Some Effects of Continued Protein Deprivation, with and without Methionine Supplementation, on Intracellular Liver Components.* (18095)

SAM SEIFTER, EDWARD MUNTWYLER, AND DAVID M. HARKNESS

From the Department of Biochemistry, State University of New York, State University Medical Center at New York, Brooklyn.

It has been reported previously from this laboratory(1) that dietary protein deprivation of rats for a 3 week period causes marked changes in the contents of nitrogen, pentosenucleic acid (PNA), and desoxypentosenucleic acid (DNA) of the various intracellular components of their livers. Since an extension of the period of dietary protein restriction results in certain compensatory changes in the liver which make its composition resemble more nearly the normal(2,3), the effects of 5 week protein depletion on the morphological constituents of the liver were studied. In addition, because methionine has been shown to exert a protective effect upon the liver under various conditions(4), the influence on the intracellular liver components of rats on a protein-free diet supplemented with this amino acid was investigated.

Experimental. Forty-two female rats of the Wistar strain, between 12 and 18 weeks of age, and weighing between 166 and 210 g, were placed on a normal diet, adequate in all respects, for a period of 7 days. At the end of this time the animals were divided into 3 groups of 14. Group I was placed on a protein-free diet for a period of 5 weeks. Group II was maintained on a protein-free diet containing 1% DL-methionine for the 35 day period. The last group served as controls and was continued on the normal diet for the same period of time.

The normal and protein-free diets were identical in composition with the corresponding diets reported in a previous study(1). The methionine-supplemented diet was similar to the protein-free diet except that it contained 1% DL-methionine which replaced a corresponding amount of glucose. The livers of the animals were obtained and prepared for analysis as described previously(1). Water and fat were determined as before, and glycogen was determined by the anthrone method(5). A 5 ml aliquot of a 10% homogenate of the liver was fractionated into so-called nuclear, mitochondrial, microsomatic, and residual cytoplasmic fractions by the differential centrifugation method of Schneider (6), and the fractions as well as the original homogenate were analyzed for nitrogen, DNA, and PNA. Nitrogen was determined by the micro-Kjeldahl procedure, the ammonia formed being steam-distilled and titrated. The DNA and PNA were determined by a modification of the method described by Ogur and Rosen(7), which is based upon the observation that cold perchloric acid extracts PNA only, whereas hot perchloric acid extracts both PNA and DNA. The essential steps of the procedure are detailed below. Each of the sedimented fractions is diluted to 10 ml with isotonic sucrose solution, and a 1 ml portion precipitated with 2.5 ml of cold 10% trichloroacetic acid. The centrifuged precipitate is then washed successively with additional amounts of trichloroacetic acid, with 70% ethanol containing 0.1% perchloric acid (PCA), then treated at 50°C for 1 minute with a 3:1 ethanol-ether mixture, and further washed with 2% PCA. Centri-

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fugation following each treatment is carried out in the cold except after the alcohol-ether extraction when the operation is performed at room temperature. The further treatment of the washed residue depends upon whether the fraction contains only PNA or both PNA and DNA.

For fractions containing only PNA (mitochondrial, microsomatic, and residual cytoplasmic): The residue is extracted 3 times with 10% PCA at 80°C for 20 minutes, and the extracts are combined and diluted to 10 ml with 10% PCA. *For "fractions" containing both PNA and DNA (nuclear fraction and homogenate)*: The residue, suspended in 10% PCA, is allowed to stand at 4°C for 18 hours, centrifuged, and washed twice by centrifugation with cold 10% PCA. The washings and original extract are combined and diluted to 10 ml with 10% PCA. The extract obtained in this manner contains PNA only.

For the extraction of DNA, the residue remaining *after* the extraction of PNA is treated as described above for fractions containing only PNA. Thus a second extract is obtained containing only DNA. The PNA or DNA contents of the various extracts were determined spectrophotometrically as described by Ogur and Rosen(7). The results obtained by this method for the PNA contents of liver homogenates and the various sedimented fractions agreed with the values obtained by the orcinol method(8) applied to trichloroacetic acid extracts(9) of the same liver samples. The DNA contents of liver homogenates as determined from the spectral absorption of PCA extracts were slightly higher than the values obtained on trichloroacetic acid extracts from the same homogenates employing the diphenylamine method (10). When applied to the nuclear fraction for the determination of DNA, the method of PCA extraction and spectrophotometric measurement gave exceedingly low values. Thus, the amount of the total liver DNA recovered in this fraction varied between 60 and 80%. It is not likely that this discrepancy is a result of partial extraction of the DNA during the extended treatment of the fraction with PCA in the cold, inasmuch as this would result in an abnormally large value for PNA in the nuclear fraction. In all cases, however, the nuclear PNA values compared favorably with values obtained by other methods.

Results. The animals maintained on the control diet for 5 weeks gained on the average 5.8 g per week. The animals subsisting on the protein-free diet and those on the protein-free, methionine-supplemented diet for this period of time lost on the average 9.8 g and 9.4 g per week respectively. The average liver weights at sacrifice of the various groups of animals were as follows: control, 7.3 g; protein-free, 5.1 g; and protein-free, methionine-supplemented, 5.2 g.

The results obtained for liver, fat, water, and glycogen on the control animals were similar to those reported previously(11). The livers of Group I animals showed no change in water content as compared with the controls. The fat content of the liver, however, increased from the control value of 26.0 to 77.8 g per kg for the protein-free group, and the glycogen content of the liver increased

TABLE I. Desoxypentosenucleic Acid Content of Liver Cell Homogenates from Control and Protein-deficient Rats.

mg per g liver	Avg	σ†	*t* value*
Control	3.52	0.40	
" N	115	10	
Protein-deficient	4.08	0.37	3.62
" " N	718	19	10.95
Protein-deficient + methionine	4.01	0.29	3.70
Protein-deficient + methionine N	170	14	9.78

* The *t* value is the expression of the significance of the difference between the means. A figure of 3 or greater is of definite significance (Hill, A. B., Principles of Medical Statistics, (1942), London, 3rd edition).

† σ represents the standard deviation.

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TABLE II. Nitrogen Contents of Liver Cell Fractions from Control and Protein-deficient Rats.

	Control		Protein-deficient			Protein-deficient + methionine		
	Avg	σ	Avg	σ	*t* value	Avg	σ	*t* value
Homogenate								
mg per g liver	30.77	1.23	23.00	1.61	14.26	23.63	1.94	11.60
Nuclear fraction								
mg per g liver	6.55	0.38	6.32	0.58	1.16	6.73	0.74	0.78
% of total	21.3	1.1	27.7	2.3	8.75	28.3	1.6	13.00
Mitochondrial fraction								
mg per g liver	5.51	0.73	3.23	0.41	9.72	3.48	0.64	7.38
% of total	18.0	2.3	14.1	1.8	4.71	14.7	2.3	3.67
Microsomatic fraction								
mg per g liver	6.71	0.50	4.42	0.45	11.86	4.44	0.34	13.62
% of total	21.2	2.4	19.3	2.1	2.07	18.8	1.4	3.12
Residual cytoplasmic fraction								
mg per g liver	12.64	0.39	9.99	0.98	8.72	9.96	0.77	11.15
% of total	41.3	1.3	43.7	1.3	4.62	42.1	2.5	1.03
% recovery	102.6	3.9	104.8	2.9		103.8	4.3	

TABLE III. Pentosenucleic Acid Contents of Liver Cell Fractions from Control and Protein-deficient Rats.

	Control		Protein-deficient			Protein-deficient + methionine		
	Avg	σ	Avg	σ	*t* value	Avg	σ	*t* value
Homogenate								
mg per g liver	7.43	0.33	7.03	0.66	2.02	7.27	0.83	0.07
" " " " N	242	18	305	28	6.88	308	24	8.25
Nuclear fraction								
mg per g liver	1.15	0.11	1.34	0.17	3.38	1.40	0.25	3.30
% of total	15.4	1.4	19.1	2.7	4.43	19.3	2.2	5.33
mg per g fraction N	176	17	213	25	4.17	208	18	4.57
Mitochondrial fraction								
mg per g liver	0.60	0.14	0.58	0.15	0.34	0.55	0.04	1.19
% of total	8.0	1.6	8.2	1.76	0.30	7.7	2.1	0.41
mg per g fraction N	106	18	181	44	5.53	163	42	4.51
Microsomatic fraction								
mg per g liver	3.75	0.53	2.84	0.38	4.96	2.92	0.31	4.87
% of total	50.4	4.9	40.3	5.7	4.78	40.5	5.2	5.03
mg per g fraction N	560	47	646	53	4.15	656	59	4.58
Residual cytoplasmic fraction								
mg per g liver	2.57	0.25	3.12	0.34	4.58	2.86	0.44	2.07
% of total	34.4	3.0	44.3	4.0	6.85	39.4	4.2	3.46
mg per g fraction N	204	20	313	21	13.33	286	33	7.57
% recovery	108.2	6.5	111.9	6.1		106.9	7.0	

from 6.16 to 7.50%. The analysis of the livers of the Group II rats for these constituents yielded results not appreciably different from those obtained on the livers of Group I animals.

Table I summarizes the results of the analyses of the liver homogenates for DNA, while those obtained for the nitrogen and PNA contents of the liver homogenates and the various intracellular fractions are presented

in Tables II and III. At the outset it should be noted that on the whole the results obtained with the methionine-supplemented animals were similar to those obtained with the protein-free group.

The liver homogenates of Group I animals showed a decrease in nitrogen, an increase in DNA, and no significant change in PNA as compared with the control group.

The nuclear fractions from livers of Group I animals showed no significant change in nitrogen content but contained significantly more PNA than did the corresponding fractions from control animals. On the other hand, there was a marked loss in nitrogen from the mitochondrial fractions of Group I livers as compared with the controls, and there was no significant change in the PNA values.

The results further show that a loss of PNA from the microsomatic fractions of Group I animals was accompanied by an increase of this constituent in the residual cytoplasmic fractions of the livers. Although there was a comparable loss of PNA from the microsomatic fractions of Group II animals, the gain of PNA by the residual cytoplasmic fractions of these same animals was not so great in magnitude as was encountered in the case of Group I animals.

Finally, there was a significant loss of nitrogen from the liver microsomatic fractions of both Groups I and II which was accompanied by a decrease in the absolute amount of nitrogen in the residual cytoplasm. However, there was a significant increase in the *percentage* of the total nitrogen located in the latter fraction in the case of the Group I animals which was not encountered in the case of the Group II rats.

Discussion. The animals maintained on a protein-free diet for 5 weeks showed a 25.2% decrease in liver nitrogen when compared to control animals, whereas animals on a similar diet for 3 weeks showed a 19.3% decrease in liver nitrogen. As in the case of the 3 week animals, the loss in liver nitrogen by the 5 week group could be accounted for by a loss of nitrogen from the mitochondrial, microsomatic, and residual cytoplasmic fractions, while the nuclear fraction was relatively unaffected. In the case of the 5 week animals, however, the loss of nitrogen tended to be more equally divided among the 3 affected fractions than in the case of the 3 week animals. However, the loss of nitrogen was greatest from the microsomatic fraction in the latter group, whereas in the 5 week animals the loss was greatest from the residual cytoplasm. For the present, the significance of this difference between the 2 groups of animals is not apparent. As was observed with the 3 week animals, the livers of the 5 week protein-deficient rats showed an increase of DNA. In the latter instance, however, the change was of unquestionable statistical significance. This increase in DNA can be interpreted as the result of an increased number of cells per unit of liver or an elevated concentration of DNA per cell nucleus. It has been shown that in other experimental situations resulting in an elevated liver DNA, the increase of this constituent can be accounted for by an increased cell density(12,13), an explanation which probably applies to conditions of protein-deficiency as well.

Although livers from the rats subsisting on the protein-free diet for the 5 week period contained slightly less PNA than did the corresponding control livers, this difference was only of probable significance. However, while the total PNA change was not marked, there was a striking redistribution of this constituent among the several cellular fractions when compared with the controls. Thus, an increased amount of PNA was present in the nuclear fraction of Group I animals, while there was a marked decrease in the PNA of the microsomatic fraction and an increase of the PNA in the residual cytoplasm. Such a redistribution of the PNA between the latter 2 fractions was previously described for 3 week protein-deficient animals(1), but the present data make it apparent that extended protein depletion magnifies this "shift." Recently, in a brief report on the effects of

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a 40 day protein-fast on the liver PNA, Vendrely and Vendrely(14) came to the conclusion that the loss of this constituent (which is apparent only when the values are expressed in terms of "initial liver weight") stems almost exclusively from the microsomes. The results reported in the present paper are generally in accord with those of the French authors inasmuch as the greatest change in PNA content occurred in the microsomatic fraction, but the interpretation presented here places the emphasis on the redistribution of PNA between this fraction and the residual cytoplasm rather than on the absolute loss from the cells.

It is important to point out that the encountered changes in the distribution of PNA and nitrogen among the various fractions seem to fall into a general pattern similar to that which has been reported by Price and co-workers(15-19), and by Cunningham, Griffin, and Luck(20) for cell fractions obtained from hepatoma tissue *per se* and from livers of rats maintained on high or low protein diets containing active carcinogenic dyes. Thus these workers have reported that hepatomas contained more DNA and nitrogen in the nuclear fraction, less PNA and nitrogen in the mitochondrial and microsomatic fractions, and considerably more PNA in the residual cytoplasm than did normal livers. Similar changes from normal were reported for liver fractions from rats fed active carcinogenic compounds although the magnitude of the changes was not so great. In the case of protein-deficient animals the livers contained more DNA and PNA, but an equal amount of nitrogen in the nuclear fraction, less nitrogen but an equal amount of PNA in the mitochondrial fraction, less PNA and nitrogen in the microsomatic fraction, and considerably more PNA in the residual cytoplasm than did the livers of control animals. The extent of these changes was related to the period of time that the animals were kept on the protein-free diet. It would appear, therefore, that the pattern of PNA and nitrogen distribution just described is more general than previously believed(19), and cannot be specifically related to existence of a pre-cancerous condition.

The only apparent difference between the results obtained with animals on the protein-free diet and those on the same diet supplemented with 1% methionine was the obviation of the increase in the PNA content of the residual cytoplasm. The significance of this effect of methionine is not at present understood.

Summary. The intracellular distribution of nitrogen and nucleic acids in the livers of rats fed a diet free of protein for 5 weeks was found to be similar to that previously observed in rats subsisting on the same diet for 3 weeks, although the changes from control values were of greater magnitude in the case of the animals depleted for the longer period of time.

The inclusion of 1% DL-methionine in the protein-free diet had no appreciable effect in protecting the liver from the observed changes due to protein depletion except in partially preventing the increase of PNA in the residual cytoplasmic fraction. It is noted that the changes encountered in the intracellular distribution of nitrogen and nucleic acids in the livers of protein-deficient animals fall into the same pattern as the changes which have been found to obtain in the livers of pre-cancerous and cancerous animals.

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