Proc. Natl. Acad. Sci. USA
Vol. 73, No. 9, pp. 3201–3205, September 1976
Cell Biology

Amino acids and control of nucleolar size, the activity of RNA polymerase I, and DNA synthesis in liver

(nucleolar hypertrophy/RNA nucleotidyltransferase I/nutrition)

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Communicated by Hewson Swift, June 15, 1976

ABSTRACT **The volume of nucleolar material per nucleus and the activity of RNA polymerase I (RNA nucleotidyltransferase I) become doubled in the liver cells of rats that are fed for several days a diet that lacks essential amino acids. Omission of methionine from a fully supplemented diet is equivalent to leaving out all the amino acids, and the responses to a deficiency of tryptophan are about 40% as great. Deprivation of one of the remaining essential amino acids gives either small responses or none at all. Supplementation of the methionine-free diet with cystine blocks the nucleolar enlargement and the enhancement of the polymerase activity that would otherwise take place, but the dispensable amino acid does not affect the responses to a deprivation of one of the other essential amino acids.**

After deprivation of all the essential amino acids or only methionine, hepatocytes make DNA when the rat is fed a meal with protein. A preparatory diet lacking in tryptophan is much less effective; a deficiency in any of the other indispensable compounds tested fails to prepare the liver for DNA synthesis.

The results give hope that elucidation of the means by which methionine deprivation affects the nucleolus will also provide information on the regulation of nuclear DNA replication in liver. One attractive possibility is that the amino acid deficiency acts by producing some imbalance in protein metabolism.

The nucleolus appears to be involved in the regulation of nuclear DNA replication. Enlargement of the organelle (probably a reflection of increased function) is one of the most constant features of the cancer cell (1). In the normal hepatocyte, nucleolar hypertrophy takes place in every case in which the liver is stimulated to make DNA. Stimuli to DNA synthesis as diverse as partial hepatectomy (2, 3), infusion of a mixture of biochemicals (4), and injection of thioacetamide (5, 6) all produce an increase in nucleolar size and an accumulation of nucleolar RNA before the onset of DNA replication.

In protein-deficient rats, nuclear DNA synthesis and mitosis ensue in some of the liver parenchymal cells soon after the animal is fed a meal containing protein (7) or some of the essential amino acids (8). Critical events take place during the period of protein deprivation that prepare the cells for the protein or amino acid stimulus (7, 8). Thus, the number of nuclei that replicate their DNA after the nutritional shift rises with the length of time the animal is deprived of protein (up to 3 or 4 days); supplementation of the deficient mash with increasing levels of protein progressively dampens the effect of the inductive meal; and well-fed animals show no enhancement in hepatic DNA formation even after a meal containing 50% protein.

The nucleolar changes that occur in the regenerating organ after partial hepatectomy also characterize the liver of the amino-acid-deficient rat: hypertrophy (9–11), an accumulation of RNA (12), and an enhanced activity of RNA polymerase I (13, 14). The aim of this work was to begin to learn about the interrelationships between dietary amino acids, on the one hand, and the regulation of nucleolar function and the preparation for nuclear DNA replication, on the other.

MATERIALS AND METHODS

Isomeric amino acids were in the L form. Female albino rats, Fischer 344, were from Microbiological Associates and were kept in a room at 23° ± 0.06. Unless otherwise specified, they were freely given pellets of Purina Laboratory Chow (24% protein) to the time of the experiment and were used when they weighed 140–150 g.

The protein-free mash (Teklad Mills, Madison, Wisc.) consisted of 64% corn starch, 8% glucose, 10% corn oil, 4% "Salt Mix, U.S.P. XIV", 2% "Vitamin Fortification Mix", and 12% nonnutritive fiber. Full amino acid supplementation was with 8.8% of a mixture of the nine essential amino acids (per 100 g of mash: histidine, 0.4 g; isoleucine, 1.15 g; leucine, 1.5 g; lysine, 1.15 g; methionine, 0.7 g; phenylalanine, 1.0 g; threonine, 1.2 g; tryptophan, 0.3 g; and valine, 1.4 g) and 8.4% of a mixture of seven of the dispensable amino acids (per 100 g of mash: 1.2 g each of alanine, arginine, asparagine, glutamine, glycine, proline, and serine).

Nucleolar size was studied by killing the rat by cervical dislocation. A portion of liver (about 100 mg) was immediately homogenized in 5 ml of a buffered solution of formaldehyde and glutaraldehyde (Karnovsky's fixative) (15). Homogenates were kept at ambient temperature for at least 4 hr, whereupon 0.3 ml was layered on 2 ml of 0.4 M sucrose. A nuclear fraction, collected by centrifugation (800 × *g*, 10 min, 3°), was washed once with 5 ml of ice-cold water and suspended in 1.2 ml of water. A portion of the nuclear suspension was then mixed on a glass microscope slide with one-half volume of stain (0.005% azure B in 0.01 M sodium citrate–0.01 M sodium phosphate buffer, pH 5.5) and the mixture was dried on a warming plate (55°). The nuclei became affixed to the slide. This procedure was an improvement over the previous method (4) in that the nucleoli were darker and had sharper borders whereas the nucleoplasm was more lightly stained. Nucleolar measurements were made at a magnification of 2500 diameters with coded slides exactly as described before (4).

RNA polymerase I was measured with liver nuclei isolated by centrifugation (100,00 × *g*, 1 hr) in 2.2 M sucrose–3.3 mM $MgCl_2$. Reaction mixtures (0.25 ml) contained 17 μmol of Tris·HCl (pH 8.3), 40 μmol of $(NH_4)_2SO_4$, 11 μmol of KCl, 1 μmol of $MnCl_2$, 6 nmol of ethylenediamine tetraacetate, 50 μmol of dithiothreitol, 0.014 ml of glycerol, 0.25 μg of α-amanitin, 150 nmol each of ATP, CTP, and GTP, 0.5 μCi of [^{3}H] UTP (5.6 nmol), and 0.1 ml of nuclear suspension (250–350 μg of DNA). After incubation (10 min, 37°), the reaction was

Table 1. Nucleolar enlargement and level of activity of RNA polymerase I as a function of time of deprivation of essential amino acids

Days on protein-free diet	Nucleolar volume (μm^3/nucleus)	RNA polymerase I* (nmol/mg of DNA)
0	4.5 ± 0.23 (6)	0.46 ± 0.04 (4)
1	4.4 ± 0.27 (6)	0.52 ± 0.07 (8)
2	5.8 ± 0.38 (6)	0.77 ± 0.09 (4)
3	7.2 ± 0.69 (6)	0.94 ± 0.08 (6)
5	8.5 ± 0.75 (6)	0.92 ± 0.11 (3)
9	9.5 ± 0.86 (6)	

The rats were fed for the indicated times the protein-free mash supplemented with the mixture of seven dispensable amino acids. Each value is the mean of the results obtained and the standard deviations. The number of animals tested is shown in parentheses.

* The α-amanitin-resistant RNA polymerase activity represented about 30 and 45% of the total nuclear activity (measured in the same test mixture but without α-amanitin) of the "0 day" and "3 day" nuclei, respectively. The activity was designated as RNA polymerase I on the basis of its insensitivity to α-amanitin and its heat lability (19). Both "0 day" and "3 day" nuclei lost 80% of their α-amanitin-resistant activity on being heated at 45° for 15 min.

stopped by the addition of 2 ml of 5% trichloroacetic acid. Insoluble material was washed with trichloroacetic acid, ethanol, and ether. Radioactivity was measured in a hyamine-phosphor solution. A blank (zero time) amounting to 5–10% of the total acid-insoluble radioactivity was subtracted from the experimental result.

Under the conditions of the assay, the rate of RNA formation was proportional to the amount of nuclei and nearly linear with time. Thus, 0.05, 0.09, and 0.17 nmol of [^{3}H]UTP was incorporated in 10 min with 0.025, 0.05, and 0.1 ml of nuclear suspension, respectively. With 0.1 ml of nuclear suspension, incorporation of [^{3}H]UTP after 2.5, 5, and 10 min of incubation was 0.055, 0.10, and 0.16 nmol, respectively.

To determine the proportions of some of the constituents of liver we homogenized a portion of the organ (150–200 mg) in 10 ml of 0.1 M citric acid. For RNA, 6 ml of trichloroacetic acid was added to 1 ml of homogenate, and the precipitate, collected on a pad of Celite, was washed with acid, ethanol, and ether. The pentose in the pad was estimated with orcinol (16). For DNA, 4 ml of homogenate was diluted with 5 ml of 0.1 M citric acid, and the suspension was centrifuged at 10,000 × *g* for 10 min. The pellet was dissolved with 0.1 ml of 1 M NaOH (80°, 10 min), 2.8 ml of 0.5 M $HClO_4$ was added, and the mixture was heated at 80° for 30 min. DNA was measured colorimetrically (17) with 1 ml of the supernatant fluid. The untreated homogenate was used to assay protein (18).

RESULTS

Nucleolar Enlargement and Elevation of RNA Polymerase I Activity as a Function of Time of Deprivation of Essential Amino Acids. No changes were detected with animals deprived of essential amino acids for only 1 day (Table 1). Thereafter, the average volume of nucleolar material per nucleus and the activity of RNA polymerase I rose gradually for several days. A doubling of nucleolar volume represented a 30% increase in diameter.

Deficiencies in Single Essential Amino Acids. Feeding a mixture of dispensable amino acids did not prevent nucleolar enlargement or the rise in the activity of RNA polymerase I in the liver, whereas a diet that contained, in addition, all the indispensable compounds was almost completely inhibitory (Table 2). Omission of methionine from the fully supplemented mash was equivalent to feeding no essential amino acids; deprivation of tryptophan gave smaller responses. A deficiency in threonine caused some enlargement of the nucleolus and may have raised slightly the activity of the polymerase, but omission of any one of the remaining essential amino acids was without effect.

Table 2. Deficiencies in single essential amino acids

Diet	Nucleolar volume (μm^3/nucleus)	RNA polymerase I (nmol/mg of DNA)
24% Protein	4.3 ± 0.26 (11)	0.46 ± 0.05 (5)
Protein-free	8.6 ± 0.88 (4)	0.90 ± 0.09 (6)
Nonessential amino acids	8.9 ± 0.65 (6)	0.93 ± 0.10 (8)
Essential amino acids	5.0 ± 0.32 (16)	
Nonessential and essential amino acids	4.5 ± 0.24 (10)	0.47 ± 0.04 (26)
−Histidine	4.6 ± 0.24 (4)	0.46 ± 0.06 (3)
−Isoleucine	4.4 ± 0.20 (6)	0.46 ± 0.04 (3)
−Leucine	4.4 ± 0.09 (4)	0.47 ± 0.05 (3)
−Lysine	4.6 ± 0.21 (10)	0.47 ± 0.04 (6)
−Methionine	9.2 ± 1.7 (15)	0.91 ± 0.09 (12)
−Phenylalanine	4.5 ± 0.22 (4)	0.48 ± 0.05 (3)
−Threonine	5.3 ± 0.25 (8)	0.52 ± 0.06 (12)
−Tryptophan	6.5 ± 0.33 (13)	0.65 ± 0.10 (14)
−Valine	4.6 ± 0.28 (5)	0.48 ± 0.03 (3)

The rats were fed for 5 days the protein-free mash supplemented, as shown, with seven nonessential and eight or nine essential amino acids. Only small differences were found in the consumption of the various diets. Thus, the average quantity of the protein-free and fully supplemented mashes eaten per day was about 10 g and, for the imbalanced diets, 6.5–10 g. Liver samples were taken on the sixth day and the means and standard deviations of the results are shown. Values in parentheses are the number of rats used. Analysis of the data on nucleolar volume by Student's *t*-test showed that all the elevated values (5.0 μm^3 or greater) were statistically different from that with the control diet (Nonessential and essential amino acids), $P < 0.001$. For the activities of RNA polymerase I, only the elevations with the diets lacking in methionine or tryptophan were significant ($P < 0.001$).

Replacement of Methionine and Tryptophan with Catabolic Products. Addition of cystine to the methionine-free mash, but not to the tryptophan-free diet, blocked nucleolar enlargement and the increase in the activity of RNA polymerase I in the liver (Table 3). Betaine and taurine were unable to substitute for the sulfur amino acids, nor could L-kynurenine replace tryptophan. The inactivity of betaine might have been anticipated since the protein-free diet provided a large amount of methyl donor (0.74% of the weight of the mash was choline dihydrogen citrate).

Losses of Hepatic RNA and Protein in Animals Deprived of an Essential Amino Acid. Omission from the diet of any one of the nine indispensable amino acids resulted in a loss of about 10% of body weight in 5 days, whereas rats fed the fully supplemented mash gained up to 5%. Yet, as Table 2 shows, deprivation of only some of the essential amino acids induced nucleolar changes. In explanation, the possibility was considered that the breakdown of muscle protein by the deficient animals provided the liver with adequate quantities of all but a small number of the indispensable compounds. This, however, was

Table 3. Effects of replacing methionine and tryptophan with catabolic products

Omission from diet	Addition to diet	Nucleolar volume (μm^3/ nucleus)	RNA polymerase I (nmol/mg of RNA)
None		4.6 ± 0.28	0.47 ± 0.04
None	Cystine	4.4 ± 0.23	
Methionine		8.6 ± 1.4	0.90 ± 0.08
	Betaine	8.5 ± 1.3	0.91 ± 0.10
	Cystine	4.7 ± 0.19	0.53 ± 0.05
	Taurine	8.9 ± 1.9	0.96 ± 0.12
Tryptophan		6.4 ± 0.31	
	Cystine	6.6 ± 0.32	0.64 ± 0.09
	L-Kynurenine	6.5 ± 0.30	

Liver samples were from rats that were fed for 5 days the protein-free mash supplemented with seven nonessential amino acids, eight or nine of the essential compounds, and, as shown, betaine or a product of methionine or tryptophan metabolism. The levels of the test compounds in the diets were 0.7% of cystine, betaine·HCl, or taurine, and 0.3% of L-kynurenine. Each value is the mean of the results with four rats; standard deviations are given.

Table 4. Losses of hepatic RNA and protein in animals deprived of an essential amino acid

Diet	RNA/DNA		Protein/DNA	
	3 days	5 days	3 days	5 days
	% of control			
24% Protein	100		100	
Amino acids	101	98	96	102
-Histidine		82		80
-Isoleucine	93	75	74	65
-Lysine	90	78	76	73
-Methionine	88	82	68	77
-Threonine	88	68	70	77
-Tryptophan	93	70	68	74

The rats were fed for 3 or 5 days the protein-free diet containing a mixture of seven nonessential amino acids and eight or nine of the essential compounds, as shown. Liver samples were taken on the fourth or sixth days. The ratios of RNA/DNA and protein/DNA for the animals fed a 24% protein diet were 4.0 and 66, respectively. Each value is the average of the results with three rats.

not the case. The liver recognized deficiencies in histidine, isoleucine, and lysine as well as in methionine, tryptophan, and threonine, since deprivation of any one of the amino acids led to losses of hepatic RNA and protein (Table 4).

In agreement with the observations of others (20–22), the protein-free diet caused no reduction in hepatic DNA nor could any decrease be detected in the animals fed the imbalanced diets of Table 4.

Nucleolar Changes in Animals Fed Amino-Acid-Deficient Diets after a Period of Starvation. To determine whether more severe deficiencies in amino acids other than methionine, tryptophan, and threonine can cause nucleolar alterations, we followed a protocol similar to that of Stenram (23). Animals were starved for 5 days before they were fed diets lacking an essential amino acid (Table 5). Prior starvation seemed to increase amino acid requirements. For nucleolar volume, the effects of mashes lacking in methionine, tryptophan, or threonine were considerably magnified, and deprivation of lysine or phenylalanine induced some hypertrophy. The activity of RNA polymerase I was now elevated by deprivation of threonine or lysine in addition to methionine or tryptophan.

The table also shows that cystine markedly reduced the responses to methionine deprivation but had no effect with diets lacking in tryptophan, threonine, or lysine. Not shown are the results that were obtained when nucleolar volume was measured after feeding a phenylalanine-free mash supplemented with 1% tyrosine. The dispensable amino acid completely prevented the enlargement of nucleoli that would otherwise have occurred.

The possibility was examined that deprivation of any indispensable amino acid can activate the mechanism that leads to nucleolar hypertrophy but that some deficiencies block a step in the process of enlargement. To explore this idea, we tested the effects of diets lacking methionine and an additional essential amino acid after 5 days of starvation. Omission of isoleucine and, to a lesser extent, of phenylalanine, depressed the nucleolar response to the methionine deficiency, but deprivation of histidine, leucine, or valine had little or no impact. Thus, with methionine-free diets lacking in histidine, isoleucine, leucine, phenylalanine, or valine, hypertrophy was 105, 27, 80, 55, and 85%, respectively, of that with a methionine deficiency alone.

DNA Synthesis. A single meal containing protein causes nuclear DNA replication in some of the hepatocytes of the protein-deficient rat (7). An injection of 3,3',5-triiodo-L-thyronine given at the time of the nutritional shift enhances the action of the inductive meal (8).

Supplementation of the protein-free mash with nonessential amino acids was without effect on the subsequent formation of DNA, but feeding the essential compounds blocked the response of the liver to the protein meal and thyroid hormone (Table 6). The table shows that DNA synthesis was the same in animals that had been deprived of methionine or all the essential amino acids, the effect of a tryptophan deficiency was much smaller, and omission of any of the other indispensable compounds tested did not permit the liver to prepare for DNA formation. It can also be seen from the table that addition of cystine to the methionine-free diet blocked the later response to the inductive treatment.

Not shown are the results that were obtained when DNA synthesis was induced only by a meal of 50% protein. DNA formation was invariably elevated in animals that had been fed diets lacking in methionine or all nine of the essential amino acids; the average was seven times higher than in the control rats.

A relatively large fraction of the hepatocyte population was able to form DNA after methionine deprivation. Thus, with three deficient rats (5 days) that were labeled with 200 μCi of [^{3}H]thymidine from 16 to 17 hr after the inductive treatment (50% protein meal and thyroid hormone), radioautographic analysis of isolated nuclei showed that 13, 29, and 35% of the nuclei were in the S phase.

DISCUSSION

The quantity of nucleolar RNA (12), the volume of nucleolar material per nucleus (9–11), and the activity of RNA polymerase I (13, 14) all become increased in the hepatocytes of rats that are deprived of amino acids. We now find that the dietary regulation of nucleolar volume and the polymerase activity in liver depend primarily upon methionine. Deficiencies in some of the other essential amino acids cause either smaller responses or none at all.

Table 5. Nucleolar volume and level of activity of RNA polymerase I in animals fed amino-acid-deficient diets after a period of starvation

Diet	Nucleolar volume (μm^3/nucleus)		RNA polymerase I (nmol/mg of DNA)	
	No cystine	+ Cystine	No cystine	+ Cystine
None (5 days starvation)	4.5 ± 0.08 (3)			
24% Protein	4.4 ± 0.05 (3)			
Nonessential amino acids	9.0 ± 0.35 (6)			
Essential amino acids	5.8 ± 0.28 (6)			
Nonessential and essential amino acids	4.6 ± 0.28 (12)	4.7 ± 0.42 (9)	0.49 (0.05) (6)	0.47 ± 0.04 (15)
−Histidine	4.6 ± 0.12 (4)			
−Isoleucine	4.5 ± 0.27 (4)			0.41 ± 0.05 (4)
−Leucine	4.5 ± 0.25 (4)			
−Lysine	7.4 ± 0.60 (8)	6.9 ± 0.42 (6)		0.53 ± 0.06 (8)
−Methionine	15 ± 2.2 (11)	7.5 ± 2.2 (9)	0.97 ± 0.11 (8)	0.45 ± 0.05 (3)
−Phenylalanine	5.6 ± 0.26 (5)	5.5 ± 0.23 (3)		
−Threonine	8.7 ± 0.75 (6)	8.6 ± 0.54 (6)		0.67 ± 0.13 (9)
−Tryptophan	10 ± 1.0 (6)	11 ± 1.6 (6)	0.63 ± 0.07 (6)	0.62 ± 0.06 (8)
−Valine	4.7 ± 0.27 (4)			

Rats that had been starved for 5 days were then fed for 5 days commercial pellets (24% protein) or the protein-free mash supplemented with eight or nine essential amino acids, the mixture of the seven nonessential compounds, and 0.7% cystine, as shown. The means and standard deviations of the results are given. Values in parentheses are the number of rats. Analysis of the data by Student's *t*-test showed that nucleolar volume was statistically increased by phenylalanine deprivation ($P < 0.001$) and that the activity of RNA polymerase I was elevated by deficiencies in lysine ($P < 0.02$), threonine, tryptophan, or methionine ($P < 0.001$).

The enlargement of the nucleolus in methionine deprivation is not due merely to an inadequate supply of ribosomal proteins and a consequent accumulation of ribosomal RNAs in the organelle. Rather, hypertrophy seems to result from enhanced nucleolar function. We have now measured the incorporation of [^{3}H]adenine into the 28S RNA of mature cytoplasmic ribosomes and have taken into account the specific activities of nuclear ATP during the labeling period. A deficiency in methionine induces about a 2-fold increase in the rate of hepatic ribosome synthesis.

Table 6. Effects of supplementation of the protein-free diet with amino acids on the inducibility of hepatic DNA synthesis

Preparative diet	DNA synthesis (cpm in thousands/mg of DNA)
Amino-acid-free	4.1 ± 0.88 (3)
Nonessential amino acids	4.4 ± 1.9 (6)
Essential and nonessential amino acids	0.24 ± 0.13 (12)
−Methionine	3.8 ± 1.45 (8)
−Tryptophan	0.52 ± 0.26 (10)
−Threonine	0.25 ± 0.11 (4)
−Lysine	0.25 ± 0.14 (3)
−Isoleucine	0.14 ± 0.05 (4)
−Valine	0.23 ± 0.08 (4)
−Methionine + cystine	0.34 ± 0.13 (3)

The rats were fed for 5 days the protein-free mash supplemented with amino acids, as shown. Cystine was added at a level of 0.7% of the diet. The preparative mashes were removed on the morning of the sixth day. At 6 p.m., all animals were injected subcutaneously with 100 μg of 3,3′,5-triiodo-L-thyronine and they were then fed a meal of the protein-free mash containing 50% ovalbumin. After 16 hr, each rat was given 0.25 μCi of [^{14}C]thymidine in the tail vein; liver samples were taken 1 hr later. The specific activities of nuclear DNA were measured as described (24). Shown are the means and standard deviations of the results. Number of animals is given in parentheses. Analysis of the data by Student's *t*-test showed that the elevated values were statistically different from that of the control (Essential and nonessential amino acids): methionine-deprived, $P < 0.001$; tryptophan-deprived, $P < 0.01$.

Cystine blocks the nucleolar alterations caused by omission of methionine from the diet. The action of the dispensable amino acid is understandable in terms of nutritional and biochemical information. Cystine cannot substitute completely for methionine in the diet (25) but it can markedly reduce the quantity of the essential amino acid that is needed for maintenance of nitrogen equilibrium (25) and for growth (26). The sparing action of cystine is considered to stem primarily from its ability to cause a reduction in the activity of cystathionine synthase (27). As a consequence of the decreased activity, the rate of irreversible drainage of homocysteine from the methionine-homocysteine cycle is reduced and the level of methionine for use by the cell is raised.

Contrary to its action in methionine deprivation, cystine has no effect on the nucleolar changes that result from feeding diets lacking in, for example, tryptophan. It would seem, therefore, that a tryptophan deficiency does not act indirectly by distorting the metabolism of the sulfur amino acids.

The results of the estimations of RNA polymerase I activities parallel almost perfectly those of nucleolar enlargement, a change *in vivo*. These correlations are consistent with the conclusion that measurements of the RNA polymerase with isolated nuclei reflect the level of enzyme function in the cell.

The liver undergoes changes during protein deprivation of the animal that permit the parenchymal cells to replicate their DNA in response to essential amino acids. Just as for the nucleolar alterations, a deficiency in methionine is the most efficacious in preparing hepatocytes for DNA synthesis. These observations lead us to hope that an understanding of the way in which a methionine deficiency affects the nucleolus will shed light, as well, on the regulation of DNA replication in liver.

The means by which a deprivation of methionine elevates nucleolar size and the activity of RNA polymerase I and prepares the liver for nuclear DNA synthesis is not known. Two

reasonable explanations are that the changes are produced by some imbalance in protein metabolism or by a disturbance in the methylation of RNA or protein molecules. Weakening the latter hypothesis is the fact that a deficiency in tryptophan causes the same, albeit smaller, changes as a deficiency in the sulfur amino acid. As regards protein metabolism, we have found no differences in the charging levels of $tRNA^{Met}$ and $tRNA_f^{Met}$ or in the proportion of mRNA-bound ribosomes in livers from well-fed and methionine-deprived rats.

A role for methionine in regulating nuclear DNA replication may not be restricted to the liver. Fitzgerald and his colleagues (28) have found that explants of embryonic rudiments of pancreas stop making DNA and undergo differentiation (as shown by the appearance of acinar cells and the formation of zymogen granules) when they are cultured in a medium containing 80 mg/liter of L-methionine. With a lower level of the amino acid, 30 mg/liter, nuclear DNA replication continues and no differentiative changes take place.

This work was supported by grants from the National Cancer Institute and the National Science Foundation.

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