11% of the total amount of acetate metabolized has to be oxidized in the HMP pathway for NADPH generation during growth with nitrate.

Similar calculations for gluconate as a carbon source reveal that a cyclic action of the HMP pathway and operation of NADP⁺-linked isocitrate dehydrogenase are not required for NADPH synthesis during growth with ammonia as the nitrogen source (yield of 0.44 g cells per g gluconic acid). This stems from the fact that the metabolism of each molecule of gluconate is obligately linked to the production of at least one molecule NADPH. In this case a mechanism for the dissimilatory oxidation of the surplus of NADPH would be required. With nitrate as the nitrogen source (yield of 0.35 g cells per g gluconic acid) the NADPH requirement is too high to be covered by this process alone and additional formation of NADPH by the HMP pathway and NADP⁺-linked isocitrate dehydrogenase is required. This requirement, however, cannot be met by the NADP⁺-linked isocitrate dehydrogenase alone. Therefore, the operation of the HMP pathway for generation of NADPH via cyclic oxidation of ribulose-5-phosphate is required during growth on gluconate + nitrate.

Also during growth on ethanol + ammonia (yield of 0.67 g cells per g ethanol) a surplus of NADPH is calculated on the basis of the assumption that ethanol is assimilated and dissimilated exclusively via the NADP⁺-linked acetaldehyde dehydrogenase. In this case, however, even during growth with nitrate (yield of 0.5 g cells per g ethanol) a surplus of NADPH is generated. Since the quantitative contribution of each of the two iso-enzymes oxidizing acetaldehyde (Fig. 1) is not known, it is unclear whether this surplus of NADPH reflects the *in vivo* situation. When only 50% of the acetaldehyde metabolized is oxidized via the NADP⁺-linked enzyme and furthermore 50% of the isocitrate is catabolized via NADP⁺-linked isocitrate dehydrogenase, the operation of the HMP pathway is essential for NADPH synthesis during growth on ethanol + nitrate.

A special case with respect to NADPH generation for biosynthesis is growth of yeasts on methanol. During growth on methanol energy is generated in the linear oxidation of methanol to CO_2 and most probably, as in methylotrophic bacteria, the tricarboxylic acid cycle only serves an assimilatory function (Veenhuis *et al.*, 1983). Therefore, NADPH generation during growth on methanol is restricted to the HMP pathway only. From the cell yields during growth on methanol with NH_4^+ (0.38 g cells per g methanol, van Dijken *et al.*, 1976;

Egli *et al.*, 1982) and the NADPH requirement for biomass formation (Fig. 3) it follows that 6% of the methanol utilized must be oxidized via the HMP pathway for NADPH generation according to equations (10)–(12) (Veenhuis *et al.*, 1983):



ATP requirement for NADPH generation; implication for $Y_{\text{ATP}}^{\text{max}}$

The equations presented above show that NADPH formation via the HMP pathway requires an expenditure of ATP. Moreover, the ATP costs of NADPH production via this pathway are dependent on the nature of the carbon source. During growth on glucose these amount to 1/6 or 1/12 ATP per NADPH, depending on the type of HMP pathway (Fig. 2*a, b*). During growth on methanol or acetate, 1/2 or 1 mol ATP per mol NADPH are required, respectively [equations (8)–(9) and (10)–(12)]. Also generation of NADPH via isocitrate dehydrogenase probably requires energy. The NADPH requirement of the cell exceeds, by an order of magnitude, the demand for 2-oxoglutarate as a precursor for glutamate synthesis. Therefore, operation of NADP⁺-linked isocitrate dehydrogenase specifically for the purpose of NADPH formation would result in an over-production of 2-oxoglutarate. As a consequence, NADPH generation by this way would require export of citrate or isocitrate from, and import of 2-oxoglutarate into the mitochondria (Fig. 1). Such an exchange of a tricarboxylic acid for a dicarboxylic acid is likely to require energy. Therefore, synthesis of NADPH will contribute to the ATP requirement for biomass formation. Since in calculations of $Y_{\text{ATP}}^{\text{max}}$ a possible ATP requirement for NADPH synthesis has so far been neglected (Stouthamer, 1973) the calculated $Y_{\text{ATP}}^{\text{max}}$ values may be over-estimated. The amount of energy consumed in the transport of isocitrate and 2-oxoglutarate over the mitochondrial membrane is not known. Only if it is assumed that NADPH formation proceeds exclusively via the HMP pathway, the energy requirement for NADPH production can be calculated. Neglecting the energy costs for NADPH formation, the $Y_{\text{ATP}}^{\text{max}}$ for yeasts growing with glucose and ammonia is calculated to be 35 (P. M. Bruinenberg, unpublished). Taking a requirement of minimal 1/12 ATP per NADPH into account leads to a small decrease in the $Y_{\text{ATP}}^{\text{max}}$ to 34. With nitrate as the nitrogen source this decrease is much higher and the $Y_{\text{ATP}}^{\text{max}}$ in this case is 32. Although the effect of an ATP requirement for NADPH synthesis is only slight during growth on glucose, it may be considerable during growth with other carbon sources. For example, neglecting an ATP requirement for NADPH synthesis, a $Y_{\text{ATP}}^{\text{max}}$ of 18 can be calculated for growth of yeasts on methanol via the xylulose phosphate pathway of formaldehyde fixation with nitrate as the nitrogen source (W. Harder & J. P. van Dijken, unpublished). However, taken into consideration that the synthesis of NADPH during growth on methanol costs 1/2 ATP per NADPH [equations (10)–(12)] the $Y_{\text{ATP}}^{\text{max}}$ decreases to 13.6.

DISCUSSION

The present state of knowledge concerning the biochemistry of intermediary carbon metabolism in yeasts points to the HMP pathway as an important source of NADPH for biosynthesis. In our calculations also a major contribution of the NADP⁺-dependent isocitrate dehydrogenase was taken into consideration. However, at present it is not known whether this enzyme is indeed functioning for the purpose of NADPH synthesis or whether it only serves to generate cytoplasmic 2-oxoglutarate for the biosynthesis of glutamate and glutamine, a process which yields only small amounts of NADPH. When it does serve to synthesize NADPH in amounts exceeding that of 2-oxoglutarate, this process must involve a transport of the excess 2-oxoglutarate into the mitochondria (Fig. 1).

Both mechanisms of NADPH synthesis in yeasts consume metabolic energy. This is probably a general property of NADPH-producing systems in living cells. As pointed out by Atkinson (1977) an equilibrium constant for the reaction, $\text{NADH} + \text{NADP}^+ \rightleftharpoons \text{NAD}^+ + \text{NADPH}$, of

approximately one and the low NADH/NAD⁺ ratio in cells as compared to NADPH/NADP⁺ make it difficult to envisage that NADPH synthesis does not require energy. It seems therefore likely that growth of micro-organisms under conditions which require large amounts of NADPH will have consequences for the energy budget of the cell and that in theoretical calculations on the ATP requirement for bacterial biomass production the Y_{ATP}^{max} may have been over-estimated.

Varying NADPH requirements under different environmental conditions must also have consequences for the activity of NADPH-producing pathways. In yeasts, a high NADPH requirement must result in increased flows over the HMP pathway and the NADP⁺-linked isocitrate dehydrogenase. In our calculations it was attempted to quantify this phenomenon. It was shown that the carbon flow over the HMP pathway must vary with the growth conditions in order to satisfy the NADPH requirement for biosynthesis. This even holds when the contribution of the NAD⁺-linked isocitrate dehydrogenase in the dissimilation of acetyl-CoA is neglected. Also growth on non-sugar substrates may require the operation of the HMP pathway as a source of NADPH, for example during growth on methanol or on acetate with nitrate as a nitrogen source. Our calculations show the importance of fructose-1,6-bisphosphatase during growth with glucose. When this enzyme is operative the NADPH yield per glucose 6-phosphate oxidized in the HMP pathway increases twofold, which may be essential for organisms which are confronted with high NADPH requirements in biomass formation (i.e. nitrate utilization, or a high fat content of the cell). However, simultaneous operation of the HMP pathway with this enzyme and glycolysis would mean futile cycling by fructose 6-phosphate and fructose-1,6-bisphosphate.

So far, little is known about the activity of the HMP pathway during growth on non-sugar carbon sources. Only for growth on glucose have estimations of its activity been performed with radiorespirometry. Although the various estimates for a single organism by different authors show substantial variation, it is nevertheless clear that the activity of the HMP pathway in different organisms shows significant differences. For *Saccharomyces cerevisiae* it has been established that 2–30% of the glucose dissimilated is oxidized in the HMP pathway (Suomalainen & Oura, 1971; Gancedo & Lagunas, 1973; Mian *et al.*, 1974). For *Candida utilis* and *Rhodotorula* yeasts higher values were obtained, 30–50% and 60–80%, respectively (Suomalainen & Oura, 1971; Mian *et al.*, 1974). Our calculations show that, during growth on glucose with NH₄⁺, at the most 10% of the dissimilatory glucose must be oxidized in the HMP pathway in order to meet the NADPH requirement for biosynthesis. Therefore, activities of the HMP pathway exceeding the required theoretical level point to a mechanism for the dissimilatory oxidation of NADPH. Experiments with isolated mitochondria and submitochondrial particles of plants and fungi indicate that this may occur *in vivo* (Schuurmans Stekhoven, 1966; Møller & Palmer, 1981; Schwitzguebel & Palmer, 1981; Palmer & Møller, 1982).

From the calculations presented in this paper it will be clear that additional radiorespirometric and enzyme studies will be needed to answer the question to what extent the NADPH requirement of biosynthesis prescribes the flow over NADPH-producing pathways in yeasts. As pointed out above, adjustment of NADPH production to the demand of biosynthesis may not occur at all when the activity of the HMP pathway or another NADPH-generating route greatly exceeds that required for biomass formation. It is evident, however, that a better understanding of quantitative and qualitative aspects of NADPH consumption and production will extend our knowledge of various aspects of biomass formation and of overflow metabolism, such as fat or polyol accumulation, both in eukaryotic and prokaryotic organisms.

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