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The Effect of Dietary Protein Deficiency on Albumin Synthesis and on the Concentration of Active Albumin Messenger Ribonucleic Acid in Rat Liver

By VIRGINIA M. PAIN,* MICHAEL J. CLEMENS† and PETER J. GARLICK*
**Department of Human Nutrition, London School of Hygiene and Tropical Medicine, London WC1E 7HT, and †Department of Biochemistry, St. George's Hospital Medical School, London SW17 0RE, U.K.*

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In rats fed on a protein-deficient diet, albumin synthesis as a percentage of total liver protein synthesis falls from the normal value of approx. 15% to about 8%. We have extracted total cytoplasmic RNA from individual rat livers and measured the concentration of active albumin mRNA by translation in a reticulocyte lysate system from which the endogenous mRNA had been removed [Pelham & Jackson (1976) *Eur. J. Biochem.* **67**, 247–256]. In this messenger-dependent system it is possible to measure the synthesis of albumin as a proportion of the overall protein synthesis promoted by the addition of the hepatic RNA. The results show that the concentration of translatable albumin mRNA in samples of total cytoplasmic RNA from livers of protein-deficient rats is decreased markedly. These findings suggest that dietary protein supply affects selectively the synthesis and/or functional stability of albumin mRNA in rat liver.

Deficiency of dietary protein is associated with a pronounced decrease in the rate of albumin synthesis by the liver in both humans and rats (James & Hay, 1968; Kirsch *et al.*, 1968; Haider & Tarver, 1969; Morgan & Peters, 1971; Jeejeebhoy *et al.*, 1973). Similar decreases in synthesis of hepatic tissue proteins have not been demonstrated. Indeed, increases have frequently been reported (Waterlow & Stephen, 1968; Haider & Tarver, 1969; Garlick *et al.*, 1975). It thus appears that there is a degree of selectivity in the nutritional regulation of synthesis of albumin relative to that of most of the other proteins made by the liver. A possible means by which such selectivity could be exerted is through changes in the availability of translatable mRNA for albumin.

It is now a common procedure to estimate the quantity of a particular mRNA in a sample of total tissue RNA by introducing the sample into a cell-free protein-synthesizing system and measuring the synthesis of the specific protein product of that RNA. Two types of cell-free system have particular advantages for this of work. The rabbit reticulocyte lysate (e.g. Palmiter, 1973) is a very efficient system, in which the rate of protein synthesis approaches that of the intact cells (Hunt & Jackson, 1974). However, this system suffers from the disadvantage that synthesis of reticulocyte proteins, mainly globin, on endogenous mRNA is very extensive, and total protein synthesis is not markedly increased by addition of the mRNA to be tested. This problem can be avoided by the use of a cell-free system from wheat germ (Roberts & Paterson, 1973), which has little or no endogenous protein-synthesizing activity and therefore produces only the proteins directed by the added mRNA. However, the wheat-germ system is less efficient than the reticulocyte lysate in completing the synthesis of larger proteins, and translation of purified albumin mRNA in this system has been shown to result in the appearance of incomplete peptides as well as the full-sized product (Taylor & Tse, 1976).

The advantages of these two systems have been combined by the development of a preincubation technique that removes most of the endogenous mRNA from a reticulocyte lysate without damaging the ability of the system to translate added mRNA (Pelham & Jackson, 1976). We have used this messenger-dependent reticulocyte lysate system to measure total translatable albumin mRNA in livers of rats fed on normal and protein-deficient diets, and to give an estimate of how the amount of active albumin mRNA varies as a proportion of total hepatic mRNA.

Experimental

Animals

Rats were males of a COBS Wistar strain (Charles River, Margate, Kent, U.K.). They were fed on a powdered diet containing 12% (w/w) casein supple-

mented with methionine, as described by Heard *et al.* (1977). When they weighed 90–100g some groups were fed *ad libitum* with an isocaloric diet containing no protein for periods of 2 or 9 days as indicated in the Tables. After 9 days on the protein-free diet, some animals were re-fed with the control diet for 2 days.

Antisera

Antisera to rat albumin were raised in either rabbits or goats by injection at multiple subcutaneous sites of electrophoretically purified albumin emulsified with complete Freund's adjuvant. The injections were repeated after 3 weeks. A 25μl sample of each of the antisera precipitated 15μg (goat antiserum) or 10μg (rabbit antiserum) of rat albumin at their respective equivalence points.

Measurement of albumin synthesis as a proportion of total hepatic protein synthesis in vivo

Rats were injected via the tail vein with 200μCi of L-[4,5-^{3}H]leucine (from The Radiochemical Centre, Amersham, Bucks., U.K.) and 0.15mmol of unlabelled leucine/100g body wt. The purpose of using a large dose of precursor was to flood the free amino acid pool of liver with leucine so that the various compartments would tend to reach more or less the same specific radioactivity (Henshaw *et al.*, 1971; Mortimore *et al.*, 1972; Scornik, 1974). The technique minimizes any problems that would arise if there were heterogeneity of the precursor pools of amino acids from which different proteins or protein populations are synthesized, as has been suggested by the observations by Ilan & Singer (1975) and Fern & Garlick (1976). The rats were killed 10min after injection and the livers homogenized in 3vol. of 0.35M-sucrose in 0.05M-Tris/HCl, pH7.4. Samples of homogenate were precipitated with 10% (w/v) trichloroacetic acid containing unlabelled leucine, and processed for measurement of radioactivity precipitable by hot 5% trichloroacetic acid. The remainder of each homogenate was made 1% with Triton X-100 and immediately centrifuged at 2000*g* for 5min to obtain the post-nuclear supernatant. The radioactivity incorporated into albumin was estimated by immunoprecipitation from the post-nuclear supernatant with a specific anti-(rat albumin) antiserum added in an excess. To eliminate non-specific precipitation, samples of post-nuclear supernatant (40 and 80μl of each) were first incubated twice with 16μg of chicken ovalbumin [Sigma (London) Chemical Co., London S.W.6, U.K.] and 0.1mg of anti-chicken ovalbumin (Miles Laboratories, Slough, Bucks., U.K.) as originally suggested by Peters (1962*a*). Radioactivity in the immunoprecipitates generated in the second treatment was negligible. The supernatants from the ovalbumin/anti-ovalbumin precipitation were then incubated with 0.1ml of rabbit anti-(rat albumin) antiserum. All immunoprecipitation reaction mixtures were incubated for 1h at 23°C and overnight at 4°C. Albumin immunoprecipitates were washed twice with phosphate-buffered saline [10mM-sodium phosphate (pH7.5)/15mM-NaCl] containing unlabelled leucine (1mg/ml) and 1% Triton X-100, then dissolved in 0.1M-NaOH and reprecipitated with hot 10% trichloracetic acid, both containing unlabelled leucine. Precipitates were dissolved in 0.1M-NaOH and counted for radioactivity in Tritosol scintillation fluid (Fricke, 1975) with an efficiency of 19%.

Extraction of total hepatic cytoplasmic RNA

Total RNA was prepared from post-nuclear supernatants of individual rat livers by the method of Shore & Tata (1977). Briefly, this involved the homogenization of the livers in about 15vol. of a buffer of high ionic strength and high pH (0.35M-sucrose, 0.2M-Tris/acetate, pH8.5 at 2°C, 0.05M-KCl, 0.01M-magnesium acetate). These conditions inhibit the activity of endogenous ribonucleases (Shore & Tata, 1977), as shown in studies with plant systems (Davies *et al.*, 1972). Post-nuclear supernatants were prepared from the homogenates by addition of Triton X-100 to a concentration of 1.3% and centrifugation at 2000*g* for 5min. RNA was then extracted from the post-nuclear supernatants by a phenol/sodium dodecyl sulphate method, precipitated with ethanol and washed with 3M-sodium acetate as described by Shore & Tata (1977). Final RNA samples were dissolved in 10mM-Tris/acetate, pH7.6, at a concentration of about 2mg/ml (estimated by A_{260} measurement; 1mg of RNA = 23 A_{260} units) and stored in liquid N_2.

Assay of albumin mRNA in the messenger-dependent reticulocyte lysate

Rabbit reticulocyte lysates were prepared as described by Clemens *et al.* (1974) and stored in batches in liquid N_2. Samples were thawed in the presence of 20μM-haemin and treated with 80 units of micrococcal nuclease (EC 3.1.4.7) (P-L Biochemicals, Milwaukee, WI, U.S.A.; 1 unit is the amount that produces 1 A_{260} unit of acid-soluble material with DNA as substrate at pH8.8 at 37°C/) ml in the presence of 1mM-$CaCl_2$, as described by Pelham & Jackson (1976). After a 15min incubation at 20°C, the nuclease was inactivated by addition of EGTA to a concentration of 2mM. Assays of mRNA from individual livers were performed in 50μl mixtures containing 25μl of lysate, up to 4μg of total cytoplasmic RNA and the following com-

ponents at the indicated final concentrations: 10mM-Tris/HCl, pH7.6, 100mM-KCl, 2mM-magnesium acetate, 1mM-ATP, 0.2mM-GTP, 4–80μM-amino acids in the ratio in which they occur in globin, 110 μCi of [^{35}S]methionine/ml (430Ci/mmol, from The Radiochemical Centre), 10μM-haemin and an energy-generating system of phosphocreatine (3.1mg/ml) and creatine kinase (EC 2.7.3.2). Samples were incubated at 26°C for 1h, after which 3μl samples were removed for assay of incorporation of [^{35}S]methionine into total protein (Clemens *et al.*, 1974) and the remaining 47μl was taken for analysis of incorporation of radioactivity into albumin. These samples were mixed with 3μg of unlabelled carried albumin and treated with an excess (25μl) of goat or rabbit antiserum to rat albumin. The immunoprecipitates were collected and washed exactly as described by Shore & Tata (1977). The final trichloroacetic acid precipitated proteins were collected on glass-fibre filters and the radioactivity was measured in 0.5% (w/v) 2,5-diphenyloxazole in toluene in a liquid-scintillation counter with an efficiency of approximately 65%, determined by internal-channels-ratio method.

Analysis of translation products by gel electrophoresis

Cell-free incubations were performed exactly as described above. Samples (6μl) were removed for analysis of total radioactive polypeptides and 41μl samples were used for immunoprecipitation of albumin as described above. To the total protein samples or the immunoprecipitates 25μl of a buffer containing 60mM-Tris/HCl (pH6.8), 2% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 70mM-2-mercaptoethanol and 0.001% Bromophenol Blue was added and the samples were heated at 90°C for 5min. Electrophoresis of the samples was performed on 10% polyacrylamide slab gels containing 0.1% sodium dodecyl sulphate, by the procedure of Laemmli (1970). Authentic rat albumin was electrophoresed in parallel with the radioactive samples as a marker. Gel slabs were stained for protein with Coomassie Blue and were then destained with methanol/acetic acid/water (5:7:88, by vol.), dried and subjected to radioautography by using Kodak AP-54 X-ray film.

Results and Discussion

Albumin synthesis as a proportion of total hepatic protein synthesis in vivo

There have been several reports of effects of protein deprivation on the rate of synthesis of albumin *in vivo* (James & Hay, 1968; Kirsch *et al.*, 1968; Jeejeebhoy *et al.*, 1973), but these have usually involved measurements of radioactivity in albumin in the plasma which are difficult to relate to hepatic protein-synthetic activity as a whole. Morgan & Peters (1971) overcame this problem by estimating albumin synthesis as a proportion of total liver protein synthesis by extracting albumin from the liver 16min after administering a tracer dose of radioactive leucine. At this time no labelled albumin had been released into the plasma. These workers reported that albumin synthesis as a proportion of total protein synthesis by the liver fell by half when rats were fed on a protein-deficient diet. Table 1 shows the results we have obtained with a slightly different labelling method intended to minimize possible problems arising from heterogeneity of precursor amino acid pools (see the Experimental section). It can be seen that feeding a protein-free diet to rats results in a decrease from 15 to 8% in the ratio of albumin synthesis to total hepatic protein synthesis. Our value of 15% for albumin synthesis as a proportion of total protein synthesis is slightly higher than values reported by others (Morgan & Peters, 1971; Keller & Taylor, 1976). A possible reason for this is our use of younger animals, since an age-related decrease in albumin synthesis was reported by Peters & Peters (1972).

Table 1. *Effects of protein deficiency and re-feeding on albumin synthesis as a proportion of total liver protein synthesis*

Diets, animals and experimental procedures were as described in the Experimental section. Results are the means ± S.E.M. of five animals per group. Results were calculated on the basis of a leucine content in albumin of 0.1071 g/g of protein (Peters, 1962*b*) and in hepatic protein of 0.0875 g/g of protein (Morgan & Peters, 1971). By Student's *t*-test: *significance of difference from control group, $P < 0.001$; †significance of difference from control group, $P = 0.05$; ‡significance of difference from 9-day protein-free group, $P < 0.001$.

Diet	Albumin synthesis (% of total liver protein synthesis)
Complete	14.6 ±0.50
Protein-free (9 days)	7.98±0.62*
Protein-free (2 days)	8.34±0.52*
Protein-free (9 days), then re-fed with complete diet (2 days)	12.7 ±0.65†‡

Table 1 also shows that 2 days of protein deficiency is nearly as effective as 9 days in decreasing the ratio of albumin synthesis to total liver protein synthesis. Likewise, the ratio increases rapidly when the animals fed on the depleted diet for 9 days are re-fed with the complete diet, but it is still significantly below the control value after 2 days of re-feeding.

Characteristics of mRNA translation in the reticulocyte lysate

To see whether these changes could be attributed to altered availability of translatable albumin mRNA, we investigated the synthesis of albumin in a messenger-dependent reticulocyte lysate directed by total liver cytoplasmic RNA. To characterize this system for assaying hepatic RNA samples, we measured the synthesis of albumin and of total protein as a function of the amount of RNA added, both in the messenger-dependent lysate (Figs. 1*a* and 1*b*) and in the same lysate without the pretreatment with nuclease (Figs. 1*c* and 1*d*). It can be seen that the background (no mRNA) radioactivity in albumin immunoprecipitates was considerably lower in the messenger-dependent system, but that efficiency of translation of albumin mRNA was not impaired by the nuclease pretreatment. Further, incorporation of radioactivity into total protein is also stimulated by addition of exogenous mRNA (Fig. 1*b*), whereas in the untreated system there is extensive protein synthesis on endogenous mRNA which shows little or no stimulation in response to addition of liver RNA (Fig. 1*d*; note change in scale). In assaying liver RNA samples in

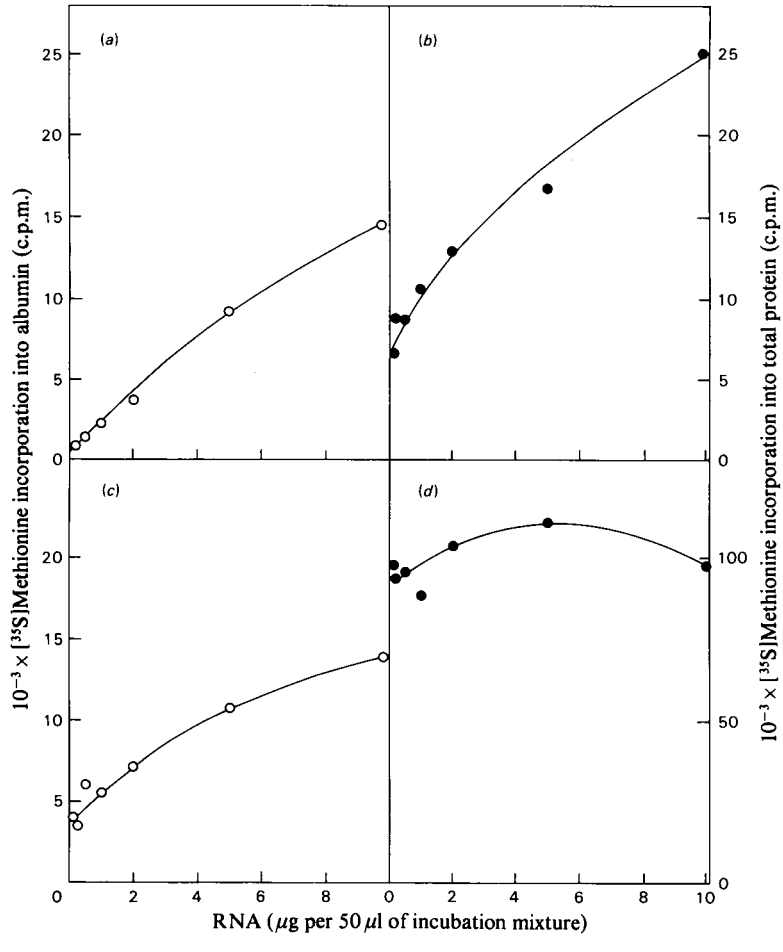

Fig. 1. *Synthesis of albumin and total protein in response to total hepatic cytoplasmic RNA in messenger-dependent and untreated reticulocyte lysates*

Total cytoplasmic RNA was extracted as described in the Experimental section and was added to either a messenger-dependent lysate (*a* and *b*), or to the same lysate without pre-treatment with nuclease (*c* and *d*). Conditions of protein synthesis were as described in the Experimental section. Incorporation of [^{35}S]methionine into albumin (○) immunoprecipitated from 47 μl of assay mixture is shown in (*a*) and (*c*), and incorporation into the total protein (●) of 3 μl of assay mixture is shown in (*b*) and (*d*).

the messenger-dependent lysate we have expressed our results in two ways: first, as the radioactivity incorporated into albumin per μg of RNA added, and, secondly, as the radioactivity in albumin as a proportion of that incorporated into total protein in response to addition of the hepatic RNA. This second value must be regarded as an approximation, however, since, at the concentration of hepatic RNA (4μg per 50μl) added to our routine assays, the response of total protein synthesis to RNA added is not completely linear.

The total products of the reaction and the immunoprecipitates have been analysed on polyacrylamide gels under denaturing conditions, and the incorporation of radioactivity into individual bands was examined by radioautography. The pattern shown for incorporation into total products (Fig. 2) is similar to that obtained by Pelham & Jackson (1976) with poly(A)-rich liver RNA. Analysis of the immunoprecipitated material shows that almost all the radioactivity in this fraction is in a band that co-migrates with an albumin marker. A very small amount of incorporation is also found in a few products of lower molecular weight, the nature of which is not known. The result in Fig. 2 was obtained with the antiserum to rat albumin raised in rabbits. A very similar pattern was obtained with the goat antiserum used in some of our experiments (G. Shore, personal communication).

Effect of protein deficiency and re-feeding on hepatic albumin mRNA concentration

Table 2 shows the effect of 9 days of protein deficiency on the concentration of albumin mRNA in samples of total cytoplasmic RNA from rat liver. The first column shows the radioactivity incorporated into albumin per μg of RNA added, and the second the radioactivity in albumin as a percentage of that incorporated into total products. The concentration of albumin mRNA in samples from livers of protein-deficient rats was about half that in control rats of the same age, whichever way the results are expressed. Since the control animals grow from about 100g to about 160g during the experimental period, we also prepared samples from a group of zero-time controls, which were killed when the experimental animals were first given the protein-free diet. The results obtained from these animals were not significantly different from those from the age controls, but, if they are used as a basis for comparison with the protein-deficient animals, they indicate a smaller proportional effect of the deficient diet (32–40% decrease in albumin mRNA). The effect of re-feeding the defi-

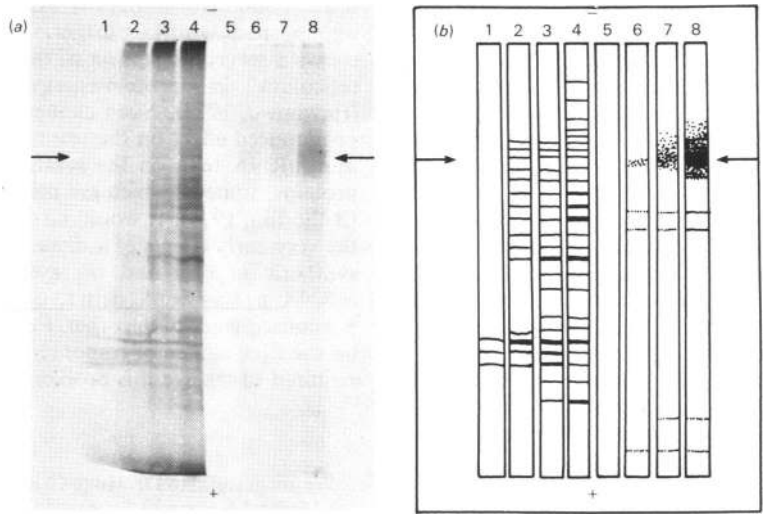

Fig. 2. *Polyacrylamide-gel analysis of radioactive cell-free translation products*

Total cytoplasmic RNA was translated in a messenger-dependent lysate as described in the Experimental section, and samples of total incubation mixtures or of anti-albumin immunoprecipitates were subjected to gel electrophoresis (*a*). The gel slab was stained, destained and dried and then radioautographed for 2 days. Tracks 1–4 show total ^{35}S-labelled polypeptides synthesized in response to the following amounts of added RNA: (1) none; (2) 0.5μg; (3) 1.3μg; (4) 2.6μg. Tracks 5–8 show immunoprecipitated products synthesized in response to the following amounts of RNA: (5) none; (6) 3.6μg; (7) 9.0μg; (8) 18.0μg. The arrow shows the position of authentic unlabelled rat albumin electrophoresed in a parallel track of the same gel slab. (*b*) Line drawings showing the most prominent bands.

Table 2. *Effect of protein deficiency and re-feeding on the concentration of translatable mRNA for albumin in samples of total cytoplasmic RNA from rat liver*

Incubations were carried out in the messenger-dependent reticulocyte lysate as described in the Experimental section. 'Age controls' represent animals fed on the complete diet and killed at the same time as the protein-deficient and re-fed rats. 'Zero-time' controls represent animals killed at the time that the experimental animals were first fed on the protein-deficient diet. Results are the means ± S.E.M. of five animals per group. *Significance of difference from age control group: $P < 0.005$; †significance of difference from zero-time control group: $P < 0.025$.

Diet	Radioactivity incorporated into albumin (10^{-3} × c.p.m./μg of RNA added)	Radioactivity incorporated into albumin (% of radioactivity incorporated into total protein)
Complete (age controls)	1.87 ±0.25	3.83 ±0.34
Protein-free (9 days)	0.98* ±0.05	1.91*† ±0.21
Protein-free (9 days), then re-fed with control diet (2 days)	1.22 ±0.29	3.11 ±0.50
Complete (zero-time controls)	1.43 ±0.25	3.34 ±0.46

cient rats for 2 days with control diet was less pronounced than that observed *in vivo* and was not statistically significant, but the results suggest that the concentration of albumin mRNA is beginning to increase towards the normal value.

The results in Table 2 also show that the radioactivity incorporated into albumin in the reticulocyte lysate is a much lower proportion of the radioactivity in total protein than is found *in vivo*. In such a complex assay system there are many possible explanations for this discrepancy, but a factor of great quantitative importance is the rarity of methionine residues in albumin (Peters, 1962*b*). Indeed, in a recent test we have found that when hepatic RNA samples from control animals are translated in the reticulocyte lysate the incorporation of radioactivity from [^{14}C]-leucine into albumin is about 20% of that incorporated into total protein.

It should be pointed out that the protein-synthesis assay *in vitro* only measures active (i.e. translatable), mRNA and therefore does not distinguish between a decline in the albumin mRNA concentration itself and a conversion of part of the mRNA into an untranslatable form. Further, a change in the concentration of a particular mRNA does not necessarily imply specific control of transcription, since effects on mRNA degradation could equally well be involved. With these reservations in mind, however, the results are of interest in that they suggest the possibility of a role for availability of specific mRNA species in the nutritional regulation of synthesis of a protein whose production constitutes a significant proportion of hepatic protein-synthetic activity. A similar correlation has been reported for the effects of hypophysectomy on albumin synthesis in rat liver (Keller & Taylor, 1976).

Our results raise the question of whether the decrease in translatable albumin mRNA is a primary effect of nutritional deprivation or whether it is secondary to effects at the level of translation or secretion. Albumin is made chiefly on ribosomes bound to the endoplasmic reticulum (see review by Rolleston, 1974), and Zahringer *et al.* (1977) have shown almost all the polyribosome-bound albumin mRNA to be associated with membrane-bound ribosomes. Preliminary data from our laboratory indicate that livers from rats fed on a protein-deficient diet for 9 days have a lower proportion of membrane-bound ribosomes (V. M. Pain & A. R. Vyas, unpublished work). Further, electron microscopy shows a severe disruption of the rough endoplasmic reticulum in protein-energy-malnourished rats (Enwonwu, 1972). Such changes could have a more pronounced effect on the translation *in vivo* of albumin mRNA than on the synthesis of hepatic tissue proteins, some of which are made on free ribosomes (Rolleston, 1974). It would be of interest to examine the very early effects of a dietary change on abumin synthesis *in vivo* and on availability of albumin mRNA, as this would help to determine whether one is a consequence of the other. Further detailed studies on the time course of response to dietary change are required to resolve this problem.

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