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The Regulation of Protein Synthesis in the Liver of Rats

MECHANISMS OF DIETARY AMINO ACID CONTROL IN THE IMMATURE ANIMAL

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Weanling (23-day-old) rats were fed either on an amino acid-deficient diet (6% of casein, which in effect represents an 'amino acid-deficient' diet) or on a diet containing an adequate amount of protein (18% of casein) for 28 days. The hepatic cells from the animals fed on the low-protein diet were characterized by low amino acid content, almost complete inhibition of cell proliferation and a marked decrease in cell volume, protein content and concentration of cytoplasmic RNA compared with cells from control rats. The lower concentration of cytoplasmic RNA was correlated with a decreased ribosomal-RNA content, of which a larger proportion was in the form of free ribosomes. The protein-synthetic competence and messenger-RNA content of isolated ribosomes from liver cells of protein-deprived animals were 40–50% of those noted in controls. At 1 hr. after an injection of radioactive uridine, the specific radioactivity of liver total RNA was greater in the group fed on the low-protein diet, but the amount of label that was associated with cytoplasmic RNA or ribosomes was significantly less than that noted in control animals. From these data it was concluded that dietary amino acids regulate hepatic protein synthesis (1) by affecting the ability of polyribosomes to synthesize protein and (2) by influencing the concentration of cytoplasmic ribosomes. It is also tentatively hypothesized that the former process may be directly related to the concentration of cellular free amino acids, whereas the latter could be correlated with the ability of newly synthesized ribosomal sub-units to leave the nucleus.

Several reports (Fleck, Shepherd & Munro, 1965; Wunner, Bell & Munro, 1966; Webb, Blobel & Potter, 1966; Sox & Hoagland, 1966; Sidransky, Bongiorno, Sarma & Verney, 1967) have indicated that in starved animals the ingestion of either a protein or a complete amino acid mixture results in a rapid increase in the degree of hepatic polyribosomal aggregation and rate of protein biosynthesis. Further, the response occurs even after inhibition of RNA synthesis by actinomycin D (Fleck *et al.* 1965; Staehelin, Verney & Sidransky, 1967). However, omission of one essential amino acid (tryptophan) from the mixture inhibits the change in the polyribosomal pattern and the stimulation of amino acid incorporation. In liver of rats fed on a tryptophan-free diet, a larger percentage of the cytoplasmic RNA is associated with the oligosomes, free ribosomes and ribosomal sub-units compared with controls (Wunner *et al.* 1966). These data have led to the conclusion that a deficiency in amino acid supply results in a breakdown of the polyribosomes and an eventual loss of cytoplasmic RNA, which can be correlated with a decreased rate of protein biosynthesis (Munro, 1966). This process can be reversed by giving the animals a balanced mixture of amino acids.

Wilson & Hoagland (1967) noted that 5 days' starvation markedly decreased the concentration of cellular ribosomes and the quantity of heavy polyribosomal material in the livers of rats. Whereas re-feeding of animals starved for 4–5 days yielded a twofold increase in total r-RNA*, there was a four- to six-fold rise in the quantity of polyribosomes. However, when the rats were fed after being treated with actinomycin D, puromycin or cycloheximide, polyribosomal re-formation did not occur, which led to the suggestion that in starved–re-fed rats both RNA and protein synthesis are necessary for the re-formation of ribosomes and polyribosomes.

If these conclusions are correct then in the liver dietary amino acids could regulate protein synthesis by influencing both the ability of a unit of polyribosomes to incorporate amino acids into protein

* Abbreviations: r-RNA, ribosomal RNA; n-RNA, nuclear RNA; m-RNA, messenger RNA; t-RNA, transfer RNA.

and the number of ribosomes/cell. The former process responds rapidly to the supply of amino acids and is independent of RNA synthesis, whereas the changes in concentration of ribosomes proceed at a lower rate and depend on the synthesis of new RNA. To evaluate this concept the following biological criteria were utilized: (1) as an immature rat grows, the hepatic cells increase in number, size and protein content (Enesco & Leblond, 1962; Mendes & Waterlow, 1958); (2) the protein content of these cells is influenced by both the quantity and quality of dietary protein (Allison, Wannemacher, Banks & Wunner, 1964), which regulates the rate of hepatic protein biosynthesis (Allison & Wannemacher, 1965; Wannemacher & Allison, 1968). Therefore variation of the protein content of the diet given to a growing rat provides a technique that is useful for studying mechanisms of subcellular control of protein synthesis. In the present study weanling rats were supplied either with a quantity of dietary amino acids that allowed a rapid hypertrophy and hyperplasia of the liver cells or a diet that allowed only maintenance of their cellular volume and number with no increase in protein content. The ribosomal concentration and distribution in hepatic cellular populations from both dietary regimes were then compared with their protein-biosynthetic ability.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]Leucine (250mc/m-mole) and [5-^{3}H]-uridine (25c/m-mole) were obtained from New England Nuclear Corp., Boston, Mass., U.S.A., and [5-^{3}H]cytidine (6c/m-mole), [8-^{14}C]adenosine (32·2mc/m-mole) and [8-^{14}C]-guanosine (29·7mc/m-mole) from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. ATP, GTP and L-amino acids were obtained through Calbiochem, Los Angeles, Calif., U.S.A. Bovine pancreatic ribonuclease was obtained from Mann Research Laboratories Inc., New York, N.Y., U.S.A.

Animals. Male weanling (23-day-old) Wistar rats were divided into groups of six and were fed on an agar-gel diet (Allison *et al.* 1964) *ad libitum* for 28 days. This diet contained either 6% of casein, which in effect represents an 'amino acid-deficient' diet, or it contained an adequate amount of protein (18% of casein). One week before they were killed all the rats were trained to consume their food between 8.30a.m. and 10.30a.m. On the day of killing, feeding was immediately followed by an intraperitoneal injection of a labelled nucleoside and the animals were killed 1hr. later. Thus all animals were killed 3hr. after ingestion of a meal. After decapitation the rats were perfused with cold 0·9% NaCl until the liver was clear of all blood. The liver was then removed, placed in cold 0·9% NaCl, weighed and passed through a tissue press; most of the connective tissue was thereby removed. When samples of this liver pulp were dissociated into isolated cells by the sodium tetraphenylboron technique of Epstein (1967), and the cells were stained with methyl green and counted in a haemocytometer, the resulting cellular population contained 93–98% of parenchymal cells. Thus the following operations were carried out on the liver pulp preparations that contained a relatively homogeneous cellular population and were performed, unless otherwise specified, at 0–4°.

Nuclear preparation. Nuclei were prepared by a modification of the procedure of Gill (1965). A 0·5–1·0g. sample of the liver pulp was diluted with 10ml. of dense sucrose solution (sp.gr. 1·28–1·29)–3mM-$CaCl_2$–1mM-acetic acid, pH6·0 at 20°, and homogenized by ten strokes at 800rev./min. in a Potter–Elvehjem homogenizer with a motor-driven Teflon pestle. The homogenate was centrifuged for 25min. at 25000rev./min. (40000*g*) in a Spinco no. 40 rotor. The pellicles and supernatant fluid were discarded; the walls were wiped clean and the crude nuclear pellet was resuspended in 10ml. of the dense sucrose medium and centrifuged for 15min. at 40000*g*. The supernatant fluid and the material adhering to the walls of the tube were removed and the nuclear pellet was resuspended in 2ml. of 0·25M-sucrose in TKM buffer (0·05M-tris–HCl, pH7·6 at 25°, 0·08M-KCl and 0·01M-$MgCl_2$). A 1ml. sample of the nuclear suspension was analysed for protein, RNA and DNA content, and another 0·5ml. sample was diluted 1:20 with 3% (v/v) acetic acid containing 0·2% of methyl green and counted directly in a haemocytometer. In adult rats fed on an adequate diet, the liver nuclear preparation contained 5–6% of the cellular RNA and 80–90% of the DNA.

Isolation of endoplasmic reticulum and free ribosomes from liver. A 1g. sample of liver pulp was diluted with 4ml. of 0·88M-sucrose and homogenized by ten strokes at 800rev./min. in a Potter–Elvehjem homogenizer with a motor-driven glass pestle. This homogenate was centrifuged for 25min. at 15000rev./min. (15000*g*) in a Spinco no. 40 rotor. The precipitate was washed twice by resuspension in 3ml. of 0·88M-sucrose and centrifugation. The supernatant fluids from all three centrifugations were pooled and utilized for the isolation of granular and agranular endoplasmic reticulum and free ribosomes by the iso-octane method of Hallinan & Munro (1965*a*). These various subfractions were suspended in 0·25M-sucrose–TKM buffer and analysed for RNA content.

Ribosomal preparation. A 1g. sample of liver pulp was diluted with 2ml. of 0·25M-sucrose–TKM buffer, homogenized by ten strokes in the motor-driven glass homogenizer and centrifuged for 15min. at 15000rev./min. (15000*g*) in a Spinco no. 40 rotor. The precipitate was washed twice with 2ml. of 0·25M-sucrose–TKM buffer and the post-mitochondrial supernatant (original plus washes) was treated with Triton X-100 (final concn. 1·0%), layered over 1·0M-sucrose–TKM buffer and centrifuged for 4hr. at 40000rev./min. (106000*g*). The resulting precipitate (ribosomes) was dissolved in 0·25M-sucrose–TKM buffer and analysed for RNA content, ability to incorporate amino acids and m-RNA concentration.

Amino acid-incorporating system. A 0·5ml. sample of the ribosomes (42μg. of RNA) was incubated at 37° with 0·1ml. of freshly prepared cell sap (0·4mg. of protein) that had been passed through Sephadex G-25 to remove inhibitors of protein synthesis (Munro, Jackson & Korner, 1964) and 0·4ml. of a medium containing 12mM-ATP, 0·25mM-GTP, 0·25mM each of the L-isomers of the 19 naturally occurring amino acids and 0·4μM-L-[U-^{14}C]leucine (250μc/μmole). After 15min. the amino acid incorporation was stopped by the addition of 4ml. of cold 0·4N-$HClO_4$

containing 1 mg. of unlabelled L-leucine/ml. After this the protein precipitate was washed twice with cold 0·2 N-$HClO_4$, dissolved in 0·3 N-KOH, incubated at 37° for 1 hr., precipitated with 60% (w/v) $HClO_4$ and washed twice with 0·2 N-$HClO_4$ to remove the RNA, dissolved in 1·0 ml. of 0·3 N-NaOH in methanol, added to the scintillation mixture (Wannemacher, Banks & Wunner, 1965) and counted in a Packard liquid-scintillation counter. Results from all samples were corrected for absolute counting rate by the internal standardization procedure of Herberg (1963) and expressed as disintegrations/min./mg. of ribosomal RNA.

Determination of m-RNA. The m-RNA content of the ribosomal preparations was determined by incubating 0·15 mg. of r-RNA with 1 mg. of ribonuclease for 5 min. at 37° and then extracting the liberated nucleotides by the procedure of Mansbridge & Korner (1966).

Incorporation of RNA precursors in vivo. Rats were injected intraperitoneally with either [5-^{3}H]uridine (100 μc/100 g. body wt.), [5-^{3}H]cytidine (80 μc/100 g. body wt.), [8-^{14}C]adenosine (8 μc/100 g. body wt.) or [8-^{14}C]guanosine (8 μc/100 g. body wt.) 1 hr. before they were killed. The uptake of radioactivity into the RNA fractions of the nucleus, granular and agranular endoplasmic reticulum and free ribosomes was measured by using the methods of RNA extraction described below. A 1 ml. sample of the extracted RNA was added to 10 ml. of the scintillation mixture and counted in a liquid-scintillation counter, and absolute counting rates were determined.

Analysis for RNA, DNA, protein and free amino acids. A 1 ml. sample of tissue homogenate (1:30 dilution in 0·25 M-sucrose–TKM buffer) or of the cellular fractions was extracted three times with 0·2 N-$HClO_4$. The resulting precipitate was dissolved in 4 ml. of 0·3 N-KOH and this solution was used for the analysis of protein by the technique of Lowry, Rosebrough, Farr & Randall (1951) and for RNA and DNA determinations by the procedure of Wannemacher *et al.* (1965).

A 1 ml. sample of the original homogenate (1:3 dilution in 0·25 M-sucrose–TKM buffer) was mixed with 3 ml. of 9% (w/v) sulphosalicylic acid and centrifuged, and the resultant supernatant fluid was analysed for free amino acids by a modification of the ion-exchange chromatographic procedure of Piez & Morris (1960).

Table 1. *Free amino acid content of livers from rats fed on either a 6% casein diet or an 18% casein diet*

Homogenates of livers from 51-day-old rats, which had been fed on either a 6% casein diet or an 18% casein diet, were treated with 9% sulphosalicylic acid. The resulting supernatant fluid was analysed for free amino acids by a modification of the ion-exchange chromatographic procedure of Piez & Morris (1960). Animals from each dietary group were killed 3 hr. after ingestion of a meal. Each value is the mean ± S.E.M. of six animals.

Amino acids	Amino acid content (mμmoles/cell) 6% Casein diet	18% Casein diet
Essential		
Lys	1·70 ± 0·17	3·25 ± 0·34
Thr	1·08 ± 0·10	7·33 ± 0·76
Val	0·43 ± 0·05	1·60 ± 0·18
Leu	0·55 ± 0·07	1·46 ± 0·18
Ile	0·20 ± 0·03	0·61 ± 0·08
Phe	0·37 ± 0·04	0·58 ± 0·12
Met	—†	3·61 ± 0·54
His*	2·14 ± 0·07	2·75 ± 0·59
Total	5·87 ± 0·50	20·77 ± 2·11
Non-essential		
Asp	5·29 ± 0·20	9·37 ± 1·17
Glu	14·16 ± 0·84	25·83 ± 3·95
Ala	10·90 ± 0·65	23·65 ± 2·67
Gly	6·70 ± 0·53	13·92 ± 1·52
Ser	7·68 ± 0·54	16·19 ± 2·25
Pro	0·41 ± 0·05	1·53 ± 0·36
Tyr	0·25 ± 0·11	0·59 ± 0·11
CyS	—†	0·20 ± 0·06
Total	43·30 ± 2·23	90·86 ± 11·22
Essential/non-essential ratio	0·130 ± 0·008	0·232 ± 0·011

* Histidine + 1-methylhistidine.

† Amino acid concentration less than 0·10 mμmole/cell.

RESULTS

Effect of amino acid deprivation on liver composition. The data in Table 1 illustrate the markedly low amino acid content of the liver cells from animals fed on a 6% casein diet. Under these conditions the essential free amino acids are decreased to 28·3% and non-essential amino acids to 47·8% of the values found in the animals fed on the higher-protein diet. The deficiency of sulphur-containing amino acids in casein is reflected by the low contents of free methionine and half-cystine in the livers of rats fed on the 6% casein diet.

Livers from rats fed on the amino acid-deficient diet are significantly smaller than the livers of those given an adequate amount of dietary protein (Table 2). In contrast, the number of cellular nuclei/g. of liver tissue is significantly greater in the former group of animals; however, owing to the smaller liver size, the total number of nuclei/liver is 72·2% of that found in the group given the 18% casein diet. In addition, the average DNA content of the nuclei from rats fed on the low-protein diet is significantly lower than that found in the controls. These results are in agreement with the findings of Mariani, Migiaccio, Spadoni & Ticca (1966). Though the liver nuclei of the deficient animals contain slightly less n-RNA (Table 2), the n-RNA/DNA ratio is significantly (0·30 ± 0·016, mean ± S.E.M., versus 0·24 ± 0·006) higher than in the control animals given the 18% casein diet. The protein concentration/nucleus varies directly with DNA content and is therefore lower in animals fed on the 6% casein diet. From the assumption of Mariani *et al.* (1966) that the cytoplasmic mass of

Table 2. *Effect of dietary content of protein on liver composition*

Livers were from 51-day-old rats that had been fed on either a 6% casein or an 18% casein diet for 28 days. Animals from each dietary group were killed 3 hr. after ingestion of a meal. Cell volume, protein and RNA content were estimated by assuming that binuclear cells had twice the cytoplasmic mass of mononuclear cells. Results are the means ± S.E.M. of six rats per group.

Diet	Wet wt. (g.)	No. of nuclei (millions/g.)	No. of nuclei (millions/total liver)	Nuclear DNA ($\mu\mu$g./nucleus)	Cell wt. (mμg./cell)	Cellular protein (mμg./cell)	Cellular RNA (mμg./cell)	Nuclear protein ($\mu\mu$g./nucleus)	Nuclear RNA ($\mu\mu$g./nucleus)
6% Casein	2·27 ± 0·08	317 ± 11	717 ± 11	10·1 ± 0·1	3·22 ± 0·29	0·38 ± 0·03	26·5 ± 1·6	34·2 ± 2·3	2·95 ± 0·13
18% Casein	6·59 ± 0·44	154 ± 17	995 ± 51	13·9 ± 1·2	6·94 ± 0·71	1·23 ± 0·14	60·2 ± 4·1	43·2 ± 2·6	3·52 ± 0·32

binuclear cells is twice that of mononucleated cells, it is possible to calculate an average cell weight by dividing the value for the total number of nuclei into liver weight, as illustrated in Table 2. This calculation indicates that the liver cells from animals fed on the low-protein diet have a mean cell weight 46·4% lower than that found in rats fed on the higher-protein diet. These data are in agreement with the observations of Wannemacher (1967) that amino acid deprivation produces cells markedly decreased in size. The smaller cell volume of the liver cells from the animals given the 6% casein diet is associated with both a lower protein content and a lower RNA content (Table 2). Protein represents 18·2 ± 0·5% (mean ± S.E.M.) of the cell weight in the group given the 18% casein diet and only 12·0 ± 0·8% in the group fed on the low-protein diet, suggesting that the effects of amino acid deprivation are greater on cellular protein than on some of the other cellular constituents. This conclusion is further supported by the fact that the protein/RNA ratio (mg./mg.) is 14·8 ± 1·2 (mean ± S.E.M.) and 20·8 ± 1·3 for the groups given the 6% casein and 18% casein diets respectively.

Cytoplasmic RNA content. In 51-day-old rats fed on the low-protein diet, the cytoplasmic RNA content of an average liver cell is 23·7 ± 1·7 (mean ± S.E.M.) $\mu\mu$g. as compared with 56·9 ± 4·4 $\mu\mu$g. in the animals given a diet containing an adequate amount of protein. This difference in cytoplasmic RNA content is significant ($P < 0·01$), and is correlated with a markedly decreased concentration of RNA associated with the rough-surfaced and smooth-surfaced vesicles and fewer free ribosomes in the hepatic cells from the amino acid-deficient group (Table 3). Free ribosomes, which are mostly monomers by the procedures used in these experiments (Wunner *et al.* 1966), comprise a significantly greater percentage of the total cytoplasmic RNA in the liver cells of the group given the 6% casein diet as compared with the controls given the 18% casein diet (Table 3). The r-RNA content (most of rough-surfaced-vesicle RNA plus free r-RNA) represents 39% of the total liver RNA of control rats. These findings are in agreement with the data of Wilson & Hoagland (1967) on the r-RNA content of normal liver. However, though the RNA content of this fraction is 23·5 ± 2·1 $\mu\mu$g. (mean ± S.E.M.)/liver cell in control animals, it is only 5·6 ± 0·4 $\mu\mu$g./cell in the protein-deprived rats, or 24% of that in the controls.

Amino acid-incorporating activity and m-RNA content of ribosomes. The amino acid-incorporating activity of ribosomes was measured on equal quantities of isolated r-RNA fractions from both dietary groups, and the data are presented in Table 4. Liver ribosomes from the amino acid-deficient animal incorporate [U-^{14}C]leucine into

Table 3. *Distribution of cytoplasmic RNA from liver cells*

Liver cytoplasm was separated into endoplasmic reticulum fractions and free ribosomes by the iso-octane method of Hallinan & Munro (1965*a*). RNA was determined by the procedure of Wannemacher *et al.* (1965). All rats were fed as described in Table 2. Each value is the mean ± S.E.M. of six animals.

	Concn. of RNA ($\mu\mu$g./cell)		% of total cytoplasmic RNA	
Fraction	6% Casein diet	18% Casein diet	6% Casein diet	18% Casein diet
Rough-surfaced vesicles	4·3 ± 0·5	21·3 ± 3·2	18·1 ± 1·9	37·4 ± 2·8
Smooth-surfaced vesicles	0·084 ± 0·009	0·324 ± 0·045	0·35 ± 0·05	0·57 ± 0·05
Free ribosomes	1·25 ± 0·23	2·23 ± 0·45	5·7 ± 0·3	3·9 ± 0·2
Total cytoplasmic RNA	23·7 ± 1·7	56·9 ± 4·4	—	—

Table 4. *Effect of diet on protein-synthetic ability and m-RNA*

Ribosomes were prepared by centrifuging the post-mitochondrial supernatant fluid through 1 M-sucrose as described in the Materials and Methods section. The protein-synthetic system *in vitro* contained 42 μg. of r-RNA, 400 μg. of cell-sap protein, 0·14 mole of sucrose, 0·08 mole of KCl, 0·01 mole of $MgCl_2$, 0·05 mole of tris–HCl buffer, pH 7·6 at 25°, 5 μmoles of ATP, 0·1 μmole of GTP, 0·1 μmole each of 19 naturally occurring amino acids and 0·16 mμmole of L-[U-^{14}C]leucine (0·04 μc). Protein-synthetic competence is the product of the rate of incorporation/unit of r-RNA times the ribosomal content/cell. The m-RNA was hydrolysed from the ribosomes by ribonuclease and the soluble nucleotides were measured by u.v. absorption at 260 mμ. Rats were fed as described in Table 2. Results are recorded as the means ± S.E.M. of six animals.

	Incorporation of [^{14}C]leucine (disintegrations/min./0·1 μg. of r-RNA)	Protein-synthetic competence (10^4 × disintegrations/min./cell)	m-RNA content	
Diet			$\mu\mu$g./cell	% of r-RNA
6% Casein	3·84 ± 0·37	2·34 ± 0·18	0·119 ± 0·015	2·16 ± 0·17
18% Casein	8·60 ± 0·59	19·41 ± 0·95	1·652 ± 0·068	7·01 ± 0·95

protein at a rate 45·6% of that found in similar preparations from liver of rats fed on the 18% casein diet. If the concentration of r-RNA is taken into consideration, the protein-synthetic competence for an average liver cell from the rats raised on the 6% casein diet is only 12·1% of that found in a liver cell from control animals. The decreased incorporation rate of the liver cells from protein-deprived animals is correlated with a 69·2% lower m-RNA content of their ribosomes compared with control rats (Table 4). Since the r-RNA content is also lower in the livers of the rats given the 6% casein diet, the m-RNA content of an average liver cell in these animals is only 7·2% of that noted in the group given the 18% casein diet. Even though these data and those of Mansbridge & Korner (1966) suggest a good correlation between protein-synthetic ability and the determination of m-RNA, as measured by the liberation of acid-soluble nucleotides after digestion of r-RNA with ribonuclease, the possibilities still remain that this technique might not measure all of the m-RNA or that the acid-soluble extracts might be contaminated by other non-m-RNA nucleotides that could be released from the ribosomal preparations.

Incorporation of precursors into nuclear and cytoplasmic RNA. Because liver cells from amino acid-deprived animals are characterized by a marked decrease in cytoplasmic RNA and ribosomes, an attempt was made to compare the rate of RNA synthesis in these cells with that in cells from control rats fed on the 18% casein diet. Table 5 records the rate of incorporation of radioactive precursors into n-RNA of livers from both dietary groups. When [5-^{3}H]cytidine is used as the precursor the specific radioactivity (disintegrations/min./μg. of RNA) of the n-RNA from the livers of the protein-deprived rats is eight times that noted in the same fraction from control animals, but if [8-^{14}C]adenosine or [8-^{14}C]guanosine is the precursor the radioactivity is only 73% and 81% respectively of the control value. The same contradictory pattern is noted whether the data are expressed as radioactivity (disintegrations/min.) in the RNA of an average hepatic nucleus or as specific radioactivity (disintegrations/min./μg. of RNA). In another experiment, a similar marked stimulation of [5-^{3}H]cytidine incorporation into hepatic n-RNA has been noted in adult rats that were starved or fed on a protein-free diet (R. W. Wannemacher, jun., unpublished work).

When another pyrimidine, [5-^{3}H]uridine, is

Table 5. *Incorporation of precursors into n-RNA*

Rats were injected intraperitoneally with a radioactive ribonucleoside 2 hr. after ingestion of a meal. All animals were killed 1 hr. later, and RNA was extracted from the liver nuclear pellets. Results are recorded as the means ± S.E.M. of six animals.

Diet	[5-^{3}H]Cytidine		[5-^{3}H]Uridine		[8-^{14}C]Adenosine		[8-^{14}C]Guanosine	
	(disintegrations/min./μg. of n-RNA)	(10^6 × disintegrations/min./nucleus)	(disintegrations/min./μg. of n-RNA)	(10^6 × disintegrations/min./nucleus)	(disintegrations/min./μg. of n-RNA)	(10^6 × disintegrations/min./nucleus)	(disintegrations/min./μg. of n-RNA)	(10^6 × disintegrations/min./nucleus)
6% Casein	905 ± 110	2312 ± 262	22·5 ± 1·2	67·9 ± 3·1	0·471 ± 0·025	1·18 ± 0·09	0·512 ± 0·031	1·05 ± 0·06
18% Casein	99 ± 14	279 ± 37	16·2 ± 0·9	54·4 ± 2·4	0·648 ± 0·032	1·84 ± 0·12	0·651 ± 0·039	1·58 ± 0·12
Difference (%)	+815	+727	+39	+25	−27	−36	−19	−34

utilized the specific radioactivity of total cellular RNA and n-RNA is again greater in the liver cells from the animals given the 6% casein diet, but the amount of radioactivity associated with certain cytoplasmic RNA fractions is significantly lower than control values (Tables 5 and 6). The RNA from smooth-surfaced vesicles of both dietary groups has the highest specific radioactivity of all the cytoplasmic fractions isolated in this study. This agrees with the results of Hallinan & Munro (1964, 1965*b*). The specific radioactivity of the RNA from smooth-surfaced-vesicular and free-ribosomal fractions is significantly lower in the animals on the 6% casein diet, whereas the radioactivity of the RNA from the rough-surfaced vesicles is greater than control values.

Since RNA is not divided equally among the various cellular subfractions, specific-radioactivity data cannot be used to determine the cellular distribution of radioactive precursors within these fractions. To make this calculation, it is necessary to multiply the specific radioactivity of a fraction by its cellular RNA content. When the results from this study are expressed in this manner, the cellular radioactivity of the RNA associated with whole-cell and nuclear fractions of the liver cells from the rats on the 6% casein diet is higher than control values, but in all the cytoplasmic RNA fractions these values are significantly lower in the amino acid-deficient cells (Table 6). In both dietary groups liver n-RNA contains 80–90% of the radioactivity associated with cellular RNA, whereas cytoplasmic RNA represents 7·1 ± 0·5 (mean ± S.E.M.) and 16·4 ± 1·2% of the total radioactivity respectively of the groups given the 6% casein and 18% casein diets. Thus there is a 56·6% decrease in the amount of newly synthesized RNA that leaves the nucleus of the liver cells from the animals on the low-protein diet compared with the controls.

DISCUSSION

Mariani *et al.* (1966) have reported that, in rats fed on a diet containing an adequate amount of protein, between the ages of 28 and 48 days there is a 36% increase in the total number of liver nuclei; however, during this time-period in animals fed on a 6% casein diet, no significant nuclear proliferation was noted. In our experiments (Table 2) the effects of dietary protein deficiency on liver-nuclei concentration are similar to those noted by Mariani *et al.* (1966). Similar effects are noted in bacterial cells that have been starved of amino acids required for growth (Pardee & Prestidge, 1956; Maaløe & Hanawalt, 1961). Further, polyploidy (which is the synthesis and accumulation of nuclear DNA without subsequent cell division) of the rat liver parenchymal cells, which increases as a function of

Table 6. *Incorporation of* [5-^{3}H]*uridine into RNA of various cellular fractions of liver*

Rats were injected with 100 μc of [5-^{3}H]uridine/100g. body wt. as described in Table 5. RNA was extracted from whole-cell, nuclear and cytoplasmic fractions of liver. Rats were fed as described in Table 2. Results are recorded as the means ± S.E.M. of six animals.

	Incorporation of [5-^{3}H]uridine					
	(disintegrations/min./μg. of RNA)		(10^6 × disintegrations/min./cell)		% of total RNA radioactivity	
RNA fraction	6% Casein diet	18% Casein diet	6% Casein diet	18% Casein diet	6% Casein diet	18% Casein diet
Nuclear	22·5 ± 1·2	16·2 ± 0·9	67·9 ± 3·1	54·4 ± 2·4	92·9 ± 3·8	83·6 ± 4·5
Rough-surfaced vesicles	0·332 ± 0·049	0·171 ± 0·01	1·43 ± 0·37	3·63 ± 0·45	1·91 ± 0·11	5·64 ± 0·32
Smooth-surfaced vesicles	1·67 ± 0·07	2·81 ± 0·034	0·15 ± 0·003	0·91 ± 0·12	0·20 ± 0·02	1·21 ± 0·10
Free ribosomes	0·156 ± 0·029	0·273 ± 0·036	0·21 ± 0·04	0·61 ± 0·08	0·27 ± 0·03	0·92 ± 0·08
Whole cell	2·78 ± 0·06	1·09 ± 0·12	73·9 ± 4·2	65·8 ± 3·1	—	—

age (Nadal & Zajdela, 1966), is inhibited by a low-protein diet, as indicated by our observation of a lower nuclear DNA content in the cells of animals fed on the 6% casein diet. Thus amino acid deprivation in the immature animal appears to inhibit both hepatic DNA synthesis and hepatic mitosis.

Liver cells from an immature rat fed on a diet containing an adequate amount of protein increase not only in number but also in size (Enesco & Leblond, 1962). This process is severely depressed by giving a low-protein diet, and liver cells from these animals have far less cellular protein than those from controls given a diet containing an adequate amount of protein (Table 2). The observation of Wannemacher & Allison (1968), that a decreased liver protein content/cell in rats fed on low-protein diets is related to a lower rate of protein synthesis *in vivo*, is supported in the present study by the finding that isolated ribosomes from amino acid-deprived hepatic cells are characterized by a decreased ability to synthesize protein (Table 4) and a markedly lower cellular ribosome concentration compared with cells from control animals. The decreased ability of these ribosomes to synthesize protein appears to be related to the low amino acid content that exists in the liver cells of an animal deprived of protein (Table 1). Several investigators (Fleck *et al.* 1965; Wunner *et al.* 1966; Webb *et al.* 1966; Sox & Hoagland, 1966; Sidransky *et al.* 1967) have shown that starvation or ingestion of an amino acid-deficient diet results in a rapid disaggregation of the hepatic polyribosomes and a decrease in their rate of protein biosynthesis. In the present work, the possible direct effect of the concentration of cellular free amino acids on polyribosomal aggregation is supported by the marked decrease in m-RNA content of the ribosomes obtained from the liver cells of rats fed on the 6% casein diet (Table 4). Since the test of protein-synthetic competence is run on a fraction that contains both bound and unbound ribosomes, it is possible that part of the difference is due to the higher proportion of free ribosomes in the preparation from the livers of the group given the low-protein diet (Table 3). Similarly, in the livers from tryptophan-deficient (Wunner *et al.* 1966) or protein-depleted (Mandel, Quirin, Bloch & Jacob, 1966) rats, a larger proportion of the total ribosomes is in the free state. These free ribosomes have a decreased ability to incorporate amino acids into protein compared with those in the bound state (Hallinan & Munro, 1965*b*).

The decreased protein-synthetic ability of ribosomes from amino acid-deprived liver cells is further complicated by the significantly lower concentration of cellular r-RNA in these cells. When both of these factors are taken into consideration, the average liver cell from the animals fed on the low-casein diet is synthesizing protein at a rate 12% of that noted in cells from control rats (Table 4). This corresponds very closely to the differences noted previously in experiments on the incorporation of radioactive amino acids *in vivo* into liver protein (Wannemacher & Allison, 1968). The lower ribosomal content of the liver cells from the amino acid-deprived rats can be explained by either an increased rate of catabolism or a decreased synthesis of r-RNA. In weanling rats on a diet containing an adequate amount of amino acids, the parenchymal cells increase in size, amount of nuclear DNA, polyploidy, number of ribosomes, proportion of polyribosomal heavy aggregates and protein-synthetic ability. This growth process is partially prevented by giving a low-protein diet. Thus, in an immature animal, an amino acid deficiency inhibits the accumulation of liver ribosomes rather than causing the breakdown of existing r-RNA.

Experiments with labelled nucleosides have indicated that most of the radioactivity in the n-RNA fraction is associated with the 28s and 18s r-RNA and its precursors sedimenting at 55s, 45s and 35s (Muramatsu, Hodnett, Steele & Busch, 1966), with maximum labelling noted 20min. after injection of the tracer (Hiatt, 1962). In contrast, cytoplasmic r-RNA accumulates the label at a much lower rate and does not obtain the maximum amounts of incorporation until 1–2hr. after an injection of the labelled nucleoside (Hiatt, 1962). When the radioactive purine nucleosides (adenosine and guanosine) are used, the results do support the previous conclusion that r-RNA is synthesized at a lower rate in the liver cells of the rats fed on the amino acid-deficient diet, but opposite results are obtained if cytidine or uridine is the precursor (Table 5). Since various RNA precursors give contradictory results, it is not possible to conclude from these data what effects amino acid deprivation has on the synthesis of hepatic RNA. These differences are probably related to the effects of dietary amino acids on the size of nucleotide precursor pools that are involved in RNA synthesis. This possibility will have to be taken into consideration in future work.

The experiments of Harris and co-workers (Harris, 1959, 1963; Watts & Harris, 1959; Harris & Watts, 1962; Harris, Fisher, Rodgers, Spencer & Watts, 1963) have demonstrated that rapidly labelled n-RNA undergoes breakdown within the nucleus to acid-soluble end products. These results have led to the conclusion that only a small proportion of the RNA made in the cell nucleus is incorporated into stable templates that enter the cytoplasm; the remainder undergoes breakdown (Harris, 1964). Our observation that 1hr. after an injection of the labelled precursor 80–90% of the labelled nucleic acids are still associated with the n-RNA fraction supports the conclusion that most of the rapidly labelled n-RNA never leaves the nucleus. In addition, these data show that an amino acid deficiency decreases the amount of rapidly labelled n-RNA that becomes stabilized and enters the cytoplasm (Table 6).

The above results from weanling rats suggest that dietary amino acid deprivation could influence hepatic protein synthesis by altering the quality and the quantity of the cytoplasmic ribosomes. The former process, which is defined as the rate of incorporation/unit of r-RNA, may be directly related to the decreased concentration of cellular free amino acids and the shifts in polyribosomal profiles that have been noted by other investigators (Wunner *et al.* 1966). Ribosomal quantity (the amount of cytoplasmic ribosomes/cell) is correlated with the ability of newly synthesized ribosomal sub-units to leave the nucleus. To obtain an accurate estimate of ribosomal competence *in vitro* it is necessary to measure both of these parameters. When both factors are considered, good agreement is obtained between the measurements of protein biosynthesis *in vivo* and *in vitro*.

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