Subcellular localization of chicken liver xanthine dehydrogenase

A possible source of cytoplasmic reducing equivalents

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Classical fractionation studies showed that xanthine dehydrogenase (EC 1.2.1.37) was exclusively cytosolic in chicken liver. Fumarase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37) were also found to have major cytosolic locations. These data indicate that urate synthesis in chicken liver produces substantial quantities of cytoplasmic NADH which may supply reducing equivalents to gluconeogenesis and other processes.

Although mammalian and avian glucose metabolism exhibit basic similarities, there are important differences in detail between the two (Pearce, 1977; Langslow, 1978). An example of these differences lies in the compartmentation and regulation of hepatic gluconeogenesis. Thus, whereas the intracellular location of the key gluconeogenic enzyme phosphoenolpyruvate carboxykinase (EC 4.1.1.32) is primarily cytosolic in rat liver, it is exclusively mitochondrial in adult-chicken liver (Utter, 1959; Chiao, 1976; Sarkar, 1977; Kung *et al.*, 1980). As a result, the flux of carbon out of the avian liver mitochondria during gluconeogenesis is in the form of phosphoenolpyruvate (Söling *et al.*, 1973). The absence of malate export, which in rat liver transports oxaloacetate and reducing equivalents from the mitochondria to the cytosol (Krebs, 1969), necessitates an alternative mechanism for the supply of cytosolic reducing equivalents if amino acids are to be utilized as gluconeogenic precursors. Amino acids are undoubtedly used *in vivo* as glucose precursors, since muscle protein is hydrolysed during starvation (Brady *et al.*, 1978) and uric acid excretion is doubled (Stirpe & Della Corte, 1965; Della Corte & Stirpe, 1967).

That the supply of reducing equivalents may be rate-limiting is suggested by experiments with chicken hepatocytes (Brady *et al.*, 1979; Dickson & Langslow, 1977; Dickson *et al.*, 1978; Mapes & Krebs, 1978) and perfused pigeon livers (Söling *et al.*, 1973), where the rate of gluconeogenesis from lactate [which supplies NADH via lactate dehydrogenase (EC 1.1.1.27)] is 5–10-fold more rapid than from pyruvate, and where single amino acids have been shown to be ineffective gluconeogenic substrates.

A possible source of cytosolic NADH *in vivo* is

from the pathway for uric acid synthesis. Uric acid is the primary end product of nitrogen metabolism in the chick and is produced by a biosynthetic sequence involving the formation of inosinic acid and its conversion into uric acid via hypoxanthine and xanthine. The last two steps are catalysed by the NAD⁺-dependent enzyme xanthine dehydrogenase (EC 1.2.1.37) (Strittmatter, 1965; Stirpe & Della Corte, 1965). Both urate excretion and hepatic xanthine dehydrogenase activity increase in chicks during starvation (Stirpe & Della Corte, 1965; Della Corte & Stirpe, 1967) and on administration of a high-protein diet (Featherston & Scholz, 1968). Thus catabolism of amino acids via purine synthesis and degradation may play an integral part in chicken gluconeogenesis by supplying NADH at the level of xanthine dehydrogenase. This NADH could be supplied not only by the action of xanthine dehydrogenase itself, but also by the subsequent metabolism of the fumarate produced during purine biosynthesis. The conversion of fumarate into oxaloacetate, before its amination to aspartate, would provide reducing equivalents via the action of fumarase (EC 4.2.1.2) and malate dehydrogenase (EC 1.1.1.37).

A requirement for the hypothesis that uricogenesis may play a role in the supply of reducing equivalents for gluconeogenesis is that the appropriate enzymes supplying the reducing equivalents must be found in the cytosol. In mammals the conversion of hypoxanthine into uric acid is catalysed by xanthine oxidase (EC 1.2.3.2), a peroxisomal enzyme (Beaufay, 1959; Baudhuin, 1969). A previous study by Scott *et al.* (1969) found xanthine dehydrogenase associated with peroxisomes and cytosol in chicken liver and raised the possibility that the cytosol enzyme may be an artefact owing to

breakage of the peroxisomes. We have therefore undertaken the subcellular localization of these enzymes in chicken liver by classical fractionation techniques.

Experimental

Animals

Female adult White Leghorn chickens were obtained from the Memorial University of Newfoundland Vivarium. They were subjected to a 16 h daily light period and either fed on a commercial diet (Masterfeed, St. John's, Newfoundland, Canada) or starved for 72 h before experimentation. Water was available at all times.

Chemicals

Acetaldehyde was obtained from Eastman Kodak Co. (Rochester, NY, U.S.A.). Triton X-100 was from Beckman Instruments (Fullerton, CA, U.S.A.). All enzyme substrates and cofactors were from Sigma Chemical Co. (St. Louis, MO, U.S.A.), as were calf thymus DNA and bovine serum albumin (fraction V).

Tissue fractionation

Liver tissue was fractionated by differential centrifugation by the scheme of de Duve *et al.* (1955). Chickens were killed by cervical dislocation, the liver was removed immediately into ice-cold sucrose medium {0.33 M-sucrose / 5 mM-MgCl₂ / 2 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid], pH 7.4} and weighed. All subsequent procedures were performed at 4°C. The tissue was minced finely with scissors and then homogenized in 3 vol. of medium with a Potter-Elvehjem-type apparatus with one downward pass of a slowly rotating pestle (clearance 0.3 mm). The homogenate was filtered through three layers of cheesecloth and then centrifuged at 600 *g*_{max} for 10 min. The supernatant was removed and the pellet rehomogenized in a further 3 vol. of medium and re-centrifuged. This process was repeated a second time. The supernatants were combined to yield a cytoplasmic extract (E), and the resuspended pellet was designated as the nuclear fraction (N). The cytoplasmic extract was further fractionated into mitochondrial, lysosomal, microsomal and cytosolic fractions by centrifugations at 5100 *g*_{max} for 10 min, 17000 *g*_{max} for 20 min and 110000 *g*_{max} for 60 min respectively. Pellets were resuspended in homogenizing medium with a Polytron homogenizer and were either assayed immediately after isolation or were stored at -20°C and assayed within 24 h. The sum of the nuclear (N) and cytoplasmic-extract (E) activities was taken as 100% for the purpose of calculating recoveries.

Enzyme and marker assays

All enzyme activities were assayed as described in the references cited, without modification unless otherwise specified.

NADPH-cytochrome *c* reductase (EC 1.6.2.5) was assayed by the method of Sottocasa *et al.* (1967). Lactate dehydrogenase (EC 1.1.1.27) was measured as described by Morrison *et al.* (1966). Glutamate dehydrogenase (EC 1.4.1.3) was assayed by the method of Brdiczka *et al.* (1968), except that 0.1% (v/v) Triton X-100 was included in the incubation medium (Schachter *et al.*, 1970). β -Glucuronidase (EC 3.2.1.31) was assayed spectrophotometrically as described by Gianetto & de Duve (1955), Triton X-100 (0.1%) also being included in the incubation medium.

DNA was extracted and measured as described by Burton (1956) after delipidation of the sample with chloroform/methanol/HCl (1000:1000:6, by vol.). Calf thymus DNA was used as a standard.

Fumarase (EC 4.2.1.2) was determined spectrophotometrically at 240 nm as described by Racker (1950), in a final incubation volume of 3 ml containing 50 mM-potassium phosphate buffer, pH 7.4, and 50 mM-sodium malate.

D-Amino acid oxidase (EC 1.4.3.3) was assayed as described by Baudhuin *et al.* (1964), except that colour formation of the dinitrophenylhydrazone product was measured spectrophotometrically at 420 nm.

Malate dehydrogenase (EC 1.1.1.37) was assayed as described by Bergmeyer *et al.* (1974), and xanthine dehydrogenase as described by Strittmatter (1965), except that 0.1 M-Tris/HCl, pH 8.2, was used, owing to the limited solubility of xanthine at neutral pH.

Protein in fractions was determined by the biuret method of Gornall *et al.* (1949) after delipidation of the samples with chloroform/methanol/HCl (1000:1000:6, by vol.) and precipitation of the protein with 10% (w/v) trichloroacetic acid.

All enzyme assays were shown to be linear with time and protein concentration. Duplicate determinations were performed on each sample.

Results and discussion

Nuclear, mitochondrial, lysosomal, microsomal and cytosolic subfractions were characterized by the markers DNA, glutamate dehydrogenase, β -glucuronidase, NADPH-cytochrome *c* reductase and lactate dehydrogenase respectively. Although a peroxisomal fraction was not isolated, D-amino acid oxidase was employed as a peroxisomal marker. Activities of enzymes and specific markers are given in Table 1 for both fed and starved birds. Activities are derived from the sum of nuclear (N) and

cytoplasmic (E) extracts and are taken as representative of the whole tissue. Enzyme recoveries were routinely in the range 90–105%. As chicken liver undergoes a drastic decrease in weight on starvation, values are expressed relative to protein rather than per g of liver. However, mean values for protein were 105 and 143 mg/g of liver in fed and starved birds respectively.

Table 1. *Activities of subcellular markers and other enzymes in whole liver tissue of fed and starved chickens*

For experimental details see the text. Activities are the sum of N + E and represent the means $\pm$ s.d. of four experiments. Enzyme units are defined as nmol of product formed/min per mg of protein at 25°C. DNA is expressed as μ g/mg of protein.

Enzyme or marker	Amount of activity	
	Fed	Starved
NADPH–cytochrome <i>c</i> reductase	18.1 $\pm$ 3.3	19.1 $\pm$ 5.0
Glutamate dehydrogenase	937 $\pm$ 158	833 $\pm$ 182
Lactate dehydrogenase	1730 $\pm$ 900	1860 $\pm$ 250
β -Glucuronidase	3.0 $\pm$ 0.8	2.6 $\pm$ 0.1
DNA	15.7 $\pm$ 3.6	30.7 $\pm$ 7.8
D-Amino acid oxidase	1.6 $\pm$ 0.5	1.4 $\pm$ 0.3
Xanthine dehydrogenase	3.6 $\pm$ 1.0	6.3 $\pm$ 2.0
Malate dehydrogenase	2540 $\pm$ 730	2120 $\pm$ 330
Fumarase	54.6 $\pm$ 11	53.7 $\pm$ 15

There are no notable changes in liver enzyme activities between fed and starved chickens, with the exception of xanthine dehydrogenase, the activity of which is increased 2-fold in the starved state. This increase has been observed previously (Stirpe & Della Corte, 1965; Della Corte & Stirpe, 1967). The actual values for control (fed) enzyme activity, expressed on a wet-weight basis (0.352 μ mol/min per g of liver) agree favourably with reported values of 0.52 (Featherston & Scholz, 1968) and 0.319 (Della Corte & Stirpe, 1967).

The subcellular localization of xanthine dehydrogenase, malate dehydrogenase and fumarase in starved birds is illustrated in Fig. 1. Similar subcellular localizations were observed in liver from fed birds.

Subfractions are defined by their respective marker enzymes. It was difficult to obtain good separation of the nuclear and mitochondrial fractions, even when lesser *g* forces were used. We found the very low *g* forces (30 *g* for 5 min) used by Scott *et al.* (1969) for separation of these fractions to be unsatisfactory. The distribution of xanthine dehydrogenase coincided exactly with that lactate dehydrogenase, and we conclude that this enzyme is entirely cytosolic. Scott *et al.* (1969) have reported that about one-third of chicken liver xanthine dehydrogenase activity occurs in the peroxisomal fraction. This might conceivably be due to an oxidase rather

Fig. 1. *Subcellular distribution of xanthine dehydrogenase, fumarase and malate dehydrogenase in 72 h-starved chicken liver*

Subcellular fractions were isolated and assayed as described in the text, and are defined as nuclear (N), mitochondrial (M), lysosomal (L), microsomal (P) and cytosolic (C) fractions. Histograms represent subcellular distributions of (a) NADPH–cytochrome *c* reductase, (b) glutamate dehydrogenase, (c) lactate dehydrogenase, (d) β -glucuronidase, (e) D-amino acid oxidase, (f) DNA, (g) xanthine dehydrogenase, (h) fumarase and (i) malate dehydrogenase. Specific activities are presented relative to the specific activity of whole liver (N + E).

than to a dehydrogenase activity under the conditions of their particular assay. We were, however, unable to demonstrate an oxidase activity in these fractions. We can therefore offer no explanation for the difference between the present data and those of Scott *et al.* (1969). However, since only minor quantities of D-amino acid oxidase were found in the cytosolic fraction, we can be confident that the degree of peroxisomal breakage was very small and thus that xanthine dehydrogenase is a true cytosolic enzyme in chicken liver. The distribution of malate dehydrogenase and of fumarase was bimodal, with about half of each enzyme occurring in the mitochondria and half in the cytosol.

The existence of xanthine dehydrogenase, fumarase and malate dehydrogenase in the cytosol of chicken liver indicates that the process of uricogenesis involves the production of substantial quantities of NADH in the cytosol. This NADH is produced by the reactions catalysed by xanthine dehydrogenase:

and by the reactions involved in the regeneration of the aspartate that is utilized in the formation of inosinic acid. Fumarate formed at the adenylo-succinate lyase (EC 4.3.2.2.) step in inosinic acid synthesis could be the precursor:

Thus the synthesis of each molecule of uric acid entails the production of three molecules of cytosolic NADH. This NADH could supply reducing equivalents for gluconeogenesis from amino acids.

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