Biochem. J. (1987) 245, 263–267 (Printed in Great Britain)

The levels of nicotinamide nucleotides in liver microsomes and their possible significance to the function of hexose phosphate dehydrogenase

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The concentrations of NAD and NADP have been determined in detergent extracts of washed rat liver microsomes. Precautions were taken during the preparation of the microsomes to remove nicotinamide nucleotides from their external surface both by hydrolysis by nucleotide pyrophosphatase (EC 3.6.1.9) and by washing them three times in 0.15 M-Tris/HCl, pH 8.0, to remove soluble proteins which bind these nucleotides. The mannose phosphatase was essentially completely latent, indicating that the microsomes were intact. Assuming these nucleotides are in the cisternae of the microsomes, the concentrations in the cisternae are 240 ± 25 μM-NAD and 55 ± 12 μM-NADP. These levels of nucleotides are compatible with both the glucose:NAD^+ and the glucose 6-phosphate:$NADP^+$ oxidoreductase activities of hexose phosphate dehydrogenase (EC 1.1.1.47). Since the organ and subcellular distributions of this dehydrogenase and glucose-6-phosphatase are similar, and P_i stimulates the glucose:NAD^+ oxidoreductase activity, it is proposed that the combined action of these two enzymes leads to the reduction of both coenzymes in the lumen of the endoplasmic reticulum. A modification of the colorimetric method of Nisselbaum & Green [(1969) Anal. Biochem. **27**, 212–217] for the determination of $NADP^+$ is described. Colour formation is linear with the concentration of $NADP^+$ and is sensitive to less than 0.3 nmol of $NADP^+$.

INTRODUCTION

One reason that the metabolic function of hexose phosphate dehydrogenase (EC 1.1.1.47) is unknown is that its physiological substrates remain unidentified. Several years ago kinetic measurements led Beutler & Morrison to suggest that the enzyme oxidizes either glucose 6-phosphate or galactose 6-phosphate by $NADP^+$ [1]. Later Horne & Nordlie suggested that the enzyme also may be a glucose: NAD^+ oxidoreductase *in vivo* since several anions increase its affinities for glucose and NAD^+ [2]. More recently a kinetic study of the enzyme led Campbell *et al.* to suggest that the enzyme may exhibit both glucose phosphate:$NADP^+$ and glucose:NAD^+ oxidoreductase activities [3]. The hypothesis that the enzyme may reduce both nicotinamide nucleotides, which was suggested by these kinetic studies, would be greatly strengthened if its nucleotide substrates were shown to be present at high enough concentrations to be substrates in the same location in the cell as the enzyme. Hexose phosphate dehydrogenase is found in the lumen of the endoplasmic reticulum or in the cisternae of microsomes [4,5]. Microsomes are permeable only to uncharged compounds and to those charged compounds with a translocase, such as P_i, carbamoyl phosphate, PP_i and glucose 6-phosphate [6–8]. Many years ago Glock & McLean found microsomes to have about four times as much NAD^+ as $NADP^+$ [9]. More recently, Hino & Minikami measured the NADP content by a novel isotopic method [10]. This paper reports measurements of the concentrations of both nicotinamide nucleotides in alkaline, detergent extracts of intact microsomes which were treated with nucleotide pyrophosphatase and washed three times in 0.15 M-Tris/HCl, pH 8.0, containing 0.1 M-nicotinamide. The procedure used to assay $NADP^+$ is a modification of the method of Nisselbaum & Green [11], which is sensitive to less than 0.3 nmol. The significance of the observed concentrations of coenzymes on the possible role of hexose phosphate dehydrogenase also is discussed.

MATERIALS AND METHODS

Chemicals

The following reagents were obtained from Sigma: NAD^+, $NADP^+$, NADH, NADPH, PES, glucose-6-phosphate dehydrogenase (catalogue no. G-4134), MTT, bovine serum albumin and nucleotide pyrophosphatase (EC 3.6.1.9) from *Crotalus atrox*.

Microsomes

Rat liver microsomes were prepared essentially by the methods of Dallner [12] and Glock & McLean [9] in the cold. Diced livers from rats that had been starved for 16 h were homogenized in 3 vol. of 0.25 M-sucrose/0.1 M-nicotinamide in a Teflon/glass homogenizer. The homogenate was centrifuged at 17600 *g* for 10 min. Microsomes were collected by centrifuging the supernatant fluid at 105000 *g* for 30 min. The microsomes

Abbreviations used: PMS, 5-methylphenazinium methyl sulphate; PES, 5-ethylphenazinium ethyl sulphate; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NAD, NAD^+ + NADH; NADP, $NADP^+$ + NADPH.

from 7 g of liver were suspended in a final vol. of 3.0 ml containing 0.15 M-Tris/HCl, pH 7.4, 0.1 M-nicotinamide, 25 mM-$MgCl_2$ and 0.33 unit of nucleotide pyrophosphatase/ml. The suspension was shaken for 30 min in an ice bath. Then 18 ml of 0.15 M-Tris/HCl, pH 8.0, containing 5 mM-EDTA and 0.1 M-nicotinamide was added to the suspensions. The microsomes were collected by centrifugation as above and then washed twice in 0.15 M-Tris/HCl, pH 8.0, containing 0.1 M-nicotinamide. The washed microsomes were suspended in 1.0 ml of 0.25 M-sucrose/0.1 M-nicotinamide per 25 g of liver used.

Analytical methods

Protein was measured by the method of Lowry *et al.* [13], succinate: cytochrome *c* reductase by the method of Fleischer & Fleischer [14], NADPH:cytochrome *c* reductase by the method of Phillips & Langdon [15], NAD by the procedure of Bernofsky & Swan [16], and NADP by the modification of the method of Nisselbaum & Green [11] described below. Calculations of the rate of cytochrome *c* reduction were made using the absorption coefficient of 18.5×10^6 $M^{-1} \cdot cm^{-1}$ [17]. All enzyme assays were done at room temperature unless otherwise specified. The latency of mannose-6-phosphatase at low substrate concentration in washed microsomes was determined at 30 °C essentially by the method of Arion *et al.* [18]. The test system contained, in 1.0 ml: 30 μmol of cacodylate, pH 6.5, 1 μmol of mannose 6-phosphate and 0.03 ml of a suspension of freshly prepared thrice-washed microsomes. The microsomes were disrupted by 0.4 mg of Tergitol NP-10. The complete system, except mannose phosphate, was incubated for 10 min at 30 °C, and 10 min after the addition of mannose phosphate the reaction was stopped by the addition of 0.68 ml of 7% (w/v) $HClO_4$ and 0.57 ml of water. The precipitated protein was removed by centrifugation. Ethylene dichloride (2 ml) was added to each supernatant solution, and the tubes were shaken for 10 min. The content of P_i in the aqueous phases was determined by the method of Marinetti [19]. Latency is used as defined by Arion *et al.* [20] as the percentage of activity of disrupted microsomes that is unexpressed in intact microsomes and is calculated as:

$$\text{latency } (\%) = 100 \times \frac{(\text{activity in disrupted microsomes}) - (\text{activity in untreated microsomes})}{(\text{activity in disrupted microsomes})}$$

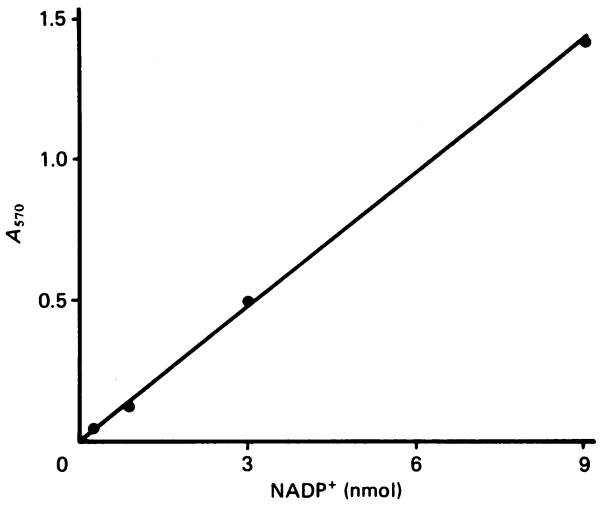

Fig. 1. Standard curve for NADP+

The system contained, in a volume of 0.8 ml: 120 μmol of Tris/HCl, pH 8.0, 0.5 μmol of MTT, 2 μmol of PES, 2 μmol of glucose 6-phosphate, 10 μg of glucose-6-phosphate dehydrogenase, 1 μmol of EDTA, 0.12 mg of bovine serum albumin and $NADP^+$ as noted. The reaction was started by the addition of PES. The tubes were incubated in the dark for 30 min at 30 °C. The reaction was stopped by the addition of 0.01 ml of 10% (w/v) SDS. The absorbance at 570 nm was determined in a colorimeter. Values were corrected for samples without added $NADP^+$.

Concentrations of NAD and NADP in detergent extracts of microsomes

In order to maximize the likelihood that the cisternae are the source of the nicotinamide nucleotides, the microsomes were treated with nucleotide pyrophosphatase to hydrolyse nucleotides bound to the external surface and washed three times with 0.15 M-Tris/HCl, pH 8.0, containing 0.1 M-nicotinamide to remove from the external surface adhering proteins capable of binding nucleotides. The excellent ability of the microsomes to retain nucleotides is indicated by the essentially complete latency of mannose phosphatase, which was measured at 30 °C [21] (Table 2). The level of detergent used to disrupt the microsomes in this experiment was that which another experiment indicated optimally stimulated mannose-6-phosphatase in these microsomes. The content of the nicotinamide nucleotides in these microsomes was measured in detergent extracts which were supplemented with EDTA and nicotinamide to reduce the destruction of the nucleotides, especially NAD^+. The extracts were centrifuged and the levels of the nicotinamide nucleotides measured in the supernatant fluid, which was treated with cold 0.04 M-NaOH which Burch *et al.* [22] found to be a suitable extraction solution for

RESULTS

Microassay for NADP+

The method of Nisselbaum & Green for the estimation of NADP depends on the reduction of MTT by NADPH generated by glucose 6-phosphate and glucose-6-phosphate dehydrogenase at rate-limiting concentrations of $NADP^+$. PMS mediates the reduction of MTT by NADH. When PMS was replaced by PES and the buffer was changed, the reductions of MTT were erratic. PES is more stable than PMS in these systems [16]. However, the addition of bovine serum albumin and EDTA to the modified system produced consistently good standard curves (Fig. 1). The data in Fig. 2(*a*) show that the time course is linear under the conditions tested. These data (Fig. 2*b*) also indicate that the rate of reduction of MTT is directly proportional to the amount of glucose-6-phosphate dehydrogenase added. The system is saturated with MTT but not PES (Table 1).

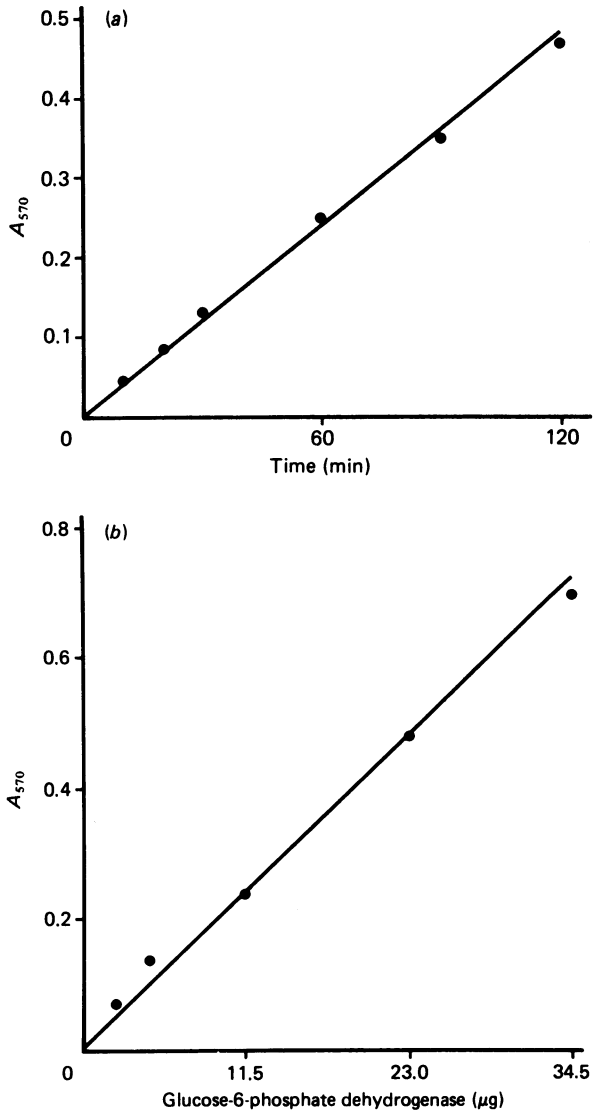

Fig. 2. Reduction of MTT as a function of time and concentration of glucose-6-phosphate dehydrogenase

(*a*) The conditions were the same as in Fig. 1 except that the tubes contained 3.6 nmol of NADP and incubations were for the identical times. (*b*) The conditions were as described in Fig. 1 except that glucose-6-phosphate dehydrogenase was added as noted.

both the oxidized and reduced forms of the nicotinamide nucleotides. Preliminary experiments showed that the alkali-treated extracts that had been centrifuged contained higher levels of both nucleotides than did water-treated, centrifuged extracts or alkali-treated extracts which were not centrifuged. The alkali will denature most proteins in the extract that may compete with the dehydrogenases used in the assays for the nucleotides. The data in Table 2 show that the recovery of added $NADP^+$ and NAD^+ in these extracts is quite good. The results in Table 2 also show that the concentration of NAD is about 4 times that of NADP in these extracts. Assuming that the luminal volume of microsomes is 2.5 μl/mg of protein [6,8,23] and that washing the microsomes with EDTA removes 30% of the protein without disrupting the lipid structure [24], the

Table 1. Effects of various concentrations of PES and MTT on the reduction of MTT

The conditions are those described in Fig. 1 except for the levels of PES and MTT, which were as noted.

[PES] (mM)	[MTT] (mM)	A_{570}
0.5	0.5	0.125
1.0	0.5	0.241
2.0	0.5	0.298
3.0	0.5	0.560
2.0	0.25	0.200
2.0	0.5	0.298
2.0	0.75	0.281
2.0	1.0	0.265

estimated concentrations in the lumen are 240 ± 25 μM-NAD and 55 ± 12 μM-NADP. The estimated concentration of NADP is about three-fourths of the value of 75 μM-NADP calculated from the data of Hino & Minikami [10] on the assumption that the cisternal volume is 2.5 μl/mg of protein. Their higher value probably can be attributed to their use of once-washed microsomes in an ionic medium with the omission of the nucleotidase treatment. The data also show that essentially all the coenzymes are extracted by a single treatment with detergent.

The concentration of NAD^+ is about 12 times higher than the K_m for NAD^+ (about 20 μM-NAD^+ for glucose: NAD^+ oxidoreductase activity of hexose phosphate dehydrogenase) in the presence of activating anion or 4 times that of the K_m (about 60 μM-NAD^+ for the glucose:NAD^+ oxidoreductase) without anions [2]. However, it should be stressed that the K_m for glucose is very high (about 0.33–1.25 M [2]). The estimated cisternal concentration of $NADP^+$ is about one-sixth of the K_m for $NADP^+$ (0.3 mM-$NADP^+$ for glucose 6-phosphate: NADP oxidoreductase [25]). Hence the nucleotide concentrations by themselves would favour the glucose: NAD^+ oxidoreductase over the glucose 6-phosphate: $NADP^+$ oxidoreductase, However, these results are consistent with either nucleotide as the substrate for hexose phosphate dehydrogenase.

DISCUSSION

Substrate specificity studies and kinetic studies have led several investigators to conclude that hexose phosphate dehydrogenase has the potential to serve both as glucose:NAD^+ and glucose 6-phosphate:$NADP^+$ oxidoreductases. The evidence that the latter activity of the enzyme is important *in vivo* is stronger than that favouring the glucose:NAD^+ oxidoreductase activity. This evidence consists of the much higher affinity of the enzyme for glucose 6-phosphate than for glucose [1], a translocase which transports glucose 6-phosphate from the cytoplasm to the lumen of the endoplasmic reticulum, which is the site of hexose phosphate dehydrogenase, the presence of NADP in microsomes [10] which is confirmed by an independent method in the present paper, and the demonstration that microsomes contain a unique 6-phosphogluconate dehydrogenase

Table 2. Concentrations of nicotinamide nucleotides in microsomal cisternae

Separate preparations of liver microsomes (0.68 ± 0.06 mg of protein) from six rats were treated with nucleotide pyrophosphatase, washed three times and suspended by brief (10 s) sonication in a total of 1 ml of a solution containing 0.1 M-nicotinamide, 2 mM-EDTA and 0.8% Tergitol NP-10. A duplicate set of these suspensions supplemented with 0.5 nmol of NAD^+ and 0.15 nmol of $NADP^+$ was prepared to measure recovery of added nucleotides. The extracts were clarified by centrifugation at 105000 *g* for 30 min. The clarified supernatant fractions (0.6 ml) were added to 0.2 ml of 0.16 M-NaOH. NAD^+ and $NADP^+$ were assayed in 0.2 ml and 30 μl aliquots respectively. Extracts were prepared at 2 °C. The concentrations of nucleotides in the microsomal cisternae were calculated on the assumption that the cisternal volume is 2.5 μl/mg of protein. Corrections were made for recoveries of added nucleotides and the loss of protein due to the wash with EDTA. The microsomes reduced cytochrome *c* with NADPH and succinate at the rates of 58 ± 25 nmol/min per mg and 2.02 ± 0.53 nmol/min per mg respectively. The mannose-6-phosphatase in the washed microsomes had a latency of 93% ± 1.1%. Mean values ± S.D. are in the Table. Microsomes from a seventh rat liver were prepared in the manner described above except that in place of the treatment with nucleotide pyrophosphatase, the unwashed microsomes were suspended in 0.2% Tergitol NP-10/0.15 M-Tris/HCl (pH 8.0)/0.1 M-nicotinamide (1.5 ml/8 g of liver) and shaken in an ice bath for 30 min. The microsomes were collected by centrifugation as described above. They were washed twice in 0.15 M-Tris/HCl, pH 8.0, containing 0.1 M-nicotinamide and suspended in 0.25 M-sucrose (1 ml/25 g of liver). The concentrations of NAD and NADP were determined in this suspension using the procedure described above. The 'cisternal' concentrations, calculated in the manner described above, were: 0 μM-NAD and 8 μM-NADP.

	Recovery of added nucleotide (%)	Concentration in microsomal cisternae (μM)
NAD	75 ± 5.2	240 ± 25
NADP	55 ± 4.6	55 ± 12

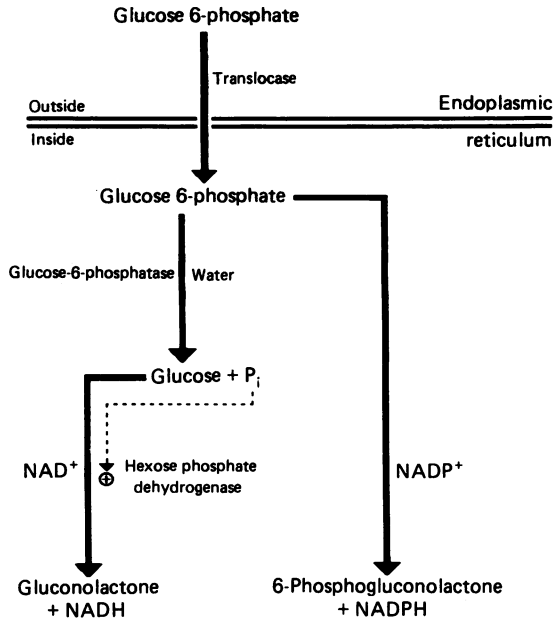

Fig. 3. Reduction of $NADP^+$ and NAD^+ in the lumen of the endoplasmic reticulum by hexose phosphate dehydrogenase and glucose-6-phosphatase

which would double the rate of reduction of $NADP^+$ in the lumen over that due to the glucose-6-phosphate dehydrogenase activity of hexose phosphate dehydrogenase alone [10,26]. On the other hand, the evidence for the importance of the glucose:NAD^+ oxidoreductase activity *in vivo* rests entirely on kinetic studies [2,3]. The observations by Horne & Nordlie [2] that bicarbonate, sulphate and phosphate increase the affinities of hexose phosphate dehydrogenase for glucose and NAD^+ offer special support for the importance of the glucose:NAD^+ oxidoreductase activity. The data presented in the present paper provide evidence that NAD is found in the same subcellular compartment as hexose phosphate dehydrogenase at high enough concentrations to be a substrate for this dehydrogenase. Since glucose freely penetrates the microsomal membrane [8], it may diffuse into the lumen of the endoplasmic reticulum. Because of the uncertainty of the intracellular role of glucose, a more likely source of glucose within the endoplasmic reticulum is the hydrolysis of glucose 6-phosphate by glucose-6-phosphatase. According to this scheme (Fig. 3), glucose 6-phosphate enters the lumen of the endoplasmic reticulum by its translocase [6]. In the lumen, glucose 6-phosphate is either oxidized by $NADP^+$ by the glucose-6-phosphate:$NADP^+$ oxidoreductase activity of hexose phosphate dehydrogenase or hydrolysed by glucose-6-phosphatase to form free glucose and P_i. Some of the glucose then may be oxidized by NAD^+ by the glucose:NAD^+ oxidoreductase activity of hexose phosphate dehydrogenase. Significantly, one of the products of the glucose-6-phosphatase reaction is the substrate for the latter activity of hexose phosphate dehydrogenase while the other product, P_i, stimulates this activity. Substrate specificity studies of this dehydrogenase have shown that the more effective reductant of $NADP^+$ is glucose 6-phosphate, whereas glucose more efficiently reduces NAD^+ [1]. This hypothesis also is supported by the parallel distributions of the phosphatase and the dehydrogenase. Both enzymes are widely distributed in animal tissues [27–29], are found in the cisternae of microsomes [4,27], are rich in the endoplasmic reticulum [5,27], and have the same regional distribution in liver [30]. Since hexose phosphate dehydrogenase is the only dehydrogenase known in the lumen which is capable of reducing either NAD^+ or $NADP^+$, it can be assumed, at least until another dehydrogenase is found in this region of the cell, that hexose phosphate dehydrogenase is reducing both nucleotides. The function of these reduced nicotinamide nucleotides within the lumen is unknown at this time. A dual function for hexose phosphate dehydrogenase in the lumen may be important in explaining the quite different ratios of glucose-6-phosphate dehydrogenase: phosphogluconate dehydrogenase found in the soluble fraction and in the microsomes. The former activity is greater in the microsomes [10] while the latter activity predominates in the soluble fraction [31].

These results also raise the question as to the

mechanism whereby these nucleotides are transported into the cisternae. Uncharged compounds of M_r up to at least 600 readily penetrate the microsomal membrane [8] but the only charged compounds which penetrate into microsomes are those which have a translocase. However, there are no known translocases for the coenzymes. Indeed, the latency of hexose phosphate dehydrogenase is due to the inability of its coenzyme substrate to penetrate into microsomes [4]. Further investigations are necessary to determine the mechanism of entry of the coenzymes into the microsomes.

This investigation was supported by grant PCM 82-04120 from the National Science Foundation. The authors thank the journal's anonymous reviewer for useful suggestions.

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Received 8 January 1987/8 April 1987; accepted 23 April 1987