Morphological changes in hepatocytes of rats deprived of dietary nucleotides

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The aim of the present study was to investigate the influence of dietary nucleotides on liver morphology. Adult rats were fed for 21 d on a nucleotide-containing diet or the same diet free of nucleotides. Liver sections were examined by light and transmission electron microscopy, as well as for nucleic acid and protein contents. Morphometric analysis was performed for different variables. Deprivation of dietary nucleotides resulted in a reduction in hepatocyte nuclear and nucleolar areas as well as in nuclear chromatin condensation. In addition, the rough endoplasmic reticulum was reduced, as were ribosome association and abundance, whereas fat accumulated. These findings portray dietary nucleotides as required nutrients for the liver under normal physiological conditions and suggest that an inadequate supply of nucleotides for a certain period of time has transient negative effects on liver ultrastructure and function.

Dietary nucleotides: Liver

Nucleotide requirements of the liver and other tissues are presumed to be met by *de novo* synthesis from amino acids as well as by the recovery of precursors such as nucleosides and nucleobases via salvage pathways (Rasenack *et al.* 1985; Kim *et al.* 1992). Salvage mechanisms could use endogenous precursors derived from the degradation of nucleic acids and exogenous precursors derived from other tissues and/or from the diet (Moyer *et al.* 1985). Nucleotides are present in food mainly as nucleoprotein from which they are liberated and very efficiently absorbed (Wilson & Wilson, 1962). The re-utilization of these nucleotide precursors spares the cost of *de novo* synthesis and is known to inhibit synthesis by feedback mechanisms (Palella & Fox, 1989). We hypothesized, therefore, that dietary nucleotides are energetically advantageous to fulfil the nucleotide needs of the liver.

There is evidence that exogenous nucleotides can influence liver function. For example, nucleotide supplementation to a parenteral nutritional solution facilitates liver recovery from ischaemia or partial hepatectomy (Ogoshi *et al.* 1985, 1990; Palombo *et al.* 1993). Whether or not the liver requires dietary nucleotides under normal physiological conditions is an open question. Some authors believe that most of the ingested nucleotides are degraded within the intestine (Witte *et al.* 1991), but it has been demonstrated also that dietary nucleotides are incorporated into liver nucleic acids (Burridge *et al.* 1976; Savaiano & Clifford, 1978; Sonoda & Tatibana, 1978; Berthold *et al.* 1995). One approach to the question of the nucleotide requirements of the liver is to study the consequences of dietary nucleotide deprivation. Using this approach, Carver (1994) has reported lipid accumulation and reduced glycogen content in the liver of rats fed on a diet lacking nucleotides for 5 weeks. We recently reported that dietary nucleotide deprivation for 10 d decreased liver soluble nucleotides and RNA and that these changes were reversed after 21 d of deprivation

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Table 1. *Composition of the semi-purified diet (g/kg)*

Ingredients			
Calcium caseinate	226	Proteins	200
Maize starch	446	Carbohydrates	670
Sugar	150	Fat	100
Cellulose	50	Minerals	25
VKO	100		
Minerals and vitamins mix*	24		
DL-Methionine	3		
Choline bitartrate	1		

VKO, mixture of (g/kg) olive oil (660), soyabean oil (230) and refined coconut oil (110).

* From American Institute of Nutrition (1977). The mineral and vitamin mixture contained (g/kg mix): calcium diphosphate 9·01, sodium chloride 2·59, potassium citrate 0·75, potassium phosphate 5·61, potassium sulphate 1·82, magnesium sulphate 3·02, manganous sulphate 0·18, ferric lactate 0·19, zinc carbonate 56 mg, cupric acetate.2 H_2O 17 mg, potassium iodate 0·35 mg, sodium selenite.5 H_2O 0·35 mg, chromic potassium sulphate.10 H_2O 19 mg, calcium citrate 0·71, thiamin 6 mg, riboflavin 6 mg, pyridoxine hydrochloride 7 mg, nicotinic acid 30 mg, calcium pantothenate 16 mg, folic acid 2 mg, biotin 0·2 mg, cyanocobalamin 0·01 mg, retinyl acetate 1·4 mg, cholecalciferol 0·025 mg, DL-α-tocopherol 5 mg, phylloquinone 0·05 mg.

(López-Navarro *et al.* 1995). In the present paper we describe the morphological and morphometric changes in nuclear and nucleolar areas, as well as in ribosome, fat and nucleic acids contents, resulting from deprivation of dietary nucleotides during the same period of time.

METHODS

Animals and diets

Adult male Wistar rats weighing 400–500 g, supplied by the Animal Service of the University of Granada (Granada, Spain), were maintained under standard environmental conditions (22 ± 1°, 12 h light–dark cycle, feed and water consumption *ad libitum*). A group of eight rats fed on a non-purified standard diet (Panlab A.04; Panlab, Barcelona, Spain) was studied as the reference group. Another group was fed on a semi-purified diet (Puleva-Abbott Laboratories, Granada, Spain) supplemented with 2·5 g/kg of each of the following nucleotides: AMP, CMP, GMP and UMP, for 2 weeks (experimental). After this adaptation period, rats in the experimental group were randomly assigned to two subgroups of sixteen rats each. One subgroup was fed on the semi-purified diet (control group) and the other subgroup was fed on the same diet but free of nucleotides (deprived group). To preclude effects of decreased feed intake, the dietary treatment was pair-fed following the recommendations of the Institute of Laboratory Animal Resources (1979) Committee on Laboratory Animal Diets. The protocol was approved by the Committee for Animal Research of the University of Granada.

The composition of the semi-purified diet is shown in Table 1. The nucleotide content of the diets was verified by HPLC (Wynants & Belle, 1985) before use. The amount of nucleotides in the semi-purified diet was undetectable, whereas the supplemented semi-purified diet contained 10 (SD 1) g/kg diet, a quantity comparable with that of the non-purified diet (11 (SD 3) g/kg diet).

Liver samples were taken after 10 and 21 d of the dietary treatment and processed for light and electron microscopic analysis. A portion of liver was homogenized (0·1 g/ml) in cold saline solution (9 g NaCl/l) and used directly for DNA and protein determinations. Another portion was homogenized in cold perchloric acid (0·6 mol/l; PCA 1 : 10, w/v). The PCA-insoluble fraction was separated by centrifugation at 3000 ***g*** for 10 min and used for RNA analysis.

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Tissue processing for light and electron microscopic analysis

A portion of the fresh liver (from the same lobe in all cases) was diced into 1 mm^3 pieces and fixed in 25 g glutaraldehyde and 20 g paraformaldehyde/l in 0·1 M-sodium cacodylate–sucrose buffer (350 mosmol/l, pH 7·4) for 4 h, and subsequently post-fixed in a solution of 20 g osmium tetroxide/l in sodium cacodylate–sucrose buffer for 2 h at 4°. After acetone dehydration, samples were embedded in Spurr (1969) resin. Semi-thin sections (2 μm) were stained in toluidine blue for light microscopic observation using an Olympus BH-2 microscope, (Olympus, Tokyo, Japan) and 50–70 nm ultrathin sections were subsequently contrasted in 40 g uranyl acetate/l for 15 min and lead citrate (1·33 g lead nitrate and 1·76 g sodium citrate in 50 ml water) for 5 min (Reynolds, 1963), for examination under a Zeiss EM 902 transmission electron microscope (Carl Zeiss, Oberkochem, Germany). For each specimen, at least ten sections were studied.

Morphometric analysis

Five microscopic views from each of the ten sections were scanned from negatives with the Sharp JX-450 scanner (Sharp, Mahwak, NJ, USA), recorded and transferred to the NIH image analyzer memory for the morphometric measurements, using a Macintosh II fx computer (Apple, Cupertino, CA, USA). Morphometric measures were taken by one of the authors before knowing the diet grouping. The images were digitalized at 300 dots per inch for the different measurements. Chromatin density was defined as the mean of the grey levels of the pixels in the nucleus, each pixel being defined by one of 256 grey levels. The content in ribosomes and fat droplets was estimated by converting the images into binary digits, in order to separate the objects from the background, after retouching to eliminate background noise; estimations were based on an average size of 200 Å and 3·14 μm^2 for ribosome and fat droplet respectively. All counts were performed using a standard square of 1·522 μm^2 in non-nuclear regions chosen at random.

Biochemical analysis

DNA concentrations were determined after chromatin dissociation by the fluorescence enhancement, using the fluorochrome Hoechst 33258 dye 2′-(4-hydroxyphenyl)-5-(4-methyl-1-piperazinyl)-2-5′-bi-1H benzimidazole trihydrochloride (Sigma Chemical Co., St Louis, MO, USA), following the method of Labarca & Paigen (1980). Calf-thymus DNA was used as a standard. The fluorescence was measured in a Shimadzu RF 5001 PC (Kyoto, Japan) spectrofluorimeter at 37°. RNA concentrations were determined following the alkaline-digestion method of Fleck & Munro (1962). Calf-liver RNA type IV was used as a standard. Protein was determined by Bradford's (1976) method using bovine albumin as a standard.

Statistical analysis

Results from the two experimental groups were analysed by two-way ANOVA (diet × time) and the *post hoc* test of Student–Newman–Keuls (Dixon *et al.* 1990), using the BMDP version PC-90 (BMDP Statistical Software, UCLA, Los Angeles, CA, USA).

RESULTS

There were no significant differences between the control and the reference groups for any of the variables studied. This finding validates our semi-purified diet as a useful control diet.

As shown in Fig. 1, the group deprived of dietary nucleotides for 10 d differed from the control group with respect to the size, shape and chromatin density of the hepatocyte nucleus; the nucleus and nucleolus became smaller and lobulated, and the nuclear chromatin showed increased density. No differences, however, were observed between these two groups at 21 d, indicating a recovery of the deprived group.

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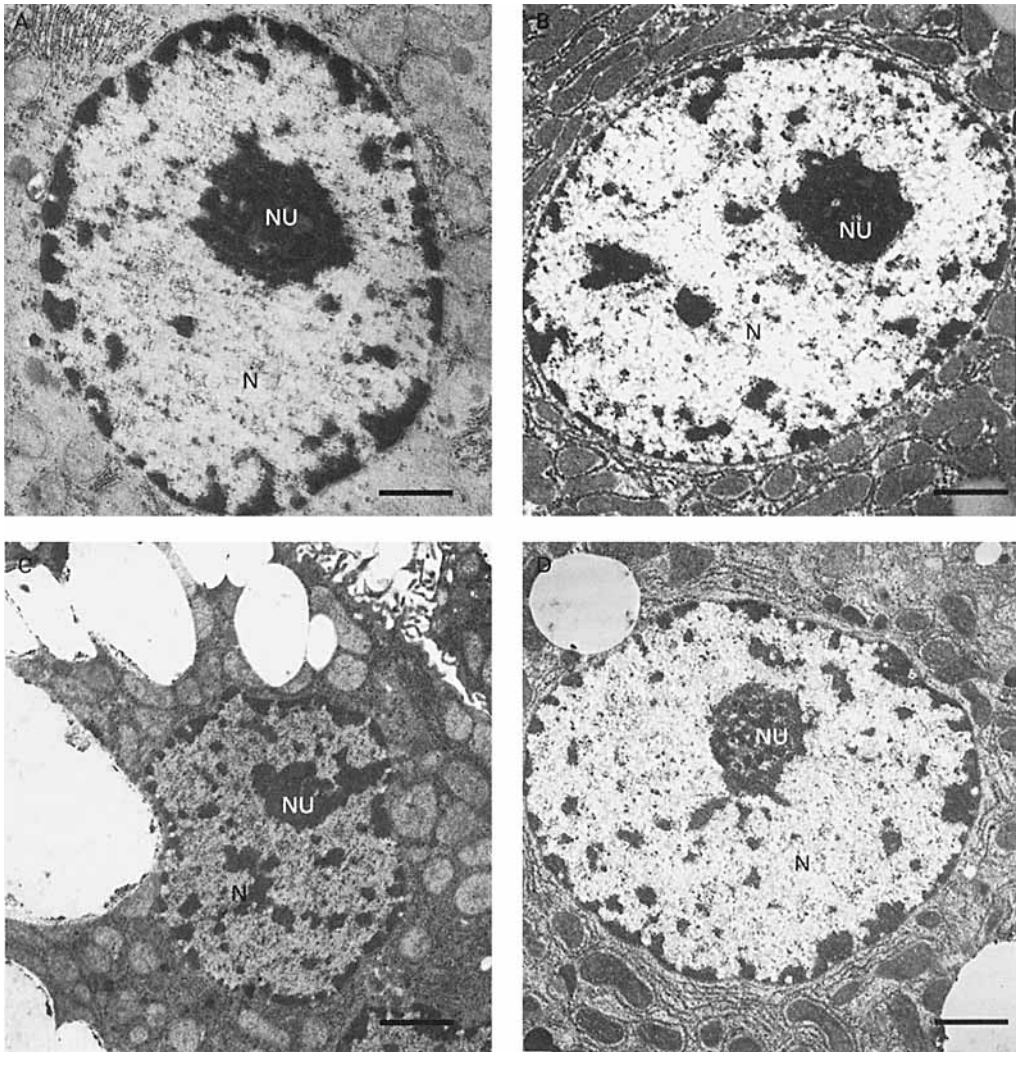

Fig. 1. Microscopic views of liver from rats fed on reference diet (standard diet; A) and experimental diets with (control; B) without (deprived) nucleotides for 10 d (C) and 21 d (D). N, nucleus; NU, nucleolus. Original magnification × 7000; ——, 1 μm. For details of diets and procedures, see Table 1 and pp. 580–581.

The quantitative morphometric results are shown in Table 2. The size reduction of the nucleus was apparent from the data of the nucleus perimeter and nucleus:cytoplasmic area index; however, the decrease in nuclear surface area did not prove to be significant. From 10 to 21 d, all these variables significantly increased to return to control values. The nucleolar surface area significantly decreased at 10 d of deprivation and recovered to control values by 21 d. The nucleolus and nucleus registered parallel reductions at 10 d, but in the subsequent recovery the nucleus showed a greater relative increase in area by 21 d, as shown by the nucleolus:nucleus area index. Chromatin within the nucleus showed higher density, reflecting a more compact distribution at 10 d, and returned to control values at 21 d. Finally, the number of binucleate hepatocytes was not altered by deprivation of nucleotides at 10 d, but increased significantly at 21 d.

As shown in Fig. 2, hepatocyte rough endoplasmic reticulum was clearly altered in the group deprived of dietary nucleotides for 10 d and partially restored after 21 d. The number

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Table 2. *Morphometric analyses of hepatocyte nucleus and nucleolus in rats receiving experimental diets with (control) or without (deprived) nucleotides*‡

(Mean values and standard deviations for eight rats)

Dietary group	Binuclearity§ (%)		Cytoplasmic surface area (C; μm^2)		Nuclear surface area (N; μm^2)		Nuclear perimeter (μm)		Nucleolar surface area (No; μm^2)		N:C area index		No:N area index		Chromatin density‖	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Reference (standard diet)	13·93	3·32	131	26	32	15	17·9	2·8	2·12	0·86	0·27	0·02	0·07	0·04	134	31
Experimental: 10 d																
Control	13·52	3·69	157	35	41	17	18·0	2·3	2·54	1·17	0·25	0·03	0·07	0·03	128	17
Deprived	16·08	3·77	158	38	27	11	13·8**	2·0	1·48**	0·67	0·16***	0·01	0·06	0·02	154**	17
Experimental: 21 d																
Control	13·87	3·44	160	31	40	14	18·2	1·9	2·44	0·98	0·24	0·01	0·06	0·03	132	18
Deprived	20·70**	1·04	162	32	43†	13	17·9††	2·3	2·09	0·97	0·25†††	0·03	0·04**	0·02	140	18

N:C, Nucleus: cytoplasm; No:N, nucleolus:nucleus.
Mean values were significantly different from control values: ** $P < 0·01$, *** $P < 0·001$.
Mean values were significantly different from those at 10 d: † $P < 0·05$, †† $P < 0·01$, ††† $P < 0·001$.
‡ For details of diets and procedures, see Table 1 and pp. 580–581.
§ Binuclearity is expressed as number of binucleate hepatocytes per 100 hepatocytes; all other measurements were made in fifty hepatocytes.
‖ Mean of the grey levels of the pixels in the nucleus, each pixel being defined by one of 256 grey levels.

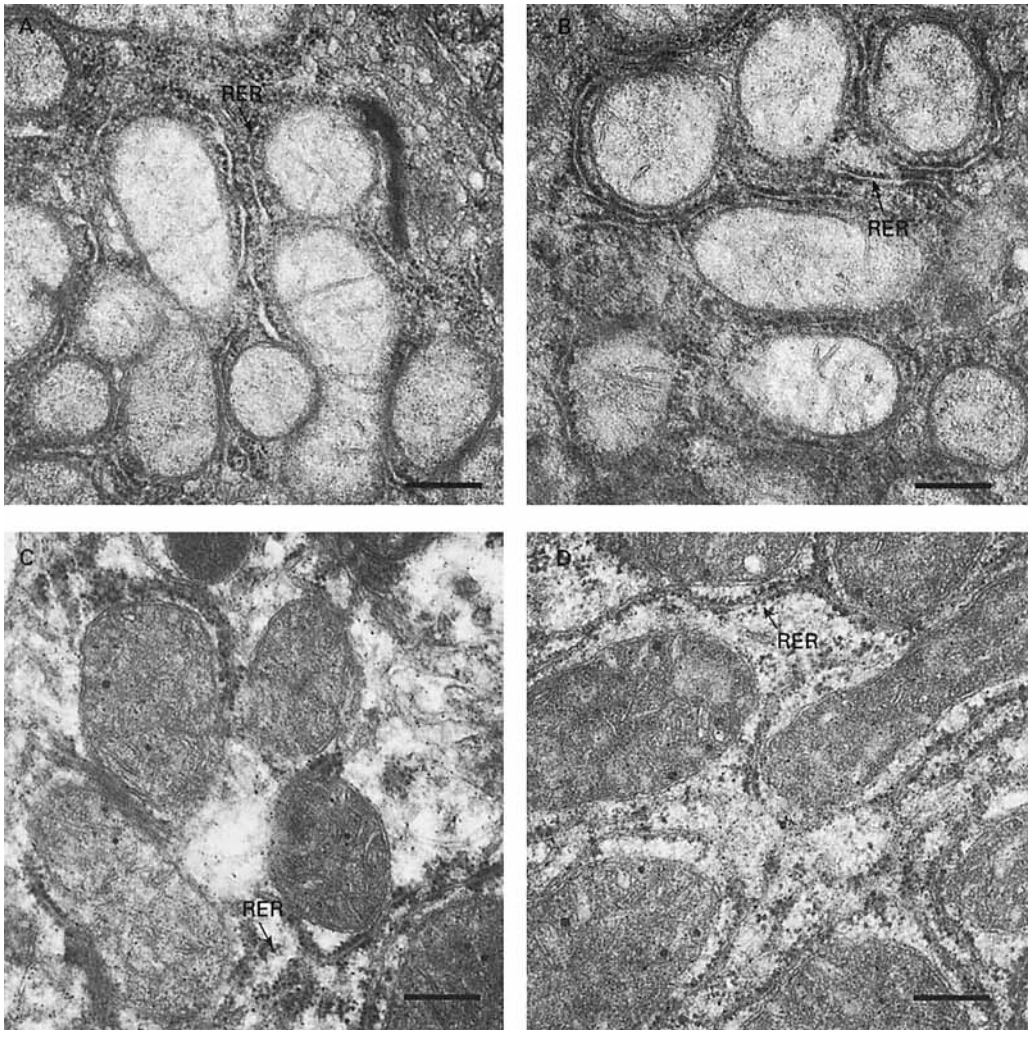

Fig. 2. Microscopic views of liver from rats fed on reference diet (standard diet; A) and experimental diets with (control; B) and without (deprived) nucleotides for 10 d (C) and 21 d (D). RER, rough endoplasmic reticulum. Original magnification × 30000; ——, 0·4 μm. For details of diets and procedures, see Table 1 and pp. 580–581.

of monoribosomes and polyribosomes (associations of two, three and four or more ribosomes) decreased significantly after 10 d of deprivation. At 21 d an increase in the number of monoribosomes was evident, with no changes apparent in polyribosomes (Table 3).

The livers of the nucleotide-deprived rats showed fat accumulation, which increased from 10 to 21 d of deprivation, due mainly to increased droplet size (Table 3).

The RNA content significantly decreased in the nucleotide-deprived group at 10 d and returned to control values at 21 d. No significant changes in the contents of DNA or protein were observed (Table 4).

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Table 3. *Morphometric analyses of hepatocyte ribosomes and fat in rats receiving experimental diets with (control) and without (deprived) nucleotides*‡

(Mean values and standard deviations for eight rats)

	Ribosome size distribution§								Fat			
	1		2		3		⩾ 4		Content‖		Size (μm^2)	
Dietary group	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Reference (standard diet)	80·0	6·9	31·0	7·0	18·5	5·0	14·5	1·6	14·7	7·5	3·22	1·29
Experimental: 10 d												
Control	72·2	4·2	26·3	1·2	16·3	0·8	13·5	1·3	23·3	11·8	4·13	1·05
Deprived	53·9***	4·4	12·9***	1·4	5·6***	0·8	7·6***	1·0	108·4***	5·9	4·35	2·03
Experimental: 21 d												
Control	71·5	4·7	23·4	1·2	13·2	1·8	17·1	8·8	22·9	12·1	4·15	1·10
Deprived	66·7†††	4·4	13·2***	2·1	5·7***	1·6	9·4***	1·4	149·6***†	24·4	7·84***††	1·35

Mean values were significantly different from control values: *** $P < 0·001$.
Mean values were significantly different from those at 10 d: † $P < 0·05$, †† $P < 0·01$, ††† $P < 0·001$.
‡ For details of diets and procedures, see Table 1 and pp. 580–581.
§ Expressed as the no. of units of ribosomes or associations per microscopic field (1·522 μm^2).
‖ Expressed as the no. of fat droplets per microscopic field (0·026 mm^2).

Table 4. *Nucleic acid and protein contents in intact liver from rats receiving experimental diets with (control) and without (deprived) nucleotides*‡

(Mean values and standard deviations for eight rats)

	Protein (mg/g)		DNA (mg/g)		RNA (mg/g)	
Dietary group	Mean	SD	Mean	SD	Mean	SD
Reference (standard diet)	155·4	15·7	4·22	0·81	8·78	2·92
Experimental: 10 d						
Control	141·9	16·6	4·90	0·48	7·92	1·35
Deprived	160·1	19·6	4·45	0·50	4·88***	1·21
Experimental: 21 d						
Control	146·7	16·5	4·00	1·12	8·09	1·30
Deprived	165·0	16·2	4·23	0·80	6·86††	1·20

Mean value was significantly different from control value: *** $P < 0·001$.
Mean value was significantly different from that at 10 d: †† $P < 0·01$.
† For details of diets and procedures, see Table 1 and pp. 580–581.

DISCUSSION

Early studies using labelled nucleobases or nucleic acids demonstrated that small but significant amounts of dietary nucleotides are incorporated into liver nucleic acids (Burridge *et al.* 1976; Savaiano & Clifford, 1978; Sonoda & Tatibana, 1978). The nutritional relevance of these findings has since been ignored in the belief that most of the orally-ingested nucleotides are catabolized and excreted. However, the issue has not been completely resolved. For example, it is known that nucleotide catabolic enzymes predominate over the anabolic enzymes in the intestine (Witte *et al.* 1991), suggesting degradation, but it is also known that extensive nucleotide salvage takes place (MacKinnon & Deller, 1973; Bissonnette, 1992), suggesting incorporation into tissues. Catabolism of purines seems to be higher than that of pyrimidines and, in fact, incorporation of intact dietary pyrimidine, but not purine, nucleosides into hepatic RNA has been found (Berthold *et al.* 1995). This difference between purines and pyrimidines may be linked to the need to stabilize the concentration of adenosine, a nucleoside that has potential toxic effects (Uauy *et al.* 1994). In addition, nutritional status seems to influence purine catabolism in the intestine; increased salvage and incorporation into tissues and decreased catabolism of dietary nucleotides has been reported in fasting (Gross *et al.* 1988; Gross & Savaiano, 1991). Using longer periods of time to evaluate the influence of dietary nucleotides, we and others have reported a significant contribution of these compounds to the liver (Carver, 1994; Lopez-Navarro *et al.* 1995). It is now believed that conditions that alter the intrahepatic nucleotide content affect liver structure and function (Matsui *et al.* 1994). The data presented in the present paper support this hypothesis.

The reduced size of the nucleus and nucleolus found here indicates diminished DNA and RNA content, under the assumption that the size of these organelles is related with their nucleic-acid content. The stronger influence on the nucleolus than on the nucleus may be a result of the higher turnover of RNA compared with DNA in the liver when cell proliferation is not high, i.e. under normal physiological conditions. RNA content was more affected than that of DNA by dietary nucleotide deprivation, a finding that agrees with previous studies (Lopez-Navarro *et al.* 1995). In addition, diet-related changes in RNA without changes in DNA have also been reported (Clifford *et al.* 1972). A larger effect

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on DNA is conceivable under conditions of rapid cell proliferation, as has been reported in the small intestine (Uauy *et al.* 1994).

The supply of dietary nucleotides seems not to be essential for hepatocyte survival as neither cell necrosis nor general alterations in liver structure were observed; however, a lack of these nutrients might have significant negative consequences for liver biosynthetic functions, the most apparent consequence being fat accumulation. Other aspects of hepatic biology that may be altered as a result of nucleotide deprivation are glycogen metabolism (Carver, 1994), eicosanoid formation (Marimoto *et al.* 1993) and biosynthesis of glycoproteins (Pels Rijcken *et al.* 1995). Deprivation of dietary nucleotides leads to a decrease in liver acid-soluble nucleotides, mainly ATP and cytidine derivatives, including CMP (Lopez-Navarro *et al.* 1995), and this decrease may influence the levels of nucleotide sugars involved in the biosynthesis and subsequent transport of secretory proteins. In agreement with our results, decreased levels of ATP and CMP as well as of CMP-N-acetylneuraminate in the Golgi lumen and, therefore, diminished sialylation of proteins, have been reported in response to uridine and cytidine treatments (Pels Rijcken *et al.* 1993, 1995).

Although the mechanism responsible for fat accumulation is not known, we believe it is the result of decreased protein synthesis. Thus, in the nucleus, in addition to the reduction in size, the higher chromatin density indicates a more tightly coiled chromatin (heterochromatin) that can lead to a reduced transcription rate. The reduction of the nucleolus suggests reduced ribosomal production as a result of lower rRNA synthesis (Warner, 1990; Mélise & Xue, 1995), a possibility supported by the fact that the content of ribosomes decreased. While there is no reason why a reduction in ribosome number means that protein synthesis is reduced, a reduction in the number of polyribosomes actively engaged in translation is clear evidence for decreased rates of protein synthesis. Other workers have reported that alterations in liver nucleotide pools (Windmueller & Levy, 1967) and in protein metabolism appear to be related to the induction of fatty liver in the rat (Oler *et al.* 1969).

In our experiment the changes in the nucleus as well as in the content of monoribosomes returned to initial values after 21 d, apparently as a result of the increase in nucleotides through the activation of *de novo* synthesis, so that the effect of deprivation appears to be transitory. These transient morphological changes showed a high correlation with the changes in RNA and acid-soluble nucleotide contents (López-Navarro *et al.* 1995). The recovery was also confirmed by the increase in binucleate hepatocytes from 10 to 21 d. Despite the recovery, however, the nucleolar area apparently remained more restricted than in controls, although this was not significant; the amount of polyribosomes persisted at significantly lower levels indicating that protein synthesis probably remained depressed, and fat accumulation increased. These findings suggest that although the liver has the capacity for *de novo* synthesis of nucleotides, the process is inadequate to restore initial levels under deprivation. One of the possible explanations is that under dietary nucleotide deprivation the loss of ATP boosted the *de novo* synthesis of nucleotides and, hence, the recovery.

Utilization of exogenous nucleotides given parenterally has proved to be beneficial in relation to various forms of liver injury in rats. Thus, a mixture of nucleotides and nucleosides improves hepatic function and promotes earlier restoration of the liver after injury or partial hepatectomy (Ogoshi *et al.* 1985, 1990). Palombo *et al.* (1993) have demonstrated also that exogenous adenosine or adenine enhance ATP recovery after cold ischaemia. In addition, Hernández-Muñoz *et al.* (1994) have reported that adenosine treatment of CCl_4-poisoned rats counteracted the effect of the hepatotoxin in the maintenance of the mitochondrial function and collagen accumulation. Furthermore,

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recent studies have shown that dietary nucleotides accelerate the structural restoration of liver recovering from thioacetamide-induced cirrhosis (Torres *et al.* 1996).

In summary, the data presented support the hypothesis that dietary nucleotides are used by the liver under normal physiological conditions. The lack of an adequate supply of dietary nucleotides for a certain period of time may have negative effects on liver structure and function.

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