Impact of starvation-refeeding on kinetics and protein expression of trout liver NADPH-production systems

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[1]*Department of Biochemistry and Molecular Biology, Faculty of Experimental Sciences, University of Jaén, E23071 Jaén;* [2]*Department of Biochemistry and Molecular Biology and* [4]*Department of Animal Biology and Ecology, Centre of Biological Sciences, University of Granada, E-18001 Granada; and* [3]*Department of Biochemistry, Cellular and Molecular Biology of Plants, Estación Experimental del Zaidín Consejo Superior de Investigaciones Científicas, E18008 Granada, Spain*

Barroso, Juan B., Juan Peragón, Constanza Contreras-Jurado, Leticia García-Salguero, Francisco J. Corpas, Francisco J. Esteban, María A. Peinado, Manuel de la Higuera, and José A. Lupiáñez. Impact of starvation-refeeding on kinetics and protein expression of trout liver NADPH-production systems. *Am. J. Physiol.* 274 (*Regulatory Integrative Comp. Physiol.* 43): R1578–R1587, 1998.—Herein we report on the kinetic and protein expression of glucose-6-phosphate dehydrogenase (G6PDH), 6-phosphogluconate dehydrogenase, and malic enzyme (ME) in the liver of the trout (*Oncorhynchus mykiss*) during a long-term starvation-refeeding cycle. Starvation significantly depressed the activity of these enzymes by almost 60%, without changing the Michaelis constant. The time response to this nutritional stimulus increased with fish weight. The sharp decline in G6PDH and ME activities was due to a specific protein-repression phenomenon, as demonstrated by molecular and immunohistochemical analyses. Also, the dimeric banding pattern of liver G6PDH shifted from the fully reduced and partially oxidized forms, predominant in control, to a fully oxidized form, more sensitive to proteolytic inactivation. Refeeding caused opposite effects in both protein concentration and enzyme activities of about twice the control values in the first stages, later reaching the normal enzyme activity levels. Additionally, the partially oxidized form of G6PDH increased. The kinetics of these enzymes were examined in relation to the various metabolic roles of NADPH. These results clearly indicate that trout liver undergoes protein repression-induction processes under these two contrasting nutritional conditions.

rainbow trout; low-fat and high-carbohydrate diet; glucose-6-phosphate dehydrogenase; 6-phosphogluconate dehydrogenase; malic enzyme; dimeric banding pattern; immunohistochemistry

THE SUPPLY OF REDUCING equivalents in the form of NADPH is one of the most important factors related to cell growth, proliferation, and detoxification (3, 23, 24). NADPH, one of the principal end products of several metabolic pathways, is also an indispensable substrate of reductive biosynthetic reactions (34). Hexose monophosphate shunt dehydrogenases, both glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), together with malic enzyme (ME), are the key cytoplasmic dehydrogenases generating reducing power in the form of NADPH.

It is also well established that in higher vertebrates the activity of hexose monophosphate dehydrogenases in various tissues changes under different nutritional and hormonal conditions (10, 16, 23, 24, 34). Nevertheless, in fish, the adaptive response of these NADPH-production enzyme systems to such conditions has not been completely characterized (2, 4, 18, 27).

As opposed to mammals, fish can survive rather prolonged starvation (4). This survival capacity is influenced by age, sex, temperature, and salinity, as well as other environmental and seasonal factors. There is evidence that this starvation tolerance is owed to processes of metabolic adaptation, regulated by the nervous and endocrine systems, in which different pancreatic and thyroid hormones play a major part (21, 30).

It is clear that the liver is the principal site of lipogenesis in teleostean fish (4, 12), whereas adipose tissue is dedicated primarily to the incorporation and storage of fatty acids produced de novo in hepatic tissue (33). In addition, starvation provokes a generalized weight loss, which translates directly as reduced cell growth. Therefore, the relationship between the production of reducing equivalents, such as NADPH, and protein synthesis (15) implies a decline in the activity of these enzyme systems during prolonged starvation.

Although in rainbow trout the behavior and role of the liver and skeletal muscle in relation to the variations in cell growth differ according to the physiological situation (2, 3), during prolonged starvation, both tissues lose significant weight, although each to a different degree. In analyzing the nature of tissue growth, it is necessary to consider two types of growth: *1*) hyperplasia and *2*) hypertrophy. The number of cells or nuclei (indicator of hyperplasia) can be estimated by determining the total DNA content, whereas the cell size or cell area controlled by a single nucleus (indicating hypertrophy) can be estimated by the protein-to-DNA ratio (39). Depending on total DNA content and the protein-to-DNA ratio, during a prolonged starvation, the liver weight loss is caused by two cumulative factors, reducing both cell number and size. Subsequent refeeding tends to restore the original values of these two cell indexes (5).

It is well known that the metabolic response of ectotherms depends on temperature and adaptational

periods (4, 37), although under different conditions these animals need long periods for intracellular enzyme activity to regain equilibrium (4). Wacke et al. (36) conclude that teleost fish, and therefore rainbow trout, which are genetically adapted to enduring prolonged food deficits, maintain relatively high and stable levels of enzymatic activities. Nevertheless, controversy surrounds the attainment and maintenance of equilibrium in these NADPH-production enzyme systems, not only during starvation-induced weight loss but also during compensatory cell growth stimulated by refeeding (2, 4, 21, 40).

In general terms, the activity of an enzyme reflects the number of enzyme molecules per cell or the regulation of the catalytic efficiency of a constant number of enzyme molecules per cell (11). We have assessed the effects of long-term starvation and refeeding on the kinetic adaptive behavior and expression of the hepatic NADPH-production systems in trout of different body sizes, determining the specific protein content by Western blot, kinetic, and immunohistochemical analysis. Our aim is to provide a detailed understanding of the main regulatory mechanisms of these enzyme systems and thereby clarify the metabolic roles of each.

MATERIALS AND METHODS

Chemicals. All biochemicals were obtained from Sigma Chemical (St. Louis, MO) or Boehringer (Mannheim, Germany). Other chemicals came from Merck and were of highest purity available.

Fish and maintenance. Juvenile rainbow trout (*Oncorhynchus mykiss*) of different body weights (30, 100, and 180 g) were obtained from a local fish farm (Riofrio, Granada, Spain). Fish were kept in 350-liter fiberglass tanks with continuous aeration, dechlorinated water with a flow rate of $1.5\ l \cdot min^{-1} \cdot kg^{-1}$ of fish at 15.0 ± 0.5°C. The light-dark period was a 12:12-h cycle. During 2 wk of acclimation, fish were fed a standard diet (composition in proteins, lipids, and carbohydrates was 45, 12, and 8 g/100 g, respectively, whereas the amount of gross energy was 14.9 kJ/g diet). For the enzyme activity experiments, the timing for starvation differed for each fish weight group, choosing those that reached a significant reduction in the enzyme activity levels. For this, the fish groups of 30, 100, and 180 g were starved for 35, 77, and 133 days, respectively. All fish were refed with a low-fat and high-carbohydrate diet (LF-HC; composition in proteins, lipids, and carbohydrates was 40, 8, and 23 g/100 g, respectively, whereas the amount of gross energy was 15.0 kJ/g diet) for 40 days. For the SDS-PAGE, immunoblot, and immunohistochemical analyses for G6PDH and ME, only fish of 30 g were used, with a starvation period of 63 days and a refeeding time of 20 days with the standard diet as previously defined.

Tissue preparation for analytic procedures. Fish were killed by a sharp blow to the head. Livers were immediately removed and homogenized (1:10, wt/vol) in 100 mM Tris·HCl containing (in mM) 250 sucrose, 1 EDTA, 0.1 NADP, and 0.57 phenylmethylsulfonyl fluoride, pH 7.6. All procedures were performed at 4°C. Homogenates were centrifuged at 105,000 *g* for 60 min. The supernatant fraction was used for biochemical and immunochemical assays.

Enzyme activity assays. G6PDH (D-glucose-6-phosphate: $NADP^+$ 1-oxido-reductase, EC 1.1.1.49) and 6PGDH (6-phospho-D-gluconate:$NADP^+$ 2-oxidoreductase-decarboxylating, EC 1.1.1.44) were determined as described by Barroso et al. (3), based on the reduction of $NADP^+$ at 340 nm in 50 mM HEPES, pH 7.6, containing 2 mM $MgCl_2$, 0.8 mM $NADP^+$, and a variable concentration of substrate. For kinetic studies, the range of substrate concentration for both G6PDH and 6PGDH was 0.005–5 mM [13 concentrations were used: 5, 7.5, 10, 12.5, 15, 20, 50, 100, 250, 500, 1,000, 2,500, and 5,000 μM; of these, 4 concentrations were below the Michaelis constant (K_m) value, 2 around its value, and 7 above K_m]. The G6PDH activity was corrected for 6PGDH activity as described Corpas et al. (8). ME (L-malate:$NADP^+$ oxidoreductase-oxaloacetate-decarboxylating, EC 1.1.1.40) was assayed according to the following protocol: the reaction mixture contained, in a total volume of 1 ml, 50 mM HEPES, pH 7.6, 2 mM $MgCl_2$, 0.4 mM NADP, and a variable concentration of L-malate. For kinetic studies the range of L-malate concentrations was 0.05–10 mM (11 malate concentrations were used: 0.050, 0.075, 0.1, 0.15, 0.175, 0.2, 0.5, 1, 2.5, 5, and 10 mM; of these, 4 concentrations were below the K_m value, 2 around its value, and 5 above K_m).

A milliunit of activity (mU) is defined as the amount of enzyme required to reduce 1 nmol NADP/min at 25°C. Proteins were determined according to the Bradford method (6) using BSA as a standard. Enzyme activity is expressed as specific activity in terms of milliunit of activity per milligram of protein.

Determination of DNA concentration. The method of Munro and Fleck (22) was used for the DNA separation, purification, and quantification. The RNA and DNA fractions were separated by digestion in alkali (0.3 N KOH) at 37°C for 1 h, followed by acidification in $HClO_4$, 1.2 N. The DNA concentration was estimated by the indole test (7).

Nondenaturing gel electrophoresis and detection of G6PDH activity. Liver samples were separated by PAGE in 5% acrylamide tube gels. Before electrophoresis, samples were prepared with 20% glycerol and 8 mM $NADP^+$ (final concentration). Samples were electrophoresed at a constant current of 1.5 mA/gel. The isoforms of G6PDH were visualized by staining of the enzyme activity after the incubation of the gel in 50 mM Tris·HCl, pH 7.6, containing (in mM) 10 G6P, 0.8 $NADP^+$, 5 EDTA, 2 $MgCl_2$, 0.24 nitroblue tetrazolium, and 65 μM phenazine methosulfate in the dark, until precipitated formazan appeared (about 15 min). The reaction was stopped by immersing the gel in 7% acetic acid. Gels were scanned using a gel scanner and then photographed.

Antibodies. Polyclonal antibodies against G6PDH and ME from rat liver were used (35).

SDS-PAGE and immunoblot analyses. Samples from high-speed liver supernatant fractions were heated to 95°C for 3 min in 62 mM Tris·HCl, pH 6.8 buffer, containing 2% (wt/vol) SDS, 10% (vol/vol) glycerol, and 10 mM 1,4-dithiothreitol. Polypeptides were separated by 7.5% SDS-PAGE using a Bio-Rad Mini-Protein II apparatus and electroblotted onto 0.2 μm polyvinylidene difluoride membrane (Immobilon-P, Millipore) using a semi-dry transfer apparatus (Hoeffer) at $1.5\ mA/cm^2$ membrane for 90 min in 25 mM Tris, 192 mM glycine, and 10% methanol, pH 9.4. The membranes were blocked with 10 mM Tris·HCl, 100 mM NaCl, pH 7.5 buffer (TBS) containing 5% nonfat dry milk and 0.05% Tween 20. The blots were then incubated overnight at 4°C with either rabbit anti-G6PDH or rabbit anti-ME antisera (diluted 1:1,000 and 1:1,500 in blocking solution, respectively). The blots were washed with TBS buffer containing 0.1% Tween 20. Immunodetection was performed using an enhanced chemiluminescence kit (Amersham). The blots were scanned with a computer-assisted videodensitometer and photographed.

Immunohistochemical studies. Fish were anesthetized in water containing 0.3 ml ethylene glycol-mono-phenylether per liter, weighed, and then heparinized through the dorsal aorta (500 IU, Rovi). For hepatic perfusion, abdominal and heart cavities were exposed, and a blunt 20-gauge cannula (Abbott) was inserted into the major tributary to the hepatic portal vein and tied securely in place. The liver was cleared of blood by an in situ perfusion with 3–4 ml of carbogenated 10 mM Na-phosphate, pH 7.4, containing 0.9% (wt/vol) NaCl (PBS), at room temperature, with a flow rate of 5.2 ml of PBS·min^{-1}·kg body wt^{-1}, using a peristaltic pump (Gilson minipuls). After the flow was initiated, a small cut was made in the tail kidney to allow the blood and perfusate to escape from the portal venous system. Livers were fixed in 100 mM Na-phosphate, pH 7.4, containing 4% paraformaldehyde, at the same flow rate. Fixed livers were removed, cut into cubes of 8–10 mm^3, and incubated for 3 h at room temperature with the previous fixative solution. Liver blocks were kept overnight at 4°C in 100 mM Na-phosphate, pH 7.4, containing 30% (wt/vol) sucrose. Blocks were covered with OCT compound and then frozen in 2-methylbutane prechilled in liquid nitrogen. Serial sections of 30 μm were prepared using a cryostat (2800 Frigocut E, Reicher-Jung). Inhibition of endogenous peroxidase was made on free-floating sections with 0.03% H_2O_2 in PBS for 30 min. After several washes in PBS, these free-floating sections were incubated with antibodies of either rabbit anti-rat G6PDH or rabbit anti-rat ME, diluted 1:50 in PBS containing 0.2% Triton X-100 overnight at 4°C, washed in PBS, and then incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories) followed by peroxidase-linked avidin-biotin complex. Peroxidase activity was detected by nickel-enhanced diaminobenzidine procedure (32). Sections were then mounted on slides using DePeX. Control procedures were carried out when the primary antibody was either omitted or replaced with an equivalent concentration of preimmune serum.

Kinetic parameters. Kinetic data were analyzed using a nonlinear regression analysis program (Enzfitter, Elsevier Biosoft) and EZ-FIT (Dupont de Nemours, Glenolden Laboratory). The activity ratio is the relationship between enzyme activity at subsaturating substrate concentration and maximum rate. Catalytic efficiency, defined as the ratio between enzyme activity and K_m, was determined at a substrate-saturating concentration. This parameter relates total enzyme concentration to the interaction between enzyme and the substrate.

Another way to express the different kinetic parameters used in the present work is by their relationship to the cell unit, given that the number of cell nuclei is represented by the total quantity of DNA (39).

The total activity corresponds to the total number of units of enzyme present in the complete organ and is expressed in total units of tissue. The activity by cell unit represents the enzyme activity per cell and is expressed in units per milligram DNA. The maximum rate per cell unit indicates the initial rate of the enzyme at substrate-saturating concentrations per cell and is determined consistently under the same experimental conditions, expressed in milliunits of activity per milligram DNA. The specific activity per cell unit reflects the specific activity of the enzyme per cell, expressed in milliunits of activity per milligram protein per milligram DNA. The catalytic efficiency of activity per cell unit corresponds to the catalytic efficiency per cell and is expressed in milliunits of activity per milligram DNA per 10^6 M.

Statistical analysis. All values are reported as means ± SE. The normal distribution of variables was analyzed using a computerized Kolmogorov-Smirnov test. This statistical test accepted the hypothesis of a normal distribution, and the results obtained for age groups and different nutritional situation were compared using the one-way ANOVA followed, in the appropriate cases, by a Duncan or Newman-Keuls multiple-range test. Also, statistical significance between means was determined using an unpaired two-tailed Student's *t*-test. The possibility of a tank effect was tested for each parameter, also using the unpaired Student's *t*-test, with no differences being found between tanks of the same experimental group (data not shown).The difference was considered significant at a level of $P < 0.05$.

RESULTS

Liver growth, protein, and DNA content. Loss of mass, a clear sign of long-term starvation, is especially critical in the liver, skeletal muscle, adipose tissue, and intestine (2). Our results indicate that, at the end of the starvation period (133 days) in the trout weighing 180 g, the liver weight diminished by 80%, with a loss of 63% of the total DNA content. At 8 days of refeeding, the liver had regained 74% of its original weight (a gain of 259% over starvation weight) without registering significant changes in the total DNA content. Cell size, represented by the relationship between the cell protein and hepatic DNA concentrations, showed opposite changes during the starvation-refeeding cycle, that is, the protein-to-DNA ratio fell 23% during starvation and rose 76% during refeeding (Table 1).

Hepatic G6PDH and 6PGDH activities. The effects of long-term starvation and refeeding on the behavior of the hepatic pentose-phosphate cycle dehydrogenases, G6PDH and 6-phosphogluconate dehydrogenase (6PGDH) in trout of different body weights are shown in Tables 2 and 3 and Figs. 1, 2, and 3. The results were qualitatively similar in the three experimental groups. Starvation significantly inhibited the rates of hepatic G6PDH and 6PGDH, although the larger the trout, the longer the response time to the nutritional regimen. In all cases, the enzymes followed a Michaelian curve for G6P and 6PG, respectively (results not shown). In the trout of 180 g, starvation did not significantly alter any of the kinetic parameters of hepatic G6PDH or 6PGDH during the first 35 days. At 98 days, activity of both dehydrogenases was significantly inhibited, reaching the highest reduction level (65%) at 133 days in specific activity as well as in maximum rate and catalytic efficiency (Tables 2 and 3, and Fig. 1), without signifi-

Table 1. *Influence of long-term starvation-refeeding cycle on liver weight, protein, and nucleic acid contents in rainbow trout*

	Control	Starved	Refed (8 days)
Liver wt	3.90 ± 0.46	0.80 ± 0.06*	2.87 ± 0.35†
Total protein, mg	581.5 ± 43.9	164.7 ± 10.9*	339.6 ± 38.6†
Total DNA, mg	9.09 ± 1.08	3.36 ± 0.25*	3.96 ± 0.490
Protein/DNA	63.95 ± 5.05	48.99 ± 3.38*	85.68 ± 9.99†

Values are means ± SE of 18 observations. Results of different nutritional conditions were tested with a 1-way ANOVA followed by both Newman-Keuls multiple-range and Student's *t*-tests using means. Significance at the $P < 0.05$ level was considered when *final starvation values were compared with control and †refeeding values were compared with starvation values.

Table 2. *Kinetic behavior of hepatic glucose-6-phosphate dehydrogenase during long-term starvation-refeeding cycle in rainbow trout of 180 g*

			Refed		
Liver	Control	Starved 133 days	8 days	20 days	40 days
Sp act	170.83 ± 18.21	67.32 ± 7.45*	120.68 ± 11.21†	369.59 ± 40.18†	171.10 ± 15.25†
V_{max}, mU	28.91 ± 2.71	12.59 ± 1.16*	17.82 ± 1.22†	62.84 ± 5.32†	25.26 ± 2.33†
K_m, μM	12.00 ± 1.37	16.32 ± 1.70	15.01 ± 1.80	16.30 ± 1.71	15.10 ± 1.56
Activity ratio, V_{ss}/V_{max}	0.31 ± 0.04	0.33 ± 0.04	0.34 ± 0.04	0.32 ± 0.04	0.31 ± 0.04
Catalytic efficiency	14.24 ± 1.52	4.13 ± 0.49*	8.04 ± 0.60†	22.67 ± 2.31†	11.33 ± 1.20†
Total activity	45.10 ± 4.96	4.03 ± 0.30*	18.87 ± 1.90†	86.43 ± 6.53†	39.21 ± 3.80†
Sp act/cellular unit	8.54 ± 0.84	7.29 ± 0.52	14.81 ± 1.42†	ND	ND
V_{max}/cellular unit	3.18 ± 0.31	3.74 ± 0.27	4.49 ± 0.42	ND	ND
Activity/cellular unit	4.96 ± 0.49	1.20 ± 0.09*	4.96 ± 0.48†	ND	ND
Catalytic efficiency/cellular unit	0.26 ± 0.03	0.23 ± 0.02	0.26 ± 0.02	ND	ND

Values are means ± SE of 6 observations. Livers from 4 trout were used in each observation. Specific activity and catalytic efficiency (V_{max}/K_m) are expressed as mU/mg protein and mU·mg protein^{-1} $10^6 \cdot M^{-1}$, respectively. ND, not determined; Sp act, specific activity; V_{max}, maximal velocity; K_m, Michaelis constant; V_{ss}, enzyme activity at a substrate-subsaturating concn. Results of the different nutritional conditions were tested with 1-way ANOVA followed by both Newman-Keuls multiple-range and Student's *t*-tests using means. Significance at $P < 0.05$ level was considered when *final starvation values were compared with control and †refeeding values were compared with the starvation values.

cant changes in K_m, compared with control values. This reduction in the enzyme activities, recorded at all substrate concentrations, was proportionally constant over the saturating curves, indicating a reduction in enzyme concentration. This idea is supported by the constancy of the activity ratio values (Tables 2 and 3).

On the contrary, during refeeding with the LF-HC experimental diet, the response of both dehydrogenases was much quicker and abrupt, both the specific activity and the catalytic efficiency rising significantly without changes in the K_m or activity rate. At 8 days of refeeding, the specific activity of both G6PDH and of 6PGDH increased by 75% with respect to the final starvation value; however, after 20 days, values rose roughly 5.5-fold, and at 40 days returned to control values (Tables 2 and 3, and Fig. 1).

In relation to the changes in tissue weight and in the concentrations of cell protein and DNA, the total activity of both dehydrogenases diminished 91% during starvation as a consequence of the decrease of 75% of the activity per cell unit (Tables 2 and 3), without significant changes in the other kinetic parameters per cell unit. At 8 days of refeeding (on the LF-HC diet), the hepatic tissue weight increased 3.6-fold with respect to starvation values and recovered 74% with respect to the initial weight (results not shown). Therefore, the total activity, both of G6PDH and 6PGDH, increased fivefold over starvation values. This sharp increase reflects a rise of twofold in the specific activity per cell unit and fourfold in the activity per cell unit.

The kinetic parameters of the hepatic enzymes in the lower-weight trout during the starvation-refeeding cycle followed a similar pattern as that described for the 180-g fish but with less response time in the former. In 100-g trout, the specific activity of both dehydrogenases fell significantly from *day 55* of starvation and from *day 35* in 30-g individuals (Fig. 1). Refeeding the LF-HC diet raised these activities significantly, even exceeding control values at 20 days, although to a lesser degree than in the 180-g weight category (Fig. 1). In all cases, at the end of refeeding (40 days), the enzyme activities values returned to the control levels.

Table 3. *Kinetic behavior of hepatic 6-phosphogluconate dehydrogenase during long-term starvation-refeeding cycle in rainbow trout of 180 g*

			Refed		
Liver	Control	Starved 133 days	8 days	20 days	40 days
Sp act	164.50 ± 18.14	67.89 ± 5.46*	118.59 ± 12.34†	338.64 ± 30.54†	167.46 ± 15.63†
V_{max}, mU	28.74 ± 2.56	12.69 ± 1.10*	18.08 ± 1.73†	59.32 ± 5.21†	26.51 ± 2.17†
K_m, μM	13.10 ± 1.16	16.00 ± 1.55	13.92 ± 1.40	13.08 ± 1.33	14.12 ± 1.38
Activity ratio, V_{ss}/V_{max}	0.33 ± 0.04	0.37 ± 0.04	0.38 ± 0.04	0.36 ± 0.04	0.37 ± 0.04
Catalytic efficiency	12.56 ± 1.31	4.24 ± 0.45*	8.52 ± 0.78†	25.89 ± 2.71†	11.86 ± 1.27†
Total activity	43.43 ± 4.28	4.06 ± 0.38*	20.76 ± 1.88†	83.21 ± 7.35†	40.18 ± 3.55†
Sp act/cellular unit	8.22 ± 0.81	7.34 ± 0.53	15.45 ± 1.85†	ND	ND
V_{max}/cellular unit	3.06 ± 0.30	3.78 ± 0.30	4.56 ± 0.55	ND	ND
Activity/cellular unit	4.78 ± 0.47	1.21 ± 0.08*	5.24 ± 0.58†	ND	ND
Catalytic efficiency/cellular unit	0.23 ± 0.02	0.23 ± 0.02	0.33 ± 0.04	ND	ND

Values are means ± SE of 6 observations. Livers from 4 trout were used in each observation. Specific activity and catalytic efficiency (V_{max}/K_m) are expressed as mU/mg protein and mU·mg protein$^{-1} \cdot 10^6$ M^{-1}, respectively. ND, not determined. Results of the different nutritional conditions were tested with 1-way ANOVA followed by both Newman-Keuls multiple-range and Student's *t*-tests using means. Significance at $P < 0.05$ level was considered when *final starvation values were compared with control and †refeeding values were compared with starvation values.

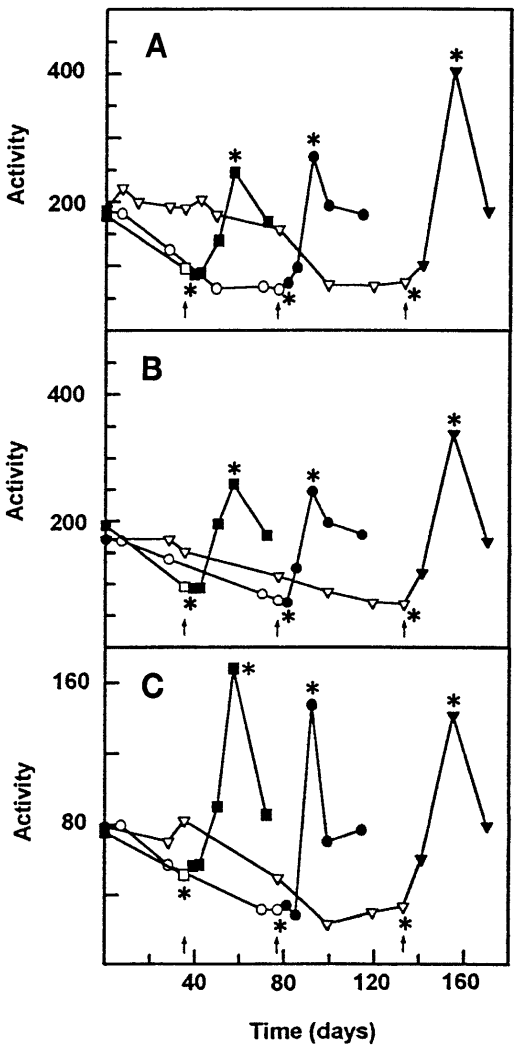

Fig. 1. Time course of hepatic glucose-6-phosphate dehydrogenase (G6PDH, *A*), 6-phosphogluconate dehydrogenase (*B*), and malic enzyme (ME, *C*) activities during the starvation-refeeding cycle. Starved rainbow trout of 30 g (□), 100 g (○), and 180 g (▽) were refed (solid symbols) a low-fat and high-carbohydrate diet at different times (arrows) when activity levels were significantly decreased. Activity results are the means of 5 experimental observations and are expressed as milliunits of activity per milligram protein. SE was always <10% and was omitted to clarify the figure. *Degree of significance ($P < 0.05$) between control and starved and control and refed fish.

When 30-g trout were refed on a standard diet (45/12/8) instead of the LF-HC (40/8/23) one, only a gradual rise to control values was recorded, without the overcompensation responses noted previously. The analysis of G6PDH by Western blot showed a single band with an apparent molecular size of 97 kDa, corresponding to the dimeric form of the protein. Figure 2 presents the evolution of the G6PDH protein content, indicating a progressive decline in the quantity of G6PDH protein from *day 35* of starvation, being 92% at 63 days and a gradual recovery of the protein levels to control values at 20 days of refeeding.

Figure 3 shows G6PDH activity on nondenaturing gel electrophoresis of high-speed supernatants of control (*lane a*), long-term starvation (*lane b*), and refeeding (*lane c*) 180-g trout. This is the first time in fish that hepatic G6PDH has been shown to exist in three dimeric forms with different electrophoretic mobility [as occurs in mammals (13, 19)], designated from 1 to 3, depending on its distance to the anode (*lane a*). Densitometric scan analysis, as well as the relative ratios expressed as percentages of total activity (results not shown) of the *bands 1*, *2*, and *3*, revealed a significant shift (about 45%) toward *band 1* in the starved fish (*lane b*) compared with control, without any significant changes in *bands 2* and *3*. During refeeding, the dimeric pattern of *bands 1* and *3* shifted selectively toward *band 2* (*lane c*).

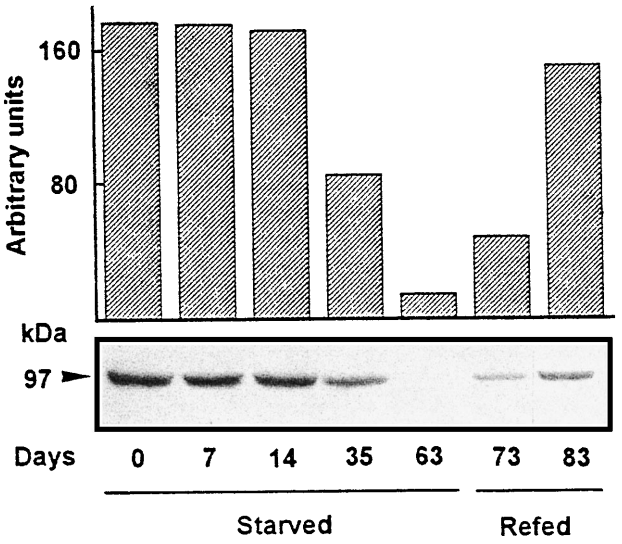

Fig. 2. Western blot analysis of G6PDH protein content from rainbow trout liver during starvation-refeeding cycle. Protein from cytosolic supernatants of trout weighing 30 g were electrophoresed and then blotted to polyvinylidene difluoride (PVDF). The 97-kDa G6PDH protein, corresponding to a dimeric form of the enzyme, was detected with specific polyclonal anti-G6PDH rabbit serum. *Bottom* shows immunoblot (80 μg/lane), indicating time-dependent effects of starvation-refeeding cycle on G6PDH protein levels. This is representative of 5 separate experiments. *Top* shows quantification by scanning densitometry of G6PDH levels; results are expressed as arbitrary densitometric units and are means of 5 observations. SE was always <5% and was omitted to clarify the figure.

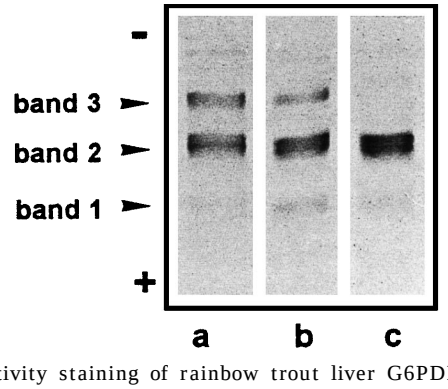

Fig. 3. Activity staining of rainbow trout liver G6PDH on a 5% polyacrylamide tube gels. *Lanes a*, *b*, and *c* contain cytosolic samples (80 μg total protein/lane) from controls, starved, and refed fish, respectively. This is representative of 5 separate experiments. A variability <5% was found.

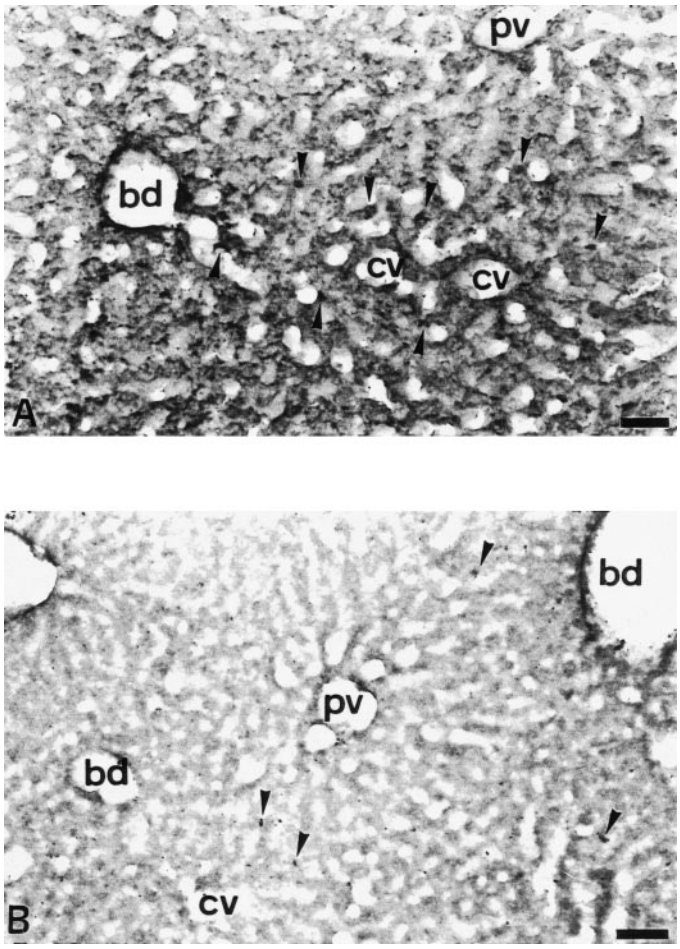

Fig. 4. G6PDH immunohistochemistry. Light micrographs of G6PDH immunoreactivity in sections 30 μm thick from control (*A*) or starved (*B*) trout liver. bd, bile duct; pv, portal vein; cv, central vein; note the immunoreaction product inside hepatocytes (arrowheads).

The results of the G6PDH immunohistochemical analysis on trout hepatocytes are presented in Fig. 4, *A* and *B*. Figure 4*A* illustrates a strong cytoplasmic immunoreaction (*p*-diaminobenzidine-nickel deposits) in control samples, although the reaction intensity showed pronounced individual cell variations, yielding a mottled appearance. The immunolabeling of the hepatocytes appears to be slightly related to venous patterns, with an increase in the perivenous areas; the bile duct epithelia are also stained. Figure 4*B* indicates a significant immunolabeling decrease in the starved fish, although some hepatocytes located in perivenous areas are still marked. The bile duct epithelia remain immunostained.

Hepatic ME activity. The results obtained followed a pattern similar to that described for the dehydrogenases of the pentose-phosphate cycle. The specific activity, maximal velocity (V_{max}), and catalytic efficiency of hepatic enzyme in the 180-g trout diminished significantly from *day 77*, registering 60% after 133 days of starvation (Table 4 and Fig. 1). These values persisted until *day 8* of refeeding (on the LF-HC diet), when values practically reached control; a fivefold increase in 20 days raised values to control levels (Table 4 and Fig. 1). Neither situation caused significant changes in the K_m or in the activity rate.

According to analysis of changes per cell unit, the total activity of ME fell 90% during starvation, due to a 74% reduction in the activity per cell unit; no significant changes were recorded in the maximum rate per cell unit, in the specific activity per cell unit, or in the catalytic efficiency per cell unit. After 8 days of refeeding (on the LF-HC diet), the total enzyme activity in the liver rose 434% over starvation values. In this sense, the catalytic activity, specific activity, and efficiency per cell unit, registered significant increases, although of less magnitude (Table 4).

In the 100-g trout, the kinetic parameters followed the same pattern described for heavier fish, although the reduction was significant from *day 70* of starvation. This period was reduced for 30-g trout, which showed significant changes in specific activity at 35 days of starvation. During LF-HC refeeding of both sizes of trout, the ME activity followed a similar pattern to that of 180-g trout (Fig. 1).

Table 4. *Kinetic behavior of hepatic malic enzyme during a long-term starvation-refeeding cycle in rainbow trout of 180 g*

			Refed		
Liver	Control	Starved 133 days	8 days	20 days	40 days
Sp act	78.65 ± 8.16	33.35 ± 4.11*	60.70 ± 7.01†	141.68 ± 12.14†	79.38 ± 8.18†
V_{max}, mU	13.31 ± 1.29	6.24 ± 0.61*	9.26 ± 0.84†	24.19 ± 2.13†	11.21 ± 1.05†
K_m, mM	0.18 ± 0.02	0.19 ± 0.02	0.19 ± 0.02	0.18 ± 0.02	0.18 ± 0.02
Activity ratio, V_{ss}/V_{max}	0.40 ± 0.04	0.43 ± 0.04	0.38 ± 0.04	0.37 ± 0.04	0.40 ± 0.04
Catalytic efficiency	0.44 ± 0.04	0.17 ± 0.02*	0.32 ± 0.03†	0.79 ± 0.08†	0.44 ± 0.04†
Total activity	20.76 ± 2.28	1.99 ± 0.15*	10.63 ± 1.27†	4.26 ± 0.41†	18.63 ± 1.62†
Sp act/cellular unit	3.93 ± 0.39	3.60 ± 0.26	7.91 ± 0.95†	ND	ND
V_{max}/cellular unit	1.46 ± 0.14	1.86 ± 0.13	2.34 ± 0.28	ND	ND
Activity/cellular unit	2.28 ± 0.22	0.59 ± 0.04*	2.68 ± 0.32†	ND	ND
Catalytic efficiency/cellular unit	8.11 ± 0.80	9.79 ± 0.70	12.31 ± 0.32†	ND	ND

Values are means ± SE of 6 observations. Livers from 4 trout were used in each observation. Specific activity and catalytic efficiency (V_{max}/K_m) are expressed as mU/mg protein and mU · mg protein^{-1} · 10^6 M^{-1}, respectively. ND, not determined. Results of different nutritional conditions were tested with 1-way ANOVA followed by both Newman-Keuls multiple-range and Student's *t*-tests using means. Significance at $P < 0.05$ level was considered when * final starvation values were compared with control and † refeeding values were compared with starvation values.

As with G6PDH and 6PGDH, refeeding 30-g trout on the standard diet instead of the LF-HC one gradually raised activity values to control, without registering the overcompensation noted previously. Western blot analysis of ME showed a single band with an apparent molecular size of 62 kDa, corresponding to the monomeric form of the protein. Figure 5 shows the evolution of the ME protein content, reflecting a progressive decline from *day 35* of starvation, reaching 77% at 63 days, as well as a gradual recovery of protein levels to control values at 20 days of refeeding.

Finally, Fig. 6 reflects ME immunohistochemical analysis of trout hepatocytes. Figure 6*A* illustrates, in the control trout, an immunoreactive distribution similar to that of G6PDH, although with a less pronounced differential venous profile and weakly reacting bile ducts. In starved fish, the cell immunoreaction significantly declined but without disappearing completely and remaining in perivenous areas and bile ducts (Fig. 6*B*).

DISCUSSION

One of the primary aspects of relationships between major metabolic pathways is metabolic change during the starvation-refeeding cycle, enabling variable fuel consumption to meet fluctuating metabolic demand. A clearly lipolytic situation such as prolonged starvation implies the mobilization of fat deposits, thereby depressing lipid biosynthesis. The supply of reducing equivalents, necessary for the synthesis of fatty acids and such processes as detoxification systems, as well as the processes of cell growth and proliferation, must also be inhibited. Higher vertebrates reportedly have an enormous adaptive capacity for dehydrogenases of the phosphogluconate cycle, altering their activity in response to different nutritional situations, for example, decreasing their activity during starvation (11, 25).

In addition, given that rainbow trout liver is the principal organ of de novo fatty-acid synthesis, a swift and abrupt reduction might be expected in the activity of the enzymes involved in lipogenesis after the beginning of starvation, for example, in the eel the levels of lipid synthesis fall some sixfold during the first week of starvation (1). Nevertheless, to clarify some of the differences found in the literature in relation to the adaptive capacity of the hepatic G6PDH, 6PGDH, and ME systems of fish during starvation, we have studied the effects on the kinetic behavior and protein concentration of these systems in trout of different sizes, noting the significant variations in the adaptive capacity of these systems in relation to the size of the fish.

Under our experimental conditions, prolonged starvation significantly diminished, at all body weights studied, the maximum rate and specific activity both of ME and of the dehydrogenases of the pentose-phosphate

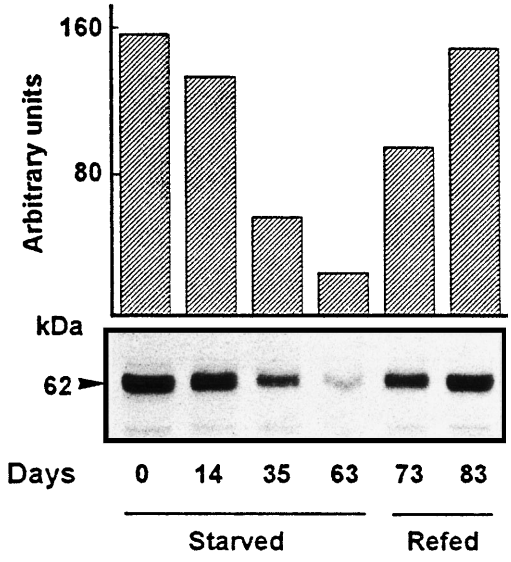

Fig. 5. Western blot analysis of ME protein content from rainbow trout liver during starvation-refeeding cycle. Protein from cytosolic supernatants of trout weighing 30 g were electrophoresed and then blotted to PVDF. The 62-kDa ME protein was detected with specific polyclonal anti-ME rabbit serum. *Bottom* shows immunoblot (40 μg/lane), indicating time-dependent effects of starvation-refeeding cycle on ME protein levels. This is representative of 5 separate experiments. *Top* shows quantification by scanning densitometry of ME levels; results are expressed as arbitrary densitometric units and are means of 5 observations. SE was always <5% and was omitted to clarify the figure.

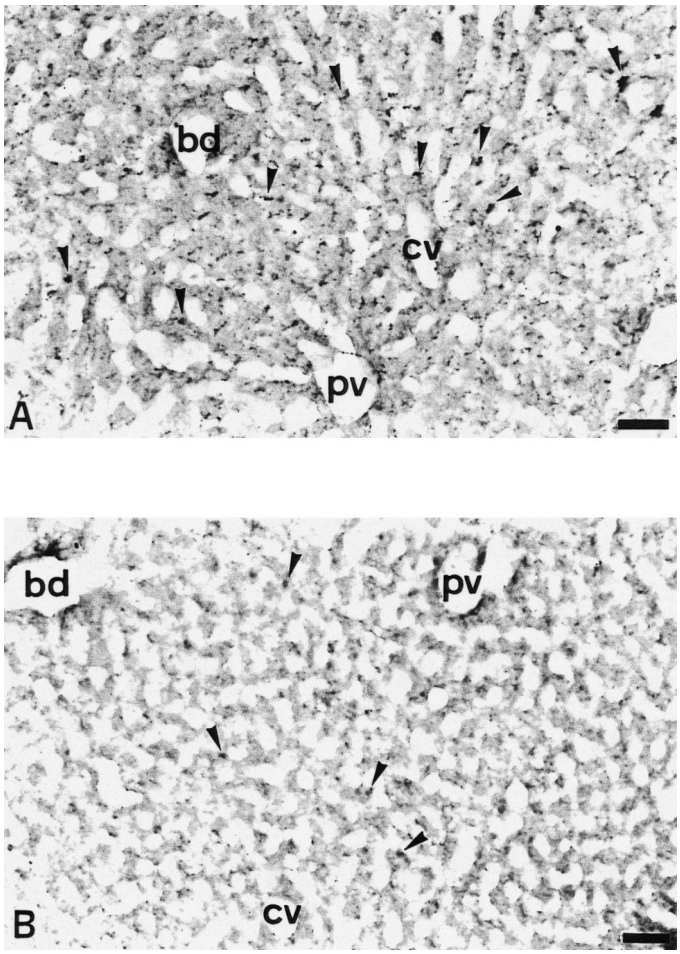

Fig. 6. ME immunohistochemistry. Light micrographs of ME immunoreactivity in sections 30 μm thick from control (*A*) or starved (*B*) trout liver. bd, bile duct; pv, portal vein; cv, central vein; arrowheads, note immunoreaction product inside hepatocytes.

cycle in hepatic tissue, without changing the K_m values and activity rate. This kinetic behavior corresponded to a clear enzyme repression, indicated by a strong decrease in both the protein G6PDH content and ME obtained by Western-blot analysis as the immunohistochemical marker for both hepatic enzymes.

In this sense, the qualitative immunohistochemical results complement the quantitative data, demonstrating that starvation decreases the accumulation of both enzymes in the trout liver. As in other salmonids, the liver of rainbow trout differs structurally from mammalian liver (14, 26), but despite these differences, the fish liver parenchyma can also function as a "metabolitostat" (28). Until now, the location of the two NADPH-generating enzymes G6PDH and ME in the trout liver has, to our knowledge, been demonstrated only by histochemical procedures (28); hence, the present study provides the first immunohistochemical detection, as well as their expression of both enzymes. In the control trout livers, the distribution patterns of G6PDH and ME reflected a slight increase in the liver parenchymal cells located in the perivenous area. Starved trout revealed that the immunohistochemical reaction decreased for both enzymes and that the remaining proteins were located mainly in perivenous areas.

These results concur with the previous general metabolic zonation of the trout liver in relation to the mammalian liver (20, 28), indicating that lipogenesis reaches its activity maxima in perivenous areas. Activity and Western blot analyses reflect a substantial decrease in the activity and total amount of G6PDH and ME, respectively, in the liver of the starved trout. The immunohistochemical results not only support these findings but also show that the low amount of these enzymes remaining in the liver are located in the perivenous areas, although we should take into account that their marked decrease corresponds to these areas also. Thus the hepatocytes located in the liver perivenous areas are mainly responsible for lipogenic changes during starvation.

In addition, this phenomenon of enzyme depression is reinforced when we analyze the evolution of total enzyme activity and the kinetic parameters as a group, expressed by cell unit. In our case both the total activity of G6PDH, 6PGDH, and ME, as well as the activity per cell unit, plunged drastically without significant changes in the specific activity per cell unit and catalytic efficiency per cell unit, reflecting a reduction both in the number and size of cells as a consequence of generalized rather than specific repression of the enzymatic proteins studied.

In this sense, on analyzing the G6PDH activity in gel, we observed that the banding pattern in the livers of 180-g trout agrees with the results of other studies concerning this enzyme (13, 19), which reported that *bands 1* and *3* represent fully oxidized and fully reduced forms of the enzyme, respectively. We also found that *band 2* (predominant in our banding pattern) represents a partially oxidized form (Fig. 3). During long-term starvation the dimeric pattern of bands moved toward *band 1* (fully oxidized), given that this dimeric form for the protein is more susceptible to proteolytic inactivation (13, 17, 19) than are *bands 2* and *3*. This shift in the dimeric banding pattern toward *band 1* reflects an early event in the degradation of G6PDH during long-term starvation, because the turnover of this enzyme involves first oxidation followed by inactivation, possibly also by a microsomal system (19).

Finally, the smallest trout showed comparable behavior in these NADPH-production systems, although the time needed for a significant fall in these activities increased markedly with the size of the trout. In addition, it is well established that in starving mammals these NADPH-production systems decline in activity, clearly as a consequence of enzyme depression. Thus Zelewski and Swierczynski (41) observed that starvation significantly depresses dehydrogenase activities of the pentose-phosphate cycle and of the ME in the liver and brown adipose tissue in the rat, whereas refeeding with a diet rich in carbohydrates significantly raises these activity levels to above control values. Similar trends were reported for fish (31), although for these NADPH-production systems the overcompensation described for mammals was not evident (4, 40).

With regard to refeeding, our results agree basically with some described above, in the significant increase of these enzymatic activities from *day 8* onward. Nevertheless, we have demonstrated the influence of the nutritional variable in the modulation of these enzyme systems during refeeding, because the low-carbohydrate diet did not lead to the overcompensation registered for the hepatic G6PDH, 6PGDH, and ME at 20 days of refeeding with the LF-HC diet. Finally, after 40 days of refeeding, the parameters studied returned to control values. In addition, our analyses of the activity values per organ and per cell of the three hepatic enzyme systems (G6PDH, 6PGDH, and ME) indicate that refeeding raised values significantly due to greater cell growth, characterized both by greater cell number (total quantity of DNA) and size (protein-to-DNA ratio). In addition, the specific activity per cell unit increased significantly during refeeding, indicating that, together with enzyme stimulation in general, there was a clear specific stimulation of these enzymes. This explains the overcompensation in the enzymatic activity found in this nutritional situation.

In this sense, when we analyzed the results of enzymatic activity in gel during the final stage of refeeding, we found a movement in the dimeric pattern of bands toward *band 2* (intermediate oxidation stage of the protein), indicating that de novo synthesis of the protein takes place in this form, before the intracellular levels of enzymatic activity reach a new equilibrium in protein turnover and consequently equilibrium between the different dimeric forms.

Therefore, we conclude that nutritional situations such as long-term starvation and refeeding significantly alter activities of the three enzymatic NADPH-production systems in the liver of rainbow trout. These kinetic alterations are intimately related to changes in

protein expression (enzyme repression or induction), the adaptive response depending on the size of the fish.

Perspectives

Factors contributing to cell growth include the synthesis and maintenance of the different elements making up the cell membranes, mainly structural lipids and proteins. In this sense, it is well established that NADPH plays a central role in the reductive biosynthesis of cholesterol and fatty acids, in the elongation and desaturation of the latter, as well as in the maintenance of cell integrity and detoxification processes. It has also been demonstrated that these reducing equivalents have a key part in the synthesis of protein, the other membrane element. As a result, NADPH is intricately involved in the growth process (29, 38). One of the most outstanding features of antagonistic nutritional situations, such as starvation and refeeding, concerns sharp changes in cell growth. In this light, the goal of our research is twofold. First, we are pursuing an in-depth understanding of the molecular mechanism behind the kinetic changes of the NADPH-generating systems in situations with significant variations in cell growth. Second, we seek to link this mechanism with the intimate relationships between the behavior of these enzyme systems and the nature of cell growth (29, 38). Only by investigating these factors in combination, can we achieve a comprehensive understanding of the molecular nature of this physiological process. Our work, recent (9) and ongoing, reflects this goal.

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