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Free and Membrane-bound Ribosomes and NADPH-Cytochrome C Reductase Activity in the Liver Cells of Protein-fed and Protein-deprived Rats

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ABSTRACT

Rats were starved for 7 days or fed a protein-free or a 25% casein diet for 7 or 14 days. Free and total ribosomes were prepared from the liver. A determination of the ratio of mg RNA of ribosomes to mg DNA of the total homogenate was made. In starved rats, RNA of free and membrane-bound ribosomes decreased to the same extent. In rats fed a protein-free diet only mg RNA of membrane-bound ribosomes decreased significantly, and after 14 days the ratio of RNA of free to total ribosomes was increased. NADPH-cytochrome c reductase activity in the liver decreased per mg DNA in protein deprivation but was unchanged per mg RNA of membrane-bound ribosomes.

INTRODUCTION

Liver nucleoli enlarge in rats fed a protein-free diet (7), probably as a result of an increased ribosomal RNA synthesis (11, 18). The total amount of liver RNA decreases, however, apparently due to a decreased half-life of the ribosomal RNA (11, 14). Ultrastructural studies reveal that the number of membrane-bound ribosomes decrease in protein deprivation, but that many free ribosomes and polysomes remain (19). A question, which cannot be answered by study of electron micrographs, is, however, whether there is an absolute decrease in the number of free ribosomes and polysomes per cell in protein deprivation. Another problem is the functional capacity of the ribosomal systems during protein deprivation. Knowledge of these parameters is of great interest in view of the world-wide deficiency in high quality protein supplies, and the ability of the liver to handle this deficiency by altered protein metabolism (23).

It is the aim of the present investigation to contribute to the answering of these questions. Quantitative changes in ribosomes per liver cell were

evaluated by determining RNA from total and free ribosomes per mg DNA after fractionation of liver tissue by ultracentrifugation. As a measure of the functional capacity of the membrane-bound ribosomal system, the activity of an enzyme synthesized in this system, NADPH-cytochrome c reductase (9), was determined per mg DNA and mg RNA of membrane-bound ribosomes.

MATERIAL AND METHODS

Forty-three male white rats (Wistar strain, Möllegaard Hansens Avls-laboratorier A/S, Denmark), weighing about 195 g, were either starved for 7 days or fed either a 25% casein diet or a protein-free diet ad libitum (17) for 7 or 14 days. The animals were given tap water ad libitum throughout the experiment. They were kept in plastic cages with saw dust on the floor.

At about 7.30 a m on the last day the rats were given an intraperitoneal injection of 0.25 µCi ^{3}H-orotic acid (The Radiochemical Centre, Amersham, England), spec act 23 Curies/mmol, per 100 g body weight, and killed by decapitation 90 min later.

Ribosomes were prepared essentially according to the method of Blobel and Potter (2). From each rat, 4 to 5 g of liver was passed through a tissue press and homogenized in a Dounce homogenizer with 10 ml 2.32 M sucrose in 0.05 M Tris-HCl buffer, pH 7.6, with 0.01 M $MgCl_2$ and 0.025 M KCl (this buffer was used throughout). Amylase (α-amylase, type 1 A, from hog pancreas, Sigma, U.S.A) was added to give 500 U/g liver. The homogenate was brought to a total volume of 4 ml sucrose buffer per g liver. The homogenate was kept at +4°C for 60 min to break down glycogen (8). 2.0 ml of the mixture was frozen for determination of nucleotides, RNA and DNA (21). The loss of RNA during amylase treatment was, if any, less than 2%. 13 ml were layered over 3 ml 2.32 M sucrose in buffer and centrifuged for 60 min at 105.000 g in a Sorvall OTD 2 ultracentrifuge AH-627 swing out rotor. The nuclear pellet was frozen for analysis of nucleotides, RNA and DNA. The pellet contained 55% of the DNA of the total homogenate. Longer centrifugation gave sedimentation of cytoplasmic material. The RNA/DNA ratio (mg/mg) of the nuclei was 0.11 - 0.18, or 3 - 5% of the total cellular RNA, the same figure as found by Blobel and Potter (2).

The supernatant and the cushion were brought to a Colworth 'stomacher' (Sewer, Bury St Edmund, England) with 32 ml buffer and, as a ribonuclease inhibitor, 144 µl heparin (AB Vitrum, Sweden) (5.000 IU/ml), and homogenized

for 2.5 min. 20 ml was brought to each of two centrifuge tubes. To the one was added 1.2 ml H_2O and to the other 1.2 ml 20% fresh sodium deoxycholate for determination of free and total ribosomes, respectively, as described by Blobel and Potter (2). The tubes were tilted and allowed to stand for 10 min. From each tube 6 ml were taken, and nucleotides, RNA and DNA determined. The remainder was brought to 20 ml by the addition of H_2O. Of this mixture 1 ml was taken for determination of membrane enzymes and frozen. From each tube 10 ml were layered over 3 ml 2.0 M sucrose with buffer and heparin (3 µl per ml). The homogenate was overlayered with buffer without sucrose so that the tubes were almost filled. The tubes were centrifuged for 24 h at 125.000 g in the ultracentrifuge described above. The overlay was removed. The pellet, the 2.0 M layer and the remaining supernatant were frozen separately for determination of RNA and enzymes. In agreement with Peterson et al (15) we found the ribosomes both in the pellet and the 2.0 M sucrose cushion. Both these fractions were therefore included in the determination of ribosomes. Membrane-bound ribosomes were calculated as described by Blobel and Potter (2) from the difference between total and free ribosomes, always prepared in the same centrifugation. Less than 1% of the total enzyme activity was found in the cushion, and none in the pellet, indicating that membrane-bound polysomes did not enter the 2.0 M sucrose layer.

NADPH-cytochrome c reductase activity was determined by the method of Ernster (6) on the total liver homogenate.

Protein was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, U.S.A), based on the Coomassie Brilliant Blue G-250 stain (3), with bovine gamma globulin as a standard on the total liver homogenate.

NADPH-cytochrome c reductase activity, mg protein and mg RNA were related to mg DNA of the total homogenate. For further ratios, see the table.

Statistical methods. Student's t-test was used (16).

RESULTS AND DISCUSSION

The liver weights decreased in starvation and protein deprivation (Table 1). However, liver weight per 100 g body weight decreased significantly only in the starved rats.

Liver protein and liver DNA was lower in both starvation and protein deprivation, compared to protein feeding, but the liver DNA per g body weight

was unchanged or even slightly increased in 2 weeks' protein deprivation. The ratios of protein/DNA, nucleotide/DNA, and RNA/DNA, all decreased in starvation and protein deprivation. All these results agree with previous studies by several authors (13).

There was a significant fall in RNA of membrane-bound ribosomes/DNA in starved as well as in protein-deprived rats. In the starved rats there was also a significant decrease in RNA of free ribosomes/DNA. The ratio of RNA of free ribosomes/RNA of total ribosomes, increased significantly in 2 weeks' protein deprivation but did not change significantly in the other groups. As no differences have been found in the RNA contents of individual liver ribosomes in protein deprivation and starvation (4, 5, 7), the changes in RNA of the ribosomes of the liver cell reflect changes in number of ribosomes. It can therefore be concluded that in starvation, free and membrane-bound ribosomes fell to the same extent. In protein deficiency, only membrane-bound ribosomes decreased significantly, substantiating our ultrastructural observations (19). It is well known that one of the main functions of the membrane-bound ribosomes of the liver is to synthesize serum albumin (12), and that this protein decreases in serum during protein depletion. This function may be less vital than that of the free ribosomes and polysomes, which mainly synthesize proteins for internal purpose of the liver cell.

In all groups, except starvation, the ratio of specific labelling of RNA of membrane-bound to RNA of free ribosomes was equal to one. In starvation, this ratio was larger than one, suggesting a more rapid labelling of membrane-bound ribosomes.

The time course of incorporation of labelled orotic acid into free and membrane-bound ribosomes has been widely discussed (10, 22). Our results agree with the generally accepted view that ribosomes enter the cytoplasm in membrane-bound form. All authors have found that there is later an identical labelling of free and membrane-bound ribosomes, suggesting an interchange of ribosomes between the free and membrane-bound states. It may take a longer time to reach equilibrium in starved rats.

The NADPH-cytochrome c reductase activity per mg DNA (or cell) decreased in protein deprivation and starvation, but was unchanged per mg RNA of membrane-bound ribosomes or even slightly increased in 2 weeks' protein deprivation. That indicates a high functional capacity of the membrane-bound ribosomes in protein deprivation. We have previously found that the liver also in protein deprivation has the ability to increase the amount of smooth-surfaced

Table 1. Effects of starvation and protein deprivation on liver protein, RNA, DNA and cytochrome c reductase activity.

Rat and liver weights are given in g. Protein is given in g/total liver and in mg/mg DNA. Nucleotide, RNA and DNA are given in mg. Cytochrome c reductase activity is measured as change in optical density /3 min/mg DNA, RNA and protein.

		1 week's experiment		2 weeks' experiment	
	Starvation	25%casein	protein free	25%casein	protein free
Initial weight of rats	204±4 s=10	195±2 s=7	204±2 s=5	197±2 s=7	199±3 s=10
Final weight of rats	138±5[a] s=13	226±3 s=8	163±4[a] s=10	263±5 s=17	151±3[a] s=9
Weight changes	-66±3[a] s=7	31±1 s=4	-41±3[a] s=7	67±4 s=13	-47±3[a] s=9
Liver weight	3.3±0.2[a] s=0.5	10.0±0.6 s=1.8	6.2±0.4[a] s=0.9	11.1±0.4 s=1.4	5.8±0.3[a] s=0.9
Liver weight / 100 g body weight	2.40±0.08[a] s=0.19	4.41±0.21 s=0.68	3.79±0.14 s=0.34	4.18±0.12 s=0.40	3.81±0.16 s=0.54
DNA/total liver	16.2±1.2[a] s=3.0	25.3±1.3 s=4.3	18.8±0.8[b] s=2.0	23.8±1.7 s=5.4	17.0±0.4[b] s=1.5
Liver DNA / g body weight	0.117±0.005 s=0.012	0.112±0.005 s=0.016	0.116±0.005 s=0.012	0.090±0.005 s=0.017	0.113±0.004[b] s=0.013
Protein/total liver	1.18±0.09[a] s=0.22	3.11±0.23 s=0.72	1.60±0.05[a] s=0.12	3.35±0.27 s=0.84	1.26±0.06[a] s=0.19
Protein/DNA	73.5±3.5[a] s=8.6	121.9±3.7 s=11.6	85.8±2.5[a] s=6.1	140.5±4.5 s=14.3	73.9±2.6[a] s=8.7
Nucleotide/DNA	0.80±0.03[a] s=0.07	1.44±0.04 s=0.11	0.99±0.03[a] s=0.08	1.68±0.04 s=0.11	1.05±0.02[a] s=0.07
Nucleotide/RNA	0.52±0.02 s=0.05	0.50±0.01 s=0.04	0.49±0.03 s=0.08	0.52±0.01 s=0.03	0.53±0.01 s=0.05
Total RNA/DNA	1.56±0.10[a] s=0.25	2.89±0.07 s=0.21	2.07±0.13[a] s=0.31	3.20±0.03 s=0.11	1.99±0.06[a] s=0.19

Table 1, continued.

$\frac{\text{RNA of free ribosomes}}{\text{DNA}}$	0.39±0.03[a] s=0.07	0.96±0.12 s=0.37	0.75±0.07 s=0.17	0.92±0.08 s=0.24	0.78±0.05 s=0.17
RNA of membrane-bound ribosomes/DNA	1.06±0.07[a] s=0.17	1.78±0.09 s=0.27	1.04±0.09[a] s=0.23	2.02±0.10 s=0.31	0.82±0.07[a] s=0.23
$\frac{\text{RNA of free ribosomes}}{\text{RNA of total ribosomes}}$	0.27±0.02 s=0.05	0.35±0.04 s=0.12	0.42±0.04 s=0.10	0.31±0.03 s=0.08	0.50±0.03[a] s=0.11
Cytochrome c reductase activity/DNA	11.2±0.9[a] s=2.3	22.1±0.8 s=2.8	13.4±0.2[a] s=0.7	26.1±1.1 s=3.7	13.6±0.5[a] s=1.9
Cytochrome c reductase activity/RNA of membrane-bound ribosomes	10.5±0.7 s=1.8	12.6±0.8 s=2.7	13.4±1.1 s=2.8	13.3±1.0 s=3.2	17.4±1.2 s=4.1
Cytochrome c reductase activity/protein	0.16±0.02 s=0.04	0.18±0.01 s=0.02	0.16±0.01[b] s=0.01	0.19±0.01 s=0.02	0.19±0.01 s=0.02
Sp act of RNA of membrane-bound ribosomes/sp act of RNA of free ribosomes	1.75±0.11[a] s=0.27	1.08±0.05 s=0.13	1.08±0.08 s=0.20	1.06±0.04 s=0.12	0.94±0.08 s=0.28
Number of rats	6	10	6	10	11

a/ $p < 0.001$ as compared to protein fed rats
b/ $0.001 < p < 0.01$ - " -

s = standard deviation of the mean.

endoplasmic reticulum on a stimulus with DDT, also a sign of this capacity (20).

A few points regarding the technique for the preparation of ribosomes deserve special comment. Starvation brings about a rapid loss of RNA within 24 h (5), which preferentially affects the free ribosomes during the first day. After 5 days' starvation the total loss is approximately the same in free and membrane-bound ribosomes (10). Fasting overnight before sacrifice should therefore be avoided in all studies concerning the effect of various diets on ribosomal and RNA metabolism, as the starvation may wipe out the effects of the diets. The advantage with the overnight fasting is that the glycogen disappears from the liver cell - another significant effect of fasting on the metabolism of the liver cell - which facilitates the separation of ribosomes during ultracentrifugation. However, incubation of the liver homogenate with amylase (8) before centrifugation breaks down glycogen and makes the fasting unnecessary.

The pressed liver homogenate should not be filtered, as we found this to give considerable losses in cellular material. To sediment the nuclei, the liver homogenate should be submitted to high speed centrifugation over a heavy sucrose cushion, as described by Blobel and Potter (2). Low speed centrifugation without a cushion, as performed by several authors, sediments much cytoplasmic material with the nuclei. In 24 h ultracentrifugation all ribosomes do not reach the bottom of the tube but can still be found in the heavy sucrose cushion (15). This fraction must therefore be included in the determination of ribosomes.

Using the method of Blobel and Potter (2) mitochondria are located in the fraction of total ribosomes. These authors consider the amount of mitochondrial RNA to be insignificant in relation to the amount of ribosomal RNA (1), a view apparently taken by most authors.

Several details are thus of uttermost importance for getting quantitative figures on free and membrane-bound ribosomes. Times and speeds of centrifugation must be checked in every laboratory to avoid penetration of cytoplasmic material into the nuclear pellet and membrane-bound ribosomes into the fractions of free ribosomes.

Technical discrepancies of these types probably explain, why it has not previously been possible (4) to detect an increased ratio of free to total ribosomes in the liver cell cytoplasm of protein-deprived rats.

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