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The mobilization of ferritin iron by liver cytosol

A comparison of xanthine and NADH as reducing substrates

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Considerable evidence suggests that the release of iron from ferritin is a reductive process. A role in this process has been proposed for two hepatic enzymes, namely xanthine oxidoreductase and an NADH oxidoreductase. The abilities of xanthine and NADH to serve as a source of reducing power for the enzyme-mediated release of ferritin iron (ferrireductase activity) were compared with turkey liver and rat liver homogenates. The maximal velocity ($V_{max.}$) for the reaction with NADH was 50 times greater than with xanthine; however, the substrate concentration required to achieve half-maximal velocity (K_m) was 1000 times less with xanthine than with NADH. NADPH could be substituted for NADH with little loss in activity. Dicoumarol did not inhibit the reaction with NADH or NADPH, demonstrating that the ferrireductase activity with those substrates was not the result of the liver enzyme 'DT-diaphorase' [NAD(P)H dehydrogenase (quinone)]. A flavin nucleotide was required for ferrireductase activity with rat and turkey liver cytosol when xanthine, NADH or NADPH was used as the reducing substrate. FMN yielded twice the activity with NADH or NADPH, whereas FAD was twice as effective with xanthine as substrate. Kinetic comparisons, differences in lability and partial chromatographic resolution of the ferrireductase activities with the two types of reducing substrates strongly indicate that the ferrireductase activities with xanthine and NADH are catalysed by separate enzyme systems contained in liver cytosol. Complete inhibition by allopurinol of the ferrireductase activity endogenous to undialysed liver cytosol preparations and the ability of xanthine to restore equivalent activity to dialysed preparations indicate that the source of reducing power for the endogenous activity is xanthine. These studies suggest that xanthine, NADH or NADPH can serve as a source of reducing power for the enzyme-mediated reduction of ferritin iron, with a flavin nucleotide serving as the shuttle of electrons from the enzymes to the ferritin iron.

INTRODUCTION

Ferritin serves a key role in iron metabolism as the primary iron-storage protein of animal tissues. In mammals ferritin is found in all tissues, but exists principally in liver, spleen and bone marrow. It accounts for the major pool of non-haem iron and approx. 25% of total body iron (Crichton & Charloteaux-Wauters, 1987). The ferritin molecule consists of two components: an inorganic hydrated ferric oxide core containing some phosphate and a multi-subunit protein shell.

The iron stored in ferritin is mobilized as needed for the synthesis of haemoglobin, myoglobin, and other important iron-containing proteins and enzymes. Neither the nature of the iron mobilizing agent *in vivo* nor the cellular site of ferritin iron mobilization have been clearly defined. *In vitro* iron can be mobilized from ferritin either by direct chelation of Fe^{3+} or by reduction of Fe^{3+} to Fe^{2+}. Direct chelation of ferritin iron by biological chelators (Mazur *et al.*, 1955; Pape *et al.*, 1968; Dognin & Crichton, 1975), synthetic chelators (Pape *et al.*, 1968; Tuffano *et al.*, 1981) and microbial siderophores (Dognin *et al.*, 1973; Crichton *et al.*, 1980; Tidmarsh *et al.*, 1983) is slow compared with the much greater rates of iron release observed with reducing agents such as dithionite, reduced flavins or thioglycollate (Sirivech *et al.*, 1974; Crichton *et al.*, 1975; Jones *et al.*, 1978; Funk *et al.*, 1985).

Reduction appears to be the more plausible mechanism *in vivo* principally because mobilization of iron from ferritin can be accomplished at physiologically significant rates. Two enzymes have been implicated in the reductive release of iron from liver ferritin stores. Green & Mazur (1957) first demonstrated that liver xanthine oxidoreductase, acting as a dehydrogenase, could reduce and liberate iron from ferritin. Several additional studies (Mazur *et al.*, 1958; Mazur & Carleton, 1965; Duggan & Streeter, 1973; Topham *et al.*, 1982; Cohen *et al.*, 1985; Bolann & Ulvik, 1987) have suggested a possible specific role for liver xanthine oxidoreductase in the release of ferritin iron, although the participation of this enzyme *in vivo* remains a matter of debate. Osaki & Sirivech (1971) identified a liver oxidoreductase that could reductively liberate iron from ferritin using NADH as the reducing substrate and FMN as a shuttle of electrons to the ferritin iron. On the basis of studies *in vivo*, Sirivech *et al.* (1977) suggested that this enzyme (designated 'NADH:FMN oxidoreductase') could be a controlling factor in the release of iron from ferritin. These workers found that allopurinol did not inhibit the activity of this enzyme and concluded that this enzyme was not xanthine oxidoreductase. However, liver xanthine oxidoreductase will oxidize NADH (Waud & Rajagopalan, 1976*a*; Cleere & Coughlan, 1975), and the oxidation of NADH by xanthine oxidoreductase is not inhibited by allopurinol. Thus the possibility remains that xanthine oxido-

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reductase could be responsible for the enzyme-mediated release of iron from ferritin with both xanthine and NADH as reducing substrates.

In view of the important role that the mobilization of iron from liver ferritin plays in the maintenance of a relatively constant plasma iron concentration and the uncertainty concerning the molecular mechanism involved in ferritin iron mobilization, we initiated an investigation of the reductive release of ferritin iron by liver homogenates. The goal of the present study was twofold: (1) to compare xanthine and NADH as reducing substrates for the enzyme-mediated mobilization of iron from ferritin catalysed by liver homogenates; and (2) to determine whether liver xanthine oxidoreductase alone, or two separate enzyme systems, are responsible for the enzymic mobilization of ferritin iron with these reducing substrates.

MATERIALS AND METHODS

Reagents

Sucrose (Grade I), horse spleen ferritin (Type I), β-NADH (Grade III), β-NADPH (Type I), β-NAD^+ (Grade III), FMN (sodium salt, synthetic), FAD (disodium salt), glucose oxidase (Type III), β-D-glucose, dicoumarol, 2,6-dichlorophenol-indophenol and Sephadexes G-25–150 were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A. Hepes (acid form) was purchased from United States Biochemical Corp., Cleveland, OH, U.S.A. 2,2′-Dipyridyl (Fisher-certified) was purchased from Fisher Scientific, Pittsburgh, PA, U.S.A. Anaerobic cuvettes (type 26G) were purchased from NSG Precision Cells, Farmingdale, NY, U.S.A.

Preparation of liver homogenates

A 20% (w/v) homogenate of rat or turkey liver was prepared in 0.25 M-sucrose. Initially the liver tissue was suspended in 0.25 M-sucrose and minced at 4 °C in a Waring Blendor at low speed for 1 min and high speed for 3 min. The resulting homogenate was transferred to a power-driven Potter–Elvehjem tissue grinder and further homogenized at 4 °C for 1 min with the pestle rotating at 1000 rev./min. The homogenate was centrifuged at 15000 *g* for 20 min. The supernatant was removed and filtered through glass wool to remove the floating lipid layer. The filtered supernatant was centrifuged at 125000 *g* for 1 h. The supernatant obtained was carefully removed, filtered through glass wool, and dialysed (overnight with 0.025 M-Hepes buffer, pH 7.4, containing 0.25 M-sucrose) to remove reducing substrates endogenous to the preparation. The dialysed samples were frozen in small portions and stored at −30 °C. These samples could be stored for several weeks without a significant loss of activity. With rat liver preparations it was important to start with freshly excised and not frozen livers when analysing activities with xanthine as reducing substrate.

When microsomes (microsomal fractions) were analysed for activity, the pellets resulting from centrifugation at 125000 *g* were resuspended in 0.025 M-Hepes buffer, pH 7.4, containing 0.25 M-sucrose. These microsomal preparations were analysed for enzyme activity immediately after resuspension of the pellet.

Assay of enzyme activities

The reductive release of iron from ferritin (referred to as 'ferrireductase activity' in the present paper) was measured continuously as the formation of the Fe^{2+}–2,2′-dipyridyl complex in anaerobic cuvettes at 530 nm with a Hewlett–Packard model 8452A or Beckman Acta C-III spectrophotometer. Unless otherwise specified, the 2.0 ml reaction mixture contained 25 mM-Hepes buffer, pH 7.4, 4.50 μM horse spleen ferritin, 1.25 mM-2,2′-dipyridyl, 0.1 mM-FMN, 55 units of glucose oxidase, 1 mM-β-D-glucose, 250 μl of the liver cytosol or microsomal preparation and 1 mM-NADH (or 1 mM-NADPH or 50 μM-xanthine). The glucose–glucose oxidase system was included in the assay to ensure that anaerobic conditions were obtained and maintained. It was previously reported (Osaki & Sirivech, 1971; Sirivech *et al.*, 1974) that the liver NADH:FMN oxidoreductase is inhibited by O_2 concentrations of greater than 3 μM. All assay components were placed in the body of the anaerobic cuvettes, with the exception of the enzyme preparation, which was placed in the side arm of the cuvette. Glucose oxidase (50 μl, 14 units) and β-D-glucose (50 μl, 0.5 μmol) were added to the side arm to make the enzyme preparation anaerobic before its addition to the assay mixture. After 5 min at 25 °C, the reaction was initiated by introduction of the enzyme preparation from the side arm. An assay in which the enzyme preparation was deleted was always run to determine the non-enzymic rate of ferritin iron reduction. An assay in which the reducing substrate (NADH or xanthine) was deleted was also run to permit the determination of any rate of ferritin iron reduction endogenous to the enzyme preparation. The endogenous rate was virtually abolished after dialysis of the liver cytosol preparations to remove endogenous reducing substrates. The non-enzymic activity and any endogenous activity remaining after dialysis were subtracted from the total activity to determine the true enzymic activity. This

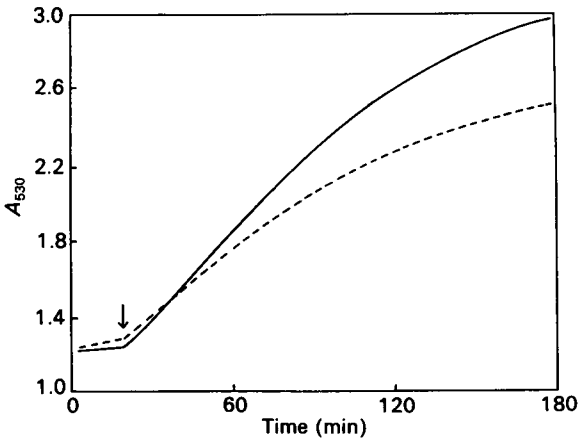

Fig. 1. Time course for the ferrireductase activity of turkey liver cytosol with xanthine and with NADH as reducing substrate

Turkey liver cytosol was prepared and ferrireductase activities with xanthine and NADH as reducing substrates were monitored as described in the Materials and methods section. ——, Time course of the reaction with 200 μM-xanthine; – – – –, time course of the reaction with 200 μM-NADH; the arrow shows the point at which turkey liver cytosol was added from the side arm of the cuvette.

assay, employing 2,2′-dipyridyl to monitor the reductive release of iron from ferritin, has been used and validated in numerous previous studies (Osaki & Sirivech, 1971; Duggan & Streeter, 1973; Sirivech *et al.*, 1974, 1977; Jones *et al.*, 1978; Funk *et al.*, 1985; Bolann & Ulvik, 1987).

The rates of FMN reduction catalysed by liver cytosol preparations with xanthine and NADH as sources of reducing power were monitored as the change in absorbance at 450 nm. In these assays, ferritin and 2,2′-dipyridyl were deleted from the reaction mixtures.

'DT-diaphorase' [NAD(P)H dehydrogenase (quinone)] activity was measured continuously as the reduction of dichlorophenol-indophenol at 600 nm with NADH or NADPH as substrate (Ernster, 1967). Assays were conducted in anaerobic cuvettes. The 2.0 ml reaction mixture contained 25 mM-Hepes buffer, pH 7.4, 55 units of glucose oxidase, 1 mM-β-D-glucose, 2.5 μl of liver cytosol and 1 mM-NADH (or 1 mM-NADPH). The reaction was initiated by the introduction of the reducing substrate (NADH or NADPH) from the side arm of the anaerobic cuvette. Diaphorase activity was inhibited by including 1 μM-dicoumarol in the final reaction mixtures.

RESULTS AND DISCUSSION

Time course, heat-lability, and cellular location of the enzyme-mediated release of ferritin with NADH and xanthine

Previous studies (Green & Mazur, 1957; Mazur *et al.*, 1958; Mazur & Carleton, 1965) suggested that the dehydrogenase form of xanthine oxidoreductase could reduce and liberate ferritin iron. *In vivo*, hepatic xanthine oxidoreductase exits as a NAD^+-linked dehydrogenase (Waud & Rajagopalan, 1976*a*). *In vitro*, the rat liver enzyme is unstable as a dehydrogenase and is gradually converted into an oxidase (Waud & Rajagopalan, 1976*b*). In contrast, avian xanthine oxidoreductases are stable as dehydrogenases (Cleere & Coughlan, 1975). Thus initial comparisons of the reductive release of ferritin iron with xanthine and NADH were conducted with cytosol prepared from turkey liver homogenates to eliminate any conversion of xanthine oxidoreductase from the dehydrogenase to the oxidase form. Fig. 1 shows the time course of the reductive release of iron from ferritin catalysed by dialysed turkey liver cytosol with equivalent concentrations (200 μM) of xanthine and NADH as reducing substrates. Similar time-course curves were obtained with rat liver cytosol prepared from freshly excised livers. Boiling of the turkey and rat liver cytosol preparations resulted in the complete loss of the ferrireductase activity with xanthine and NADH.

In their initial report of the identification of a liver enzyme system that could catalyse the reductive release of iron from ferritin using NADH as a source of reducing power, Osaki & Sirivech (1971) indicated that the enzyme activity was mainly localized in the soluble fraction of the liver cell. In later publications concerning the proposed role of the enzyme in iron metabolism, they referred to this enzyme as a 'microsomal system' (Frieden, 1973; Frieden & Osaki, 1974). In the present study these two cellular fractions were examined to determine the relative ferrireductase activities with both xanthine and NADH as reducing substrates. Virtually all (> 95%) of the activity with both reducing substrates was localized in the cytosol fraction after the final centrifugation of rat and turkey liver homogenates at 125000 *g* for 1 h.

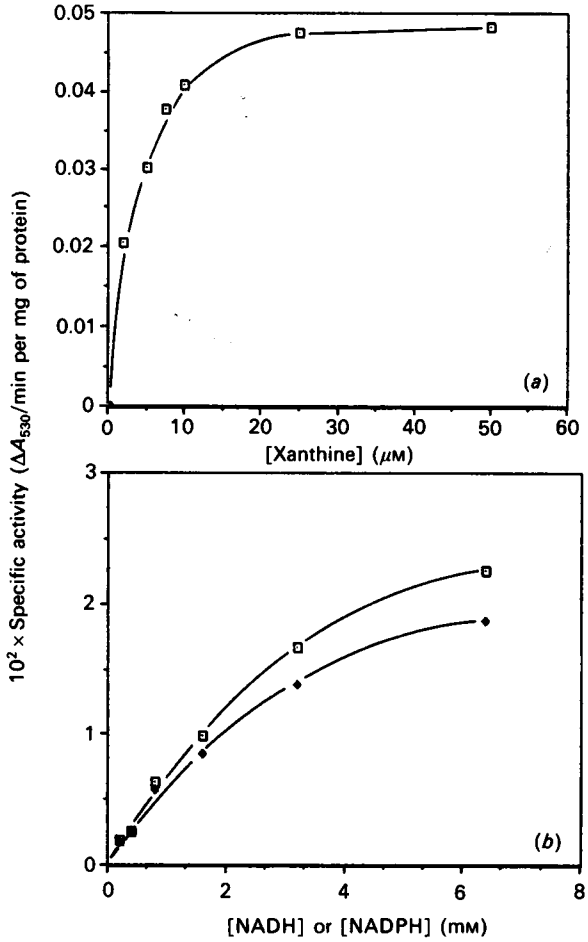

Fig. 2. Dependence of the rate of the ferrireductase reaction on the concentration of reducing substrate with rat liver cytosol

Rat liver cytosol was prepared from homogenates of freshly excised livers and dialysed to remove endogenous reducing substrates as described in the Materials and methods section. The initial rates of the ferrireductase activity of rat liver cytosol were measured with (*a*) 2–50 μM-xanthine and (*b*) 0.2–6.4 mM-NADH (□) or NADPH (◆), as described in Materials and methods section.

Substrate specificity and steady-state kinetic analyses of the enzyme-mediated release of ferritin iron

Saturation kinetics were observed with both xanthine and NADH as reducing substrates for the ferrireductase reaction catalysed by liver cytosol preparations (Fig. 2). NADPH could be substituted for NADH in the assay system and yielded rates of reaction only slightly less than those obtained with NADH (Fig. 2). Linear-regression analyses of double-reciprocal plots of the kinetic data yielded the values for K_m and $V_{max.}$ shown in Table 1. In the case of both liver cytosols, $V_{max.}$ for the ferrireductase reaction was approached at much lower concentrations with xanthine as reducing substrate than with NADH or NADPH; however, the $V_{max.}$ obtained with NADH and NADPH was approx. 50 times greater than that achieved with xanthine. A comparison of

Table 1. Steady-state kinetic parameters for the ferrireductase reaction

Initial rates for the ferrireductase activities of turkey liver cytosol and rat liver cytosol were measured with 2–50 μM-xanthine and 0.2–6.4 mM-NADH or NADPH as reducing substrate. The K_m and $V_{max.}$ values for the reaction with each reducing substrate were computed by linear-regression analyses of double-reciprocal kinetic plots.

Reducing substrate	Enzyme system	K_m (mM)	$V_{max.}$ (ΔA_{530}/min per mg of protein)	$\frac{V_{max.}}{K_m}$
Xanthine	Rat liver cytosol	3.42×10^{-3}	5.38×10^{-4}	1.57×10^{-1}
	Turkey liver cytosol	3.18×10^{-3}	5.81×10^{-4}	1.82×10^{-1}
NADH	Rat liver cytosol	2.79	2.58×10^{-2}	9.38×10^{-3}
	Turkey liver cytosol	4.00	2.91×10^{-2}	7.28×10^{-3}
NADPH	Rat liver cytosol	2.39	2.04×10^{-2}	8.54×10^{-3}
	Turkey liver cytosol	3.46	2.79×10^{-2}	8.06×10^{-3}

Table 2. Dependence of the liver ferrireductase activity on the presence of a flavin nucleotide

Rat liver and turkey liver cytosol were prepared as described in the Materials and methods section. Ferrireductase assays were conducted with 50 μM-xanthine or 1 mM-NADH or 1 mM-NADPH as reducing substrate. In assays containing flavin nucleotide, the concentration of FMN or FAD was 100 μM.

Reducing substrate	Enzyme system	Ferrireductase activity (ΔA_{530}/min per mg of protein)		
		+FMN	+FAD	− (FMN and FAD)
Xanthine	Rat liver cytosol	4.35×10^{-4}	7.63×10^{-4}	0.172×10^{-4}
	Turkey liver cytosol	5.65×10^{-4}	8.71×10^{-4}	0.169×10^{-4}
NADH	Rat liver cytosol	5.61×10^{-3}	3.30×10^{-3}	0
	Turkey liver cytosol	6.41×10^{-3}	3.10×10^{-3}	0
NADPH	Rat liver cytosol	4.97×10^{-3}	2.66×10^{-3}	0
	Turkey liver cytosol	5.59×10^{-3}	2.75×10^{-3}	0

values of $V_{max.}/K_m$ for the various reducing substrates, which may be a better measure of their relative efficiency in promoting the enzyme-mediated release of iron from ferritin, suggests that xanthine is about 20 times more efficient than NADH or NADPH.

The observation that NADPH could be substituted for NADH as a source of reducing power led to an investigation of the possibility that the ferrireductase reaction with these substrates might be mediated by the highly active DT-diaphorase known to exist in liver cytosol preparations (Ernster, 1967). Among inhibitors of DT-diaphorase, dicoumarol is one of the most potent (Ernster, 1967). The presence of 1 μM-dicoumarol in the assay completely inhibited the DT-diaphorase activity without inhibiting at all the ferrireductase activity of rat and turkey liver cytosol. This finding strongly suggests that the ferrireductase activity with NADH and NADPH is not the result of the action of liver DT-diaphorase.

Flavin-nucleotide-dependence of the ferrireductase reaction with xanthine and NADH

Several studies (Sirivech *et al.*, 1974; Jones *et al.* 1978; Funk *et al.*, 1985) have shown that biological reductants such as thiols, ascorbate, and reduced flavins are capable of reducing and releasing iron from ferritin; however, only reduced flavins mediate complete iron release at a rate sufficient to be physiologically significant (Sirivech *et al.*, 1974; Jones *et al.*, 1978). Osaki & Sirivech (1971) and Sirivech *et al.* (1974) found that FMN was an essential component in the mobilization of iron from ferritin by the NADH:FMN oxidoreductase they identified in liver tissue and proposed that FMN was serving as a shuttle of electrons from the enzyme to the iron in the core of ferritin. Thus, in our comparison of the efficiency of NADH and xanthine as reducing substrates for the ferrireductase reaction catalysed by liver cytosol preparations, FMN was routinely included as a component in the assay system. As shown in Table 2, when FMN was deleted from the assay system, the rate of enzyme-mediated iron release from ferritin was diminished by more than 96% in the case of xanthine, NADH and NADPH. The substitution of FAD for FMN in the assay system resulted in a 2-fold decrease in the rate with NADH and NADPH, but a 2-fold increase in the rate with xanthine. These results indicate that the presence of a flavin nucleotide is essential for the ferrireductase reaction with all three reducing substrates. FMN is more efficient when NADH or NADPH is the

Table 3. Comparison of the initial rates of FMN reduction and the release of ferritin iron with liver cytosol preparations

Initial rates of FMN reduction and the reductive release of ferritin iron with liver cytosol preparations and xanthine or NADH as reducing substrate were determined as described in the Materials and methods section. These assays were conducted with 50 μM-xanthine or 1 mM-NADH as reducing substrate and with 100 μM-FMN. FMN reduction was monitored at 450 nm; an absorption coefficient of 12500 $M^{-1} \cdot cm^{-1}$ (Koziol, 1971) was used to calculate the initial rate of FMN reduction. The reductive release of ferritin iron was monitored at 530 nm as Fe(II)–2,2′-dipyridyl formation; an absorption coefficient of 8430 $M^{-1} \cdot cm^{-1}$ (Jones *et al.*, 1978) was used to calculate the initial rate of ferritin iron release (ferrireductase reaction).

Reducing substrate	Enzyme system	Fe released (μM/min per mg of protein	FMN reduced (μM/min per mg of protein)	Ratio Fe/FMN
Xanthine	Rat liver cytosol	0.0947	0.0527	1.80
	Turkey liver cytosol	0.119	0.0593	2.01
NADH	Rat liver cytosol	0.472	0.248	1.90
	Turkey liver cytosol	0.402	0.206	1.95

source of reducing power, whereas FAD is more efficient when xanthine is providing the reducing power.

The rate of enzyme-mediated FMN reduction was compared with the rate of the reductive release of iron from ferritin (measured as Fe^{2+}–2,2′-dipyridyl formation) with xanthine and NADH as reducing substrates. The ratio of the enzyme-mediated rates of ferritin iron release and FMN reduction was approx. 2 in the case of both liver cytosol preparations and both reducing substrates (Table 3). This observation is consistent with the fact that FMN is capable of accepting two electrons, each of which could be used reductively to release an iron atom from ferritin.

Evidence for two liver enzyme systems that can catalyse the reductive release of iron from ferritin

Allopurinol completely inhibited the ferrireductase activity of rat liver and turkey liver homogenates with xanthine as reducing substrate, but no inhibition was observed with NADH. These findings do not establish that different enzymes are catalysing the ferrireductase reaction with the two reducing substrates. Turkey liver and rat liver xanthine oxidoreductase catalyse the oxidation of NADH as well as xanthine and the oxidation of NADH by these enzymes is not inhibited by allopurinol. Thus it is possible that xanthine oxidoreductase could be responsible for the ferrireductase activity of liver cytosol with both xanthine and NADH as reducing substrates.

Several observations argue against this possibility. Avian xanthine oxidoreductases exhibit the greatest activity with NADH (Rajagopalan & Handler, 1967; Cleere & Coughlan, 1975); even so, the $V_{max.}$ for the oxidation of NADH is, at best, comparable with that for the oxidation of xanthine. As Table 1 shows, the $V_{max.}$ for the ferrireductase reaction with NADH was approx. 50 times that with xanthine. Even though rat liver xanthine oxidoreductase is much less efficient than turkey liver xanthine oxidoreductase in catalysing the oxidation of NADH, the values of $V_{max.}$ for the ferrireductase reaction with NADH as reducing substrate were comparable for rat liver and turkey liver cytosol preparations. The contrasting behaviour with xanthine and NADH in respect to substitution of FAD for FMN (Table 2) also argues against a single enzyme catalysing the ferrireductase reaction with the two types of reducing substrates.

Partial resolution of the ferrireductase activities exhibited by liver cytosol was accomplished by calcium phosphate gel chromatography. As shown in Table 4, addition of a small amount (4 ml) of a slurry of calcium phosphate gel to turkey liver cytosol (30 ml) resulted in a decrease of greater than 90% in the ferrireductase activity with xanthine as reducing substrate but only a decrease of 46% in the ferrireductase activity with NADH as substrate. Removal of greater than 90% of the ferrireductase activity with NADH as reducing substrate required the addition of 12 ml of the calcium phosphate gel. The ferrireductase activity with the two substrates was also eluted from the gel with a different profile when the gel, with adsorbed protein, was treated with phosphate buffers of increasing concentration (Table 4). A significant amount of the ferrireductase activity with NADH was eluted from the gel with the phosphate buffer of lowest concentration; whereas very little of the ferrireductase activity with xanthine was removed with this buffer. Significant amounts of the ferrireductase activity with xanthine were not eluted until the gel was treated with the 0.25 M- and 0.5 M-phosphate buffers. Similar results in respect of adsorption and elution of xanthine oxidoreductase from calcium phosphate gel were obtained with an authentic sample of xanthine oxidoreductase in the present study and in previous studies where calcium phosphate gel was employed to purify xanthine oxidoreductase from rat and turkey liver homogenates (Waud & Rajagopalan, 1976*a*,*b*; Cleere & Coughlan, 1975). These findings strongly suggest that two enzymes with different affinities for calcium phosphate gel are responsible for the ferrireductase activity with the two reducing substrates.

The labilities of the ferrireductase activities of liver cytosol preparations with xanthine and NADH as reducing substrates were quite different. When turkey liver cytosol was stored at 4 °C, the ferrireductase activity with NADH decreased much more rapidly than the activity with xanthine. The difference in the labilities of the ferrireductase activities with the two reducing substrates was magnified upon heat treatment of the cytosol at 60 °C.

Source of the endogenous ferrireductase activity with liver cytosol preparations

As previously indicated, it was necessary to dialyse freshly prepared liver cytosol preparations to make the ferrireductase activity strictly dependent on the exogenously added reducing substrates. This finding suggested that reducing substrates were endogenous in freshly prepared liver cytosols. As Table 5 shows, dialysis

Table 4. Calcium phosphate gel treatment of turkey liver cytosol

To a sample of 30 ml (1260 mg of protein) of turkey liver cytosol (prepared as described in the Materials and methods section), successive 4 ml aliquots of calcium phosphate gel were added. After each addition of gel, the sample was gently stirred for 20 min at 4 °C and then centrifuged. A sample of the supernatant was assayed for protein content and for ferrireductase activity with 200 μM-xanthine and with 200 μM-NADH as described in the Materials and methods section. After the final addition of gel, the gel with adsorbed protein was collected by centrifugation. Protein was removed from collected gel by the successive resuspension of the gel in 20 ml of potassium phosphate buffers, pH 7.4, of increasing concentration. After each resuspension, the resulting slurry was stirred for 20 min at 4 °C and then centrifuged. A sample of the supernatant obtained after each buffer treatment was assayed for protein content and ferrireductase activity with 200 μM-xanthine or with 200 μM-NADH.

(*a*) Adsorption

Total calcium phosphate gel (ml)	Total protein (mg)	Total activity with xanthine (ΔA_{530}/min)	Decrease (%)	Total activity with NADH (ΔA_{530}/min)	Decrease (%)
0	1260	1.692	0	0.874	0
4	513	0.150	91.1	0.476	45.5
8	109	0.0432	97.5	0.113	87.0
12	33	0	100	0	100

(*b*) Elution

Concn. of elution buffer (phosphate, pH 7.4) (M)	Total protein (mg)	Total activity with xanthine (ΔA_{530}/min)	Activity (% of original)	Total activity with NADH (ΔA_{530}/min)	Activity (% of original)
0.10	274	0.0240	1.42	0.0968	11.1
0.25	336	0.424	25.1	0.144	16.5
0.50	166	0.192	11.4	0	0
1.00	35.8	0	0	0	0

or the addition of allopurinol reduced the ferrireductase activity endogenous to the turkey liver cytosol to the non-enzymic activity obtained in the absence of reducing substrate or cytosol. Similar results were obtained with rat liver cytosol. Allopurinol is known to inhibit potently xanthine oxidation by xanthine oxidoreductase and was shown in the present studies to inhibit the ferrireductase activity with xanthine, but not the activity with NADH. Thus the virtually complete inhibition of the endogenous ferrireductase activity by allpurinol strongly suggests that this activity is the result of residual xanthine in the liver cytosol preparation. To substantiate further the origin of the endogenous activity, an assay was conducted in which the ferrireductase reaction catalysed by liver cytosol in the absence of exogenous reducing substrate was permitted to reach completion. At this point, xan-

Table 5. Source of the ferrireductase activity endogenous to turkey liver cytosol

Turkey liver cytosol (37.5 mg/ml) was prepared as described in the Materials and methods section. A portion of the cytosol preparation was dialysed for 15 h at 4 °C against 25 mM-Hepes buffer, pH 7.4, containing 0.25 M-sucrose to remove endogenous reducing substrates. Ferrireductase activity was analysed as described in the Materials and methods section. In assays containing allopurinol, 100 μl of a saturated solution of allopurinol were included in the assay mixture.

Reducing substrate	Enzyme system	Rate of ferrireductase reaction (ΔA_{530}/min)
None	Undialysed cytosol	9.00×10^{-3}
	Undialysed cytosol + allopurinol	1.28×10^{-3}
	Dialysed cytosol	1.34×10^{-3}
	None	1.19×10^{-3}
Xanthine (50 μM)	None	1.25×10^{-3}
	Dialysed cytosol	10.5×10^{-3}

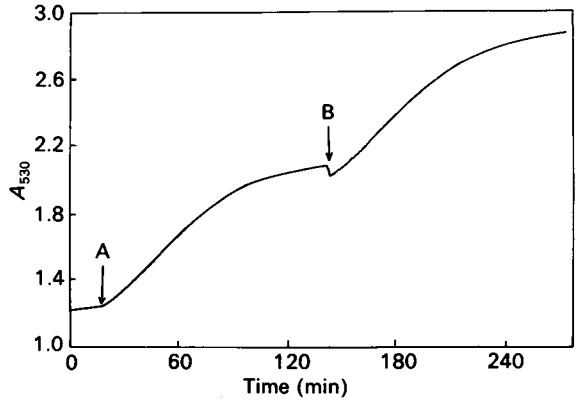

Fig. 3. Comparison of the endogenous and xanthine-promoted ferrireductase activity of turkey liver cytosol

Turkey liver cytosol was prepared and assayed for ferrireductase activity as described in the Materials and methods section. A, point at which undialysed liver cytosol was added to the assay mixture containing no xanthine or NADH; B, point at which xanthine was added to the assay mixture to give a final concentration of xanthine in the assay of 50 μM.

thine was added to the assay and the ferrireductase reaction monitored (Fig. 3). The rate of the ferrireductase reaction after the addition of near-saturating amounts of xanthine was equivalent to the rate observed during the depletion of endogenous substrate. Furthermore, addition of xanthine to dialysed liver cytosol preparations resulted in a rate of reaction only slightly greater than that endogenous to the undialysed preparation. These findings provide additional evidence that xanthine is the reducing substrate responsible for the endogenous reaction and indicate that the amount of xanthine endogenous to the cytosol preparation must be sufficient to drive the ferrireductase activity of xanthine oxidoreductase at close to $V_{max.}$.

Conclusions

The results of this investigation indicate that there are at least two enzyme systems in liver cytosol that can promote the reductive release of iron from ferritin. One of these uses NADH or NADPH as the source of reducing power, whereas the other, presumably xanthine oxidoreductase, uses xanthine as the source of reducing power. Both enzyme systems require the presence of a flavin nucleotide, apparently to shuttle electrons from the reduced enzymes to the iron contained in the ferritin molecule. The kinetic data suggest that xanthine is the more efficient substrate and that xanthine is the reducing substrate responsible for the activity endogenous to undialysed liver cytosol preparations.

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