INTERFERENCE OF AUTOLYTIC ACTIVITY WITH THE DETERMINATION OF CELL NUCLEUS COMPOSITION UNDER DIFFERENT CONDITIONS OF PROTEIN DEFICIENCY[1,2]

ROBERTO UMAÑA

Institute of Nutrition of Central America and Panama (INCAP), Guatemala, Central America

Received June 8, 1965

Abstract

The changes produced by protein-deficient diets and starvation on the composition of rat liver nuclei have been studied. It was found that the histone fraction and the histone to DNA ratio remained remarkably constant during the different experimental conditions studied. The rest of the protein fractions showed variations which can probably be ascribed to an increase in the autolytic activity of the preparations. It appears that the degree of autolysis varies with the type of dietary insult applied to the animal.

Introduction

Previous work in our laboratory (1, 2) has shown that protein deficiency in the rat produces in the liver cells an increase in the polyploid type of nuclei with a consequent increase in the average DNA content of the isolated nuclei, and secondarily an increase in the DNA concentration of homogenates of the liver. Similar increases were also reported in the DNA content of homogenates made from the livers of children who had died of protein malnutrition (3). This increase has been interpreted as the result of alterations in the mechanism of cell division, most probably due to an impairment in the mechanisms of protein synthesis.

It has been our purpose to study the effect of protein deficiency on the nuclear proteins and to try to correlate this with the impairment of cell division in protein malnutrition (1–3). Since the block in cell division seems to be located somewhere beyond phase S, it was decided to study preparations highly enriched with the polyploid type of nuclei.

The results presented in this article show that protein deficiency produces a degree of autolytic activity which tends to obscure the changes in protein composition of the nuclei. It seems that the degree of autolytic changes might be related to the type of nutritional insult applied to the animal.

Methods

Adult male rats (200–250 g) of the Sprague–Dawley strain were divided into nine groups, matched according to weight. For one week, all the animals

[1]This investigation was supported by grants AM-0981 and AM-09126-01 from the National Institutes of Health.

[2]INCAP Publication I-356.

A brief summary of this article was presented at the meeting of the Federation of American Societies for Experimental Biology in Atlantic City, N.J., April 9–14, 1965.

were fed ad libitum a 20% casein diet prepared as previously described (2). At the end of this period, one group was killed to serve as the control and the others were placed on a nitrogen-free diet prepared as described elsewhere (2). These groups were killed at 24-hour intervals after the nitrogen-free regimen was started, except for the last one which was killed after two weeks.

Two more groups of rats were used. One was fed a 5% corn protein diet (2) for two weeks and the other was starved for six days. The livers obtained after the experimental period were homogenized in 0.44 *M* sucrose solution with enough citric acid to obtain a pH of 5.8, and the nuclei were isolated and purified by differential centrifugation as described by Dounce and Umaña (4), with the following modification. After the preliminary purification in 0.44 *M* sucrose, the nuclear preparation was suspended in 45 ml of a 2.2 *M* sucrose solution (density = 1.28) and centrifuged at 25,000 × *g* for 30 minutes in a 34° angular rotor, instead of at 95,000 × *g* for the same period of time. This change allowed the separation of the polyploid type of nuclei which were collected in the bottom of the tube.

After the nuclei had been isolated in a pure form, the different protein fractions were extracted as described by Monty and Dounce (5). The method used permits the extraction of the following protein fractions: globulins (extractable with 0.14 *M* NaCl solution), histone (extractable with 0.1 *N* HCl after the extraction of the globulin fraction), and the residual protein (insoluble protein residue which is obtained after the extraction of the previous fractions and DNA). The histones were further fractionated into precipitable histones (isoelectrically precipitated at pH 10.5) and soluble histones (precipitated from the supernatant of the previous fraction with three volumes of 95% ethanol). The DNA was extracted by the method of Schneider and determined by the method of Dische as previously described (2).

The total number of nuclei in a given preparation was determined by direct counting of a very dilute suspension of nuclei in 0.05 *M* citric acid in a Levy-type hemocytometer chamber. Three freshly loaded chambers were counted for each preparation of nuclei. The total number of nuclei counted was about 1000 per preparation and the reproducibility of the countings fell within 5%.

The results were expressed as micromicrograms of constituent per nucleus. The statistical significance of the differences between mean values was determined by the *t* test.

Results

The results of the analyses of the nuclei isolated from the livers of the rats fed the nitrogen-free diet are given in Table I. These data show that during the first 96 hours on the diet, no significant change could be detected in the composition of the nuclei. After 120 hours on the diet, the globulin, the total protein, and the DNA content suddenly decreased significantly, whereas the histones and the residual protein remained constant. After 168 hours on the diet the globulins remained unchanged but the content of histone, residual protein, and total protein showed a significant decrease. After two weeks on

TABLE I

Effect of a nitrogen-free diet on the composition of isolated rat liver nuclei*

	Globulin		Precipitable histones		Soluble histones		Total histones		Residual proteins		Total protein		DNA		Total histones/DNA	
Time†	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Control	17.5	2.9	20.8	2.9	4.3	1.4	25.1	4.0	27.2	4.6	69.8	8.0	21.2	4.8	1.2	0.4
24 hours	16.9	1.8	18.6	2.8	4.4	0.6	23.2	3.0	26.6	5.1	66.8	5.9	20.1	3.0	1.2	0.3
48 hours	16.8	1.3	19.8	1.8	5.0	1.1	24.8	2.4	25.9	1.8	67.5	3.6	19.6	—	1.3	—
72 hours	15.9	3.0	20.1	3.2	4.0	1.4	24.9	4.1	26.7	4.8	70.4	11.8	20.4	1.7	1.2	0.1
96 hours	15.6	1.8	21.1	0.5	4.0	0.6	24.9	1.0	27.3	2.4	67.8	3.3	21.6	5.4	1.2	0.3
120 hours‡	13.3‖	1.7	19.6	3.3	3.5	0.7	22.9	2.6	25.0	2.2	60.9‖	2.9	16.6§	1.7	1.4	0.8
168 hours	14.1‖	1.0	17.4	1.7	3.1	0.5	20.5‖	2.1	18.9‖	2.8	55.0§	10.4	25.8§	2.5	0.7	0.6
2 weeks	21.2	2.9	21.8	4.4	4.2	1.2	26.1	4.8	23.6§	3.9	70.9	9.5	—	—	—	—

*The average is from three replicate experiments in which two rats were killed at one time.
†Six animals per group, except the control which had 10.
‡This was repeated with 8 more rats, similar results being obtained.
§$P < 0.05$.
‖$P < 0.01$.
NOTE: Results expressed as $\mu\mu$g per nucleus.

the diet, the globulin content rose in contrast with the decrease previously described. The histone content remained remarkably constant throughout the experimental period.

The average DNA content per nucleus in all the groups studied was found to be greater than previously reported by this laboratory (1, 2). The reason for this apparent discrepancy will be discussed in the following section. The ratio of total histone to DNA was found to remain constant and to be close to one.

It was not possible to determine, with the method of Schneider and Dische, the DNA content of the nuclei isolated from the livers of rats fed on the nitrogen-free diet for two weeks, the results obtained in several trials being extremely high. It is suspected that under the experimental conditions used, some metabolites could be produced that might interfere with the color produced by the reagent of Dische. The interfering substances were not determined.

The data that show the effect of the protein-deficient diets (5% corn protein diet and nitrogen-free diet) for two weeks and of the six-day period of starvation on the composition of the nuclei are presented in Table II. The diet with corn protein apparently did not produce any changes in the composition of the nuclei. The nitrogen-free diet produced an increase in the globulin fraction and a decrease in the residual protein. The starvation period produced no change in the globulin content and a significant decrease in the residual protein fraction.

Since the changes in globulin and residual protein content did not seem to follow a definite pattern, it was suspected that they were produced by autolytic degradation (6). To test this possibility, the following experiment was carried out. Three groups of rats were fed different diets, the first group (control) a 20% casein diet for two weeks, the second group a nitrogen-free diet for 72 hours, and the third a nitrogen-free diet for two weeks. After the stated periods of time, the animals were killed and the nuclei isolated from the livers by the regular procedure. Each preparation was divided into two equal parts. One half was immediately extracted by the regular procedure and the other was left at 0 °C for one hour. The results are given in Table III. It can be seen that the normal preparation lost some of the globulin fraction, but that the protein-deficient preparations lost not only a great deal of globulins but also some of the residual proteins.

When attempts were made to characterize the globulin fraction of liver-cell nuclei from animals fed the nitrogen-free diet for 120 hours, some indirect evidence was obtained that the degradative change in the nuclear proteins under conditions of protein deficiency was autolytic in nature. The method used was the microzone electrophoresis on cellulose acetate (pH 8.6, ionic strength 0.075, 7 mA (average) for 20 minutes, staining with Ponceau-S). The scanning was done with the Beckman R B analytrol provided with a model R-102 microzone scanning attachment. Figure 1 represents the electrophoretic

TABLE II

Effect of protein deficiency on the composition of isolated rat liver nuclei

Diet*	Globulin		Precipitable histones		Soluble histones		Total histones		Residual proteins		Total protein		DNA		Total histones/DNA	
	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.	$\bar{x}$	S.D.
Control 2 weeks (10)	17.5	2.9	20.8	2.9	4.3	1.4	25.1	4.0	27.2	4.6	69.9	8.0	21.2	4.8	1.2	0.4
5% Corn protein 2 weeks (4)	16.1	1.8	19.2	1.4	4.9	0.5	24.0	1.1	26.9	2.1	67.0	2.5	23.7	1.2	1.0	0.1
Nitrogen free 2 weeks (6)	21.2†	2.9	21.8	4.4	4.2	1.2	26.1	4.8	23.6	3.9	70.9	9.5	—	—	—	—
Starvation 6 days (4)	17.1	2.0	19.2	0.9	4.4	1.0	23.6	1.5	21.2‡	2.0	61.8†	3.5	21.7	1.3	1.1	0.1

*Number in parentheses is the number of animals per group.
†$P < 0.05$ when compared with the control.
‡$P < 0.01$ when compared with the control.
NOTE: Results expressed as $\mu\mu$g per nucleus.

TABLE III

Degradation of nuclear proteins after standing in 1% gum arabic, pH 5.8, for 1 hour at 0° C

Diet	Globulin	Precipitable histones	Soluble histones	Total histones	Residual proteins	DNA	Total histones/DNA
Control casein	17.6	23.1	5.3	28.4	27.1	21.2	1.3
Casein after standing	13.3	22.0	5.8	27.8	25.2	—	—
Control nitrogen free (72 hours)	17.6	21.9	3.7	25.6	25.0	18.8	1.3
Nitrogen free after standing (72 hours)	13.3	21.8	3.0	24.8	16.6	14.3	1.7
Control nitrogen free (2 weeks)	24.0	23.4	3.5	26.9	23.8	—	—
Nitrogen free after standing (2 weeks)	14.0	21.3	6.4	27.7	21.0	—	—

NOTE: Average of two preparations. Results expressed as $\mu\mu$g per nucleus.

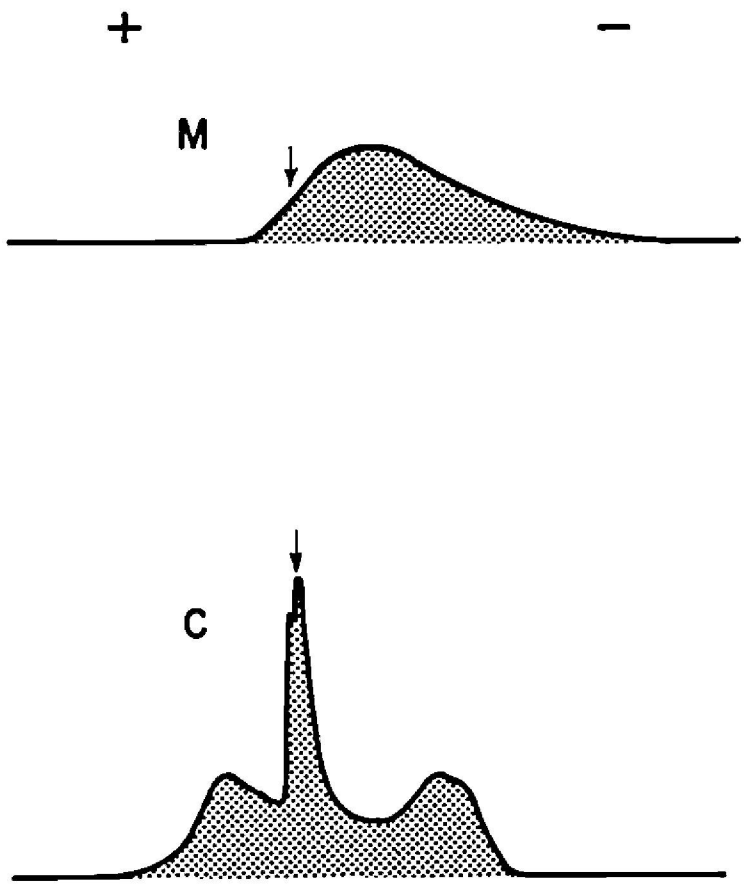

FIG. 1. Electrophoretic patterns of liver nuclei globulins obtained from normal rats (C) and from rats fed a nitrogen-free diet for 120 hours (M). The arrow corresponds to the origin.

pattern of globulins obtained from the normal and the protein-deficient nuclei. It is evident that the globulin fraction from the latter has lost all its specific characteristics, the pattern obtained corresponding to an indefinite smear.

The total histone to DNA ratio remained constant, with values close to one.

Discussion

Previous reports from this laboratory (1, 2) have given the average amount of DNA in nuclei isolated in dilute citric acid from normal rat livers as 10.8±2.5* μμg/nucleus. Table I shows an average DNA content of 21.2±4.8* μμg/nucleus for the control group. This apparent discrepancy is due to the fact that these preparations are highly enriched with nuclei of the polyploid type. Evidence for this is that the DNA content of the nuclei entrapped in the cell debris, when floated out with the 2.2 *M* sucrose, was found in two different preparations to be 7.4 and 9.7 μμg/nucleus. This agrees closely with the values reported for diploid type nuclei (1, 7, 8). The DNA content of the organelles used in the present study corresponds with the values reported for tetraploid type nuclei (7, 8). When the purification step in 2.2 *M* sucrose solution was omitted, the average DNA content of two different preparations was found to be 8.9 and 10.6 μμg/nucleus, which is in good agreement with our previous results (1, 2). The total histone to DNA ratio of the nuclei recovered from the bottom of the tubes is close to one, as was previously

*Standard deviation.

shown for nuclei isolated from tissues in which most of the cells are in a premitotic stage, with a predominance of polyploid type nuclei (9).

This laboratory has also reported (1, 2) that protein deficiency produces an increase in the average DNA content of nuclei isolated from livers of animals subjected to protein-deficient diets. This increase has been ascribed to a higher proportion of polyploid type nuclei in the preparation. It cannot be determined at this time whether this accumulation is due to a block in protein synthesis at the G-2 phase and (or) at the M phase of the cell life cycle, or to a higher rate of progression of the cells into the G-2 phase. At any rate, it is evident that under the present experimental conditions it could not be expected that an increase would be shown in the average DNA content of the protein-deficient nuclei, since a population already selectively enriched with polyploid type nuclei was studied.

The changes found in the protein fractions of the nuclei isolated from the livers of rats on the protein-deficient diets are difficult to interpret at the present time, due to two factors. In the first place, they do not follow a definite pattern. Secondly, the fact that some autolytic changes seem to have taken place under the experimental conditions used, makes it impossible at this time to differentiate between the changes produced by protein deficiency per se and the autolytic artifacts.

Studies by Wilkins *et al.* (11), Wilkins (12), and Zubay and Doty (13) have indicated that histones are linear proteins wrapped around the DNA, occupying the large groove formed by the helical structure of this molecule. Studies by Lloyd and Peacocke (14) have shown that one molecule of DNA (molecular weight 7×10^6) complexes with approximately 1.2 times its mass of histone, therefore giving a histone to DNA ratio very close to one.

Analyses of isolated chromosomes (15–18) and of nuclei isolated from tissues in which a large number of cells are about to divide (9, 10), have shown a histone to DNA ratio very close to one. In the light of the work of Lloyd and Peacocke (14), it could be assumed that in chromosomes and in nuclei about to divide all the histone is complexed with DNA, probably occupying the large groove of this molecule as suggested by Wilkins (11, 12) and by Zubay and Doty (13). On the other hand, in nuclei isolated from tissues in which most of the cells are in the G-1 phase of interphase, the histone to DNA ratio was found to be very close to two (9, 10). It follows then, that if a histone to DNA ratio of one indicates that all the histone is complexed with the available DNA, a histone to DNA ratio of two would indicate that an amount of histone similar to that already complexed with DNA exists, with a looser relationship to the nucleic acid.

The fact that nuclei in G-2 phase, and therefore about to divide (9, 10), have a histone to DNA ratio of one, indicates that the loosely bound histone, which is synthesized in early G-1 phase (10), complexes with the newly synthesized DNA during the S phase, reducing the histone to DNA ratio from two in G-1 phase to one in G-2 phase.

The constancy of the histone fraction and of the histone to DNA ratio in the nuclei of cells isolated from the livers of protein-deficient rats might then be explained by the concept that the position of histone in the large groove of DNA might sterically hinder the action of the autolytic enzymes.

In summary, the data presented in this article permit the following conclusions. (*a*) Protein deficiency seems to produce an increase in the autolytic activity of the nucleus of the liver cell. (*b*) Different dietary conditions seem to produce different levels of autolytic activity, as judged by the different results obtained from experiments with starvation, a nitrogen-free diet, and a 5% corn protein diet. (*c*) The possibility of autolytic degradation of the proteins extracted from the tissues of protein-deficient animals should be taken into consideration in experiments aimed at the study of the metabolic effects of protein malnutrition.

Acknowledgment

The author gratefully acknowledges the skillful technical assistance of Mr. Daniel Fernández.

References

1. R. UMAÑA. Can. J. Biochem. **43**, 125 (1965).
2. R. UMAÑA. J. Nutr. **85**, 169 (1965).
3. R. UMAÑA. To be published.
4. A. L. DOUNCE and R. UMAÑA. Biochemistry, **1**, 811 (1962).
5. K. J. MONTY and A. L. DOUNCE. J. Cellular Comp. Physiol. **53**, 377 (1959).
6. R. UMAÑA and A. L. DOUNCE. Exptl. Cell Res. **35**, 277 (1964).
7. M. FUKUDA and A. SIBATANI. Exptl. Cell Res. **4**, 236 (1953).
8. R. Y. THOMSON, F. C. HEAGY, W. C. HUTCHISON, and J. N. DAVIDSON. Biochem. J. **53**, 460 (1953).
9. R. UMAÑA, S. UPDIKE, and A. L. DOUNCE. Federation Proc. **21**, 156 (1962).
10. R. UMAÑA, S. UPDIKE, J. RANDALL, and A. L. DOUNCE. *In* The nucleohistones. *Edited by* J. Bonner and P. Ts'O. Holden–Day, Inc., San Francisco. 1964. p. 200.
11. M. H. F. WILKINS, G. ZUBAY, and H. R. WILSON. J. Mol. Biol. **1**, 179 (1959).
12. M. H. F. WILKINS. *In* Nucleoproteins. *Edited by* R. Stoop. Intersience Publishers, Inc., New York. 1959. p. 45.
13. G. ZUBAY and P. DOTY. J. Mol. Biol. **1**, 1 (1959).
14. P. H. LLOYD and A. R. PEACOCKE. Biochem. Biophys. Acta, **95**, 522 (1965).
15. A. E. MIRSKY and H. RIS. J. Gen. Physiol. **31**, 7 (1947).
16. A. E. MIRSKY and H. RIS. J. Gen. Physiol. **34**, 475 (1951).
17. A. E. MIRSKY and H. RIS. Nature, **163**, 666 (1949).
18. H. J. CRUFT, C. M. MAURITZEN, and E. STEDMAN. Phil. Trans. Roy. Soc. London, Ser. B. **241**, 93 (1957).