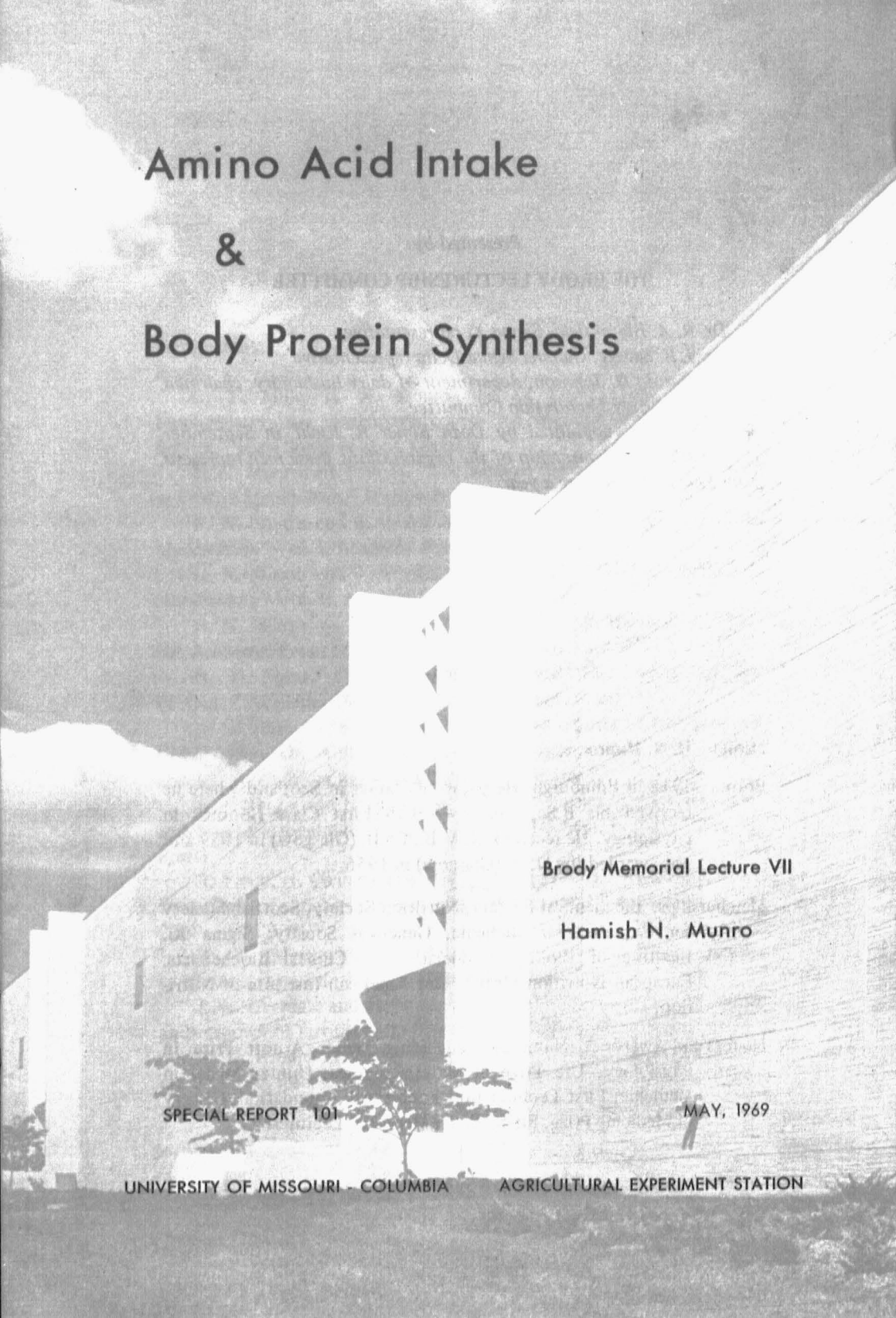
Amino Acid Intake & Body Protein Synthesis



Brody Memorial Lecture VII

Hamish N. Munro

SPECIAL REPORT 101 MAY, 1969

UNIVERSITY OF MISSOURI - COLUMBIA AGRICULTURAL EXPERIMENT STATION

Presented by:

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This committee, appointed by Dean Elmer R. Kiehl in September, 1967, with joint sponsorship of the organizations these men represent, plans at least one lecture a year.

BIOGRAPHY

Name: **H. N. Munro**

Born: 1915 in Edinburgh. He began his career in Scotland where he received his B.Sc. (Glasgow) with First Class Honours in physiology. He received his M.B., Ch.B. (Glasgow) in 1939 and was awarded the D.Sc. (Glasgow) in 1956.

Memberships: Biochemical Society, Nutrition Society, Scottish Society for Experimental Medicine, Genetical Society, Sigma Xi. Institute of Biology, Association of Clinical Biochemists, European Nutrition Group, and American Institute of Nutrition.

Honors and Awards: Numerous, including Senior Arnott Prize in Physiology, Ure Prize in Materia Medica, Hunter Medal in Medicine, First Lecturer under the Fleck Foundation, History of Medicine Prize, First Allison Memorial Lecturer.

Publications:

Books:

G. T. Mills, H. N. Munro and G. Leaf (1954). "Practical Biochemistry. An elementary course for students of medicine and science." John Smith & Sons, Glasgow.

H. N. Munro, ed. (1964). "The Role of the Gastro-Intestinal Tract in Protein Metabolism." Blackwells, Oxford.

H. N. Munro and J. B. Allison, eds. (1964). "Mammalian Protein Metabolism." Vol. I, Academic Press, New York.

H. N. Munro and J. B. Allison, eds. (1964). "Mammalian Protein Metabolism." Vol. II, Academic Press, New York.

H. N. Munro, ed. (1968). "Mammalian Protein Metabolism," Vol. III, Academic Press, New York. (In Preparation)

H. N. Munro (1968). "The Nutritional Biochemistry of the Proteins." Academic Press, New York. (In Preparation)

In addition to the numerous books and reports of International Committees, Dr. Munro has published approximately 150 journal articles. Some of the more recent publications are:

W. McIndoe and H. N. Munro (1966): "Species of RNA from liver cell fractions separated on agarose gels." Biochem. Biophys. Acta (In Press)

A. Fleck, N. Chandra and H. N., Munro (1966). "Albumin in liver: the effects of variations in dietary protein content." Scot. Med. J., 11, 226.

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Amino Acid Intake and Body Protein Synthesis

BRODY MEMORIAL LECTURE*

By H. N. MUNRO

It is a constant source of regret to me that I never met the late Dr. Samuel Brody, but I fell under the spell of comparative studies on mammals when I worked at the University of Illinois some 20 years ago in Dr. H. H. Mitchell's laboratory. One of Mitchell's standard sources of basic data was Brody's "Bioenergetics and Growth" (1) and ever since my period at Illinois this great book has seldom been far from my desk.

As a student of protein metabolism, I was particularly struck by the way in which Dr. Brody demonstrated from a wealth of published data culled from both well-known and obscure sources that endogenous output of nitrogen per unit of body weight becomes progressively less as the body size of the mammal increases. Indeed, it is expressed by the 0.72 power of body weight, a quotient similar to that relating basal energy metabolism of mammals to their body size. This means that when a rat weighing 200 gm is given a protein-free diet, it continues to excrete five times the amount of urinary nitrogen per kg. of body weight that a man of 70 kg does under similar circumstances. When a property so fundamental as endogenous nitrogen output changes in this way, it is reasonable to look for a general slowing down of protein metabolism in larger mammals. An investigation of the literature confirmed for me that this is the case. If you arrange the half lives of plasma albumin of different mammals according to their body weight (Table 1), you find that the mouse has a half-life of one day, the rat of three days and so on to the cow, with a half-life of 20 days. This made me curious to know whether this progressive decline in turnover would be associated with changes in the machinery in the liver for synthesizing plasma albumin. Accordingly, we (2) analyzed the livers of a series of adult mammals ranging in size from 30 gm to 500 kg in weight for RNA, DNA, and phospholipid constant (2). (Table 1) The table shows that the amount of RNA per liver cell (provided by the RNA/DNA ratio) declines progressively as we ascend the scale of species size. We thus have a correlation between the amount of RNA in the liver cell and the rate of synthesis of plasma

* Lecture presented Feb. 7, 1967, at the University of Missouri - Columbia.

TABLE 1--PLASMA ALBUMIN TURNOVER, LIVER CELL COMPOSITION PER MG. DNA AND COMPOSITION OF LIVER MICROSOMES OF DIFFERENT MAMMALS ARRANGED IN ASCENDING ORDER OF WEIGHT

(Reproduced from Munro and Downia, 1964)

Species	Half-life of Plasma Albumin (days)	Whole Liver Composition		Microsome Fraction (percent of constituents)	
		$\frac{\text{RNA}}{\text{DNA}}$	$\frac{\text{Phospholipid}}{\text{DNA}}$	RNA	Phospholipid
Mouse	1.2	4.45	14.2	9.6	24.2
Rat	2.5	3.05	9.8	7.3	22.9
Rabbit	5.7	2.44	11.6	6.2	29.2
Dog	8.2	1.69	9.4	-	-
Cow	20.7	1.29	8.7	5.9	31.3

albumin. The liver microsomes were also analyzed and demonstrated a similar loss of RNA content in the larger animals. This means that there are fewer ribosomes on the microsomal membranes. You will note that the membranes themselves are not affected, since the amount of phospholipid per cell or per microsome unit is not significantly diminished in the large animals. We thus have a picture of adaptation of protein synthesis in the liver cell to size of species. Although the liver of the large mammal has fewer cells than the liver of the small mammal, the size of each cell is about the same in the two groups of species; moreover, the membranous component of the endoplasmic reticulum is about equally abundant in each cell. The difference lies in the number of membrane-attached ribosomes, which is considerably less in the larger animals, and thus may be the controlling factor in the slower rate of protein synthesis observed in the larger species.

Recently, we have examined the effect of species size on the amount of RNA in the thyroid gland (3) and in skeletal muscles (4). In the case of the thyroid epithelial cells, the amount of RNA per cell declines as we progress up the scale of body weight, and the concentration of RNA in skeletal muscle is similarly affected by species size. However, the percentage of muscle in the body is fairly constant at 45 per cent in various species of mammal, whereas the liver represents a diminishing proportion of body weight as we pass from the shrew (6 per cent liver) to the elephant (0.5 per cent liver). Consequently, the *total* amount of RNA in the liver and in all the skeletal muscles does not bear a constant relationship to one another in different mammals. Thus, in the mouse, muscle and liver contain about the same total amount of RNA, whereas in the horse, the liver represents only one-fifth of the RNA of muscle (4). This implies that protein synthesis in muscle may be a relatively more important consumer of available amino acids in large mammals than in small ones.

We have not progressed further with the problem of the mechanism by which size of species regulates rate of protein metabolism. However, one factor which has to be considered is that the total food intake per unit of body weight becomes progressively less as one ascends the scale of body size: and in consequence the intake of protein is generally much smaller relative to body size in the case of the large mammals. This brings us to a consideration of the relationship between amino acid intake and body protein synthesis, the main subject of this discourse. In this field, my collaborators and I have been studying for some years the impact of protein intake on the RNA metabolism of rat liver, and we have now reached the point at which we can speculate on the nature of the mechanisms linking liver protein synthesis, liver RNA metabolism, and intake of amino acids from dietary protein. I shall present this work in three parts; first, the background to our present studies; second, the methods we are currently using to investigate the problem; and last, the results obtained and their significance.

It has long been recognized that level of protein intake affects the amount of protein in the body and that different tissues respond differently to protein deprivation. One-hundred years ago, Carl Voit demonstrated (5) that the starving

cat loses little tissue substance from its brain and heart, that its skeletal muscle is moderately affected, but that its liver is extensively depleted. This pattern has been repeatedly confirmed, both in starving animals and in those depleted by administra administration of protein-deficient diets. The first modern survey of this phenomenon was carried out by Addis and his colleagues. They showed that rats receiving a protein-free diet lose about one-quarter of their liver protein within the first two days. Kidney protein is also rapidly though less extensively affected, but the carcass undergoes a slow depletion. On feeding a protein-rich diet, the liver and kidney respond promptly, whereas carcass protein is only slowly repleted. We made a study some years ago (6) of the effects of protein depletion on cell composition in the liver, kidney, and intestinal mucosa. All three tissues lose protein rapidly during depletion. In the case of the liver and the kidney, Table 2 shows that the amount of protein per cell was reduced, whereas the intestinal mucosa retained a constant cell composition. Further investigation showed that the rate of cell

TABLE 2--CELL COMPOSITION OF LIVER (L), KIDNEY (K), AND INTESTINAL MUCOSA (L) OF THE RAT UNDER VARIOUS DIETARY CONDITIONS

(Reproduced from data of Munro and Goldberg, 1964)

DIET	Protein N/mg DNAP			RNAP/mg DNAP		
	L	K	I	L	K	I
Protein Free	74	56	33	3.32	1.47	2.02
Adequate Protein (fasting)	97	66	33	4.03	1.79	1.94
Adequate Protein (fed protein)	106	65	36	4.41	1.76	1.97

division in the mucosa, normally a rapid process, was retarded in the protein-depleted rats. Thus, tissues lose protein during depletion for different reasons. The liver and kidney, where the cells divide infrequently, undergo a loss of cytoplasm per cell; whereas the mucosal cells retain the same cell composition but replicate themselves more slowly; this presumably disturbs the normal relationship between cell production and cell loss from the intestinal mucosa. The moral of this study is that we should not expect to find one universal mechanism explaining the effects of protein depletion on the tissues and cells of the body. What I shall say now is therefore strictly confined to the response of the liver cell to amino acid intake and may not have application to other tissues.

The loss of protein from the liver cell of the depleted animal is accompanied by other changes. Kosterlitz demonstrated more than 20 years ago that there is also a loss of phospholipid and of nucleic acid which parallels the disappearance of protein. It was later shown by him and by us that the nucleic acid lost is entirely

RNA. The questions which we must now try to answer are why do we find a loss of RNA when the amino acid supply from the diet is cut off, and what effect does this have on the capacity of the liver to form protein? In order to study primary changes during protein withdrawal from the diet, we have limited our investigations to rats suffering deprivation of protein for only a few days (7). Loss of RNA from the liver cell takes place rapidly, much of it occurring within the first 24 hours on the protein-free diet (Fig. 1); the same rapid loss can be achieved simply by starving the animal for 24 hours. After about two days on the protein-free diet, the RNA

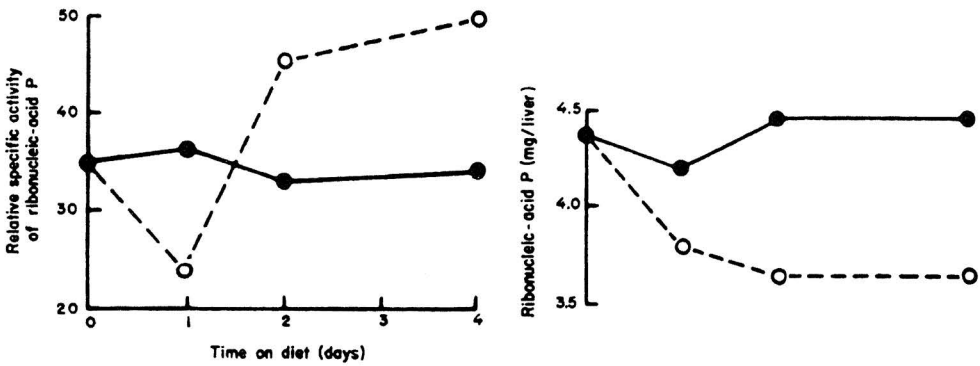

Fig. 1. Influence of protein intake on the amount of RNA in the liver (two curves on the right) and on its uptake of ^{32}P (two curves on the left). The rats were given either a normal diet (solid lines) or a protein free diet (broken lines) and received ^{32}P 24 h before killing. Uptake of ^{32}P is expressed relative to the activity in the free inorganic phosphate pool. (From Munro *et al.* *[7]*)

content of the liver attains a new plateau about 25 percent below that of a well-nourished rat. On refeeding the animal with protein, the RNA content of the liver cell quickly rises to a higher level again. We thus have three phases during which liver RNA metabolism can be explored: (a) the fully depleted state after a few days on the protein-free diet; (b) the initial period of rapid loss of RNA from the liver; (c) the response to refeeding with protein. At first sight, it might seem obvious that protein deficiency causes loss of liver RNA by reducing its rate of synthesis. However, when ^{32}P is injected into depleted rats, uptake into liver RNA is considerably increased above the level observed in control animals fed adequate amounts of protein (Fig. 1). It would thus appear that the diminished amount of RNA in the liver of the protein-depleted animal develops a compensatory increase in its rate of replacement, so that the absolute rate of RNA synthesis remains independent of dietary protein intake.

Since this approach provides no evidence that rate of RNA formation is controlled by amino acid intake, it is logical to look for a change in the rate of RNA degradation. A clue to this was provided by the experiment illustrated in Figure 1. The diagram shows that the compensatory increase in RNA turnover does

not take place immediately on withdrawal of protein from the diet. On the contrary, there is a considerable depression in ^{32}P incorporation into liver RNA on the first day of protein withdrawal, coinciding exactly with the time when the RNA content of the liver is falling precipitously. This picture was more dramatically illustrated when we used ^{14}C-glycine as a precursor of the purine bases of RNA (8). Uptake of this precursor into RNA from the free glycine pool of the liver was greatly reduced by fasting for 18 hours, and rose sharply again within three hours of feeding a meal of protein. These observations with ^{32}P and with ^{14}C-glycine suggested to us that during the period of rapid reduction in liver RNA content following withdrawal of protein from the diet, RNA breakdown products enter the pool of nucleotides from which RNA is synthesized and dilute the label of recently synthesized nucleotides (Fig. 2). Using ^{14}C-glycine, we were able to confirm that the extensive changes we had observed in the liver RNA in its response to fasting and to refeeding with protein were reflections of changes occurring in the labelling of the precursor pools of nucleotides. We came to the conclusion that the stability of at least a part of the RNA of the liver cell depends on the supply of amino acids.

Subsequent experiments have shown that liver RNA metabolism is sensitive to the nutritional completeness of an amino acid mixture fed by stomach tube to rats (9). When we omitted tryptophan from the mixture, the rate of labelling of RNA purine bases by ^{14}C-glycine and of pyrimidine bases with ^{14}C-orotic acid was significantly reduced. This was once more found to be a reflection of changes occurring in the precursor nucleotide pool. We can conclude that the mechanism regulating RNA stability is sensitive to the quality of the amino acid mixture absorbed from the intestine. In the experiments subsequently described in this discourse, we have used this amino acid mixture, either complete or with tryptophan omitted, to study the relationship between amino acid supply and RNA metabolism. Since changes in RNA labelling due to each mixture could be detected within 90 minutes after feeding, we have confined our studies to short periods (one or a few hours) after giving the amino acid mixture by stomach tube. Using rats fed either the complete or the tryptophan-deficient amino acid mixture, we have attempted to explore the factors causing a difference in RNA metabolism.

Ribonucleic acid occurs both in the nucleus and the cytoplasm, but the loss of a quarter of the cell RNA during protein depletion must represent mainly ribosomal RNA, since this makes up more than 80 percent of the total RNA of the cell. Accordingly, we explored initially the cytoplasmic locations of RNA (10). The traditional fractionation of the cytoplasm after removal of mitochondria is achieved by spinning the homogenate at 100,000 times gravity for one hour to obtain a microsomal pellet and a clear cell sap supernatant. Both of these fractions are complex. The microsome fraction contains the polysomes (ribosomes attached to messenger RNA strands). Some of these messenger RNA-ribosome complexes are quite small and we will refer to them as oligosomes. In addition, the microsome fraction can trap free ribosomes. The cell sap preparation has been shown by us to contain appreciable amounts of ribosomal RNA, apparently in the form of separate

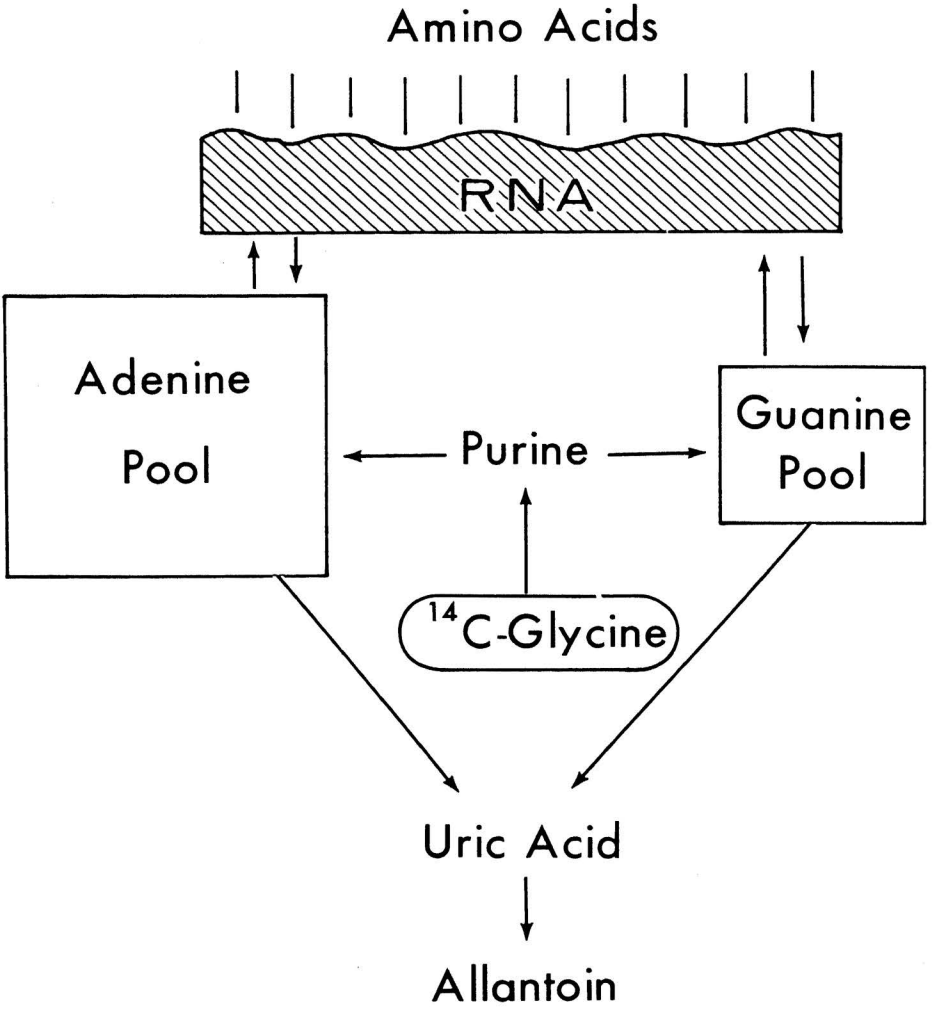

Fig. 2. Diagram to show relationship of RNA and its purine precursors to amino acid supply. (Reproduced from Clark *et al. [8]*)

subunits formed by ribosome dissociation. It also contains transport RNA (sRNA) and the enzymes for activating amino acids and transferring them to the polysomes, The quantities of RNA in all of these locations in the cell cytoplasm were examined for animals receiving the complete amino acid mixture or the tryptophan-deficient mixture. Before describing the findings, a more detailed survey of these methods of separating cytoplasmic fractions is called for. The scheme is summarized in Figure 3.

Most of the polysomes of the liver cell appear to occur attached to the lipoprotein membranes of the endoplasmic reticulum. Consequently, the post-

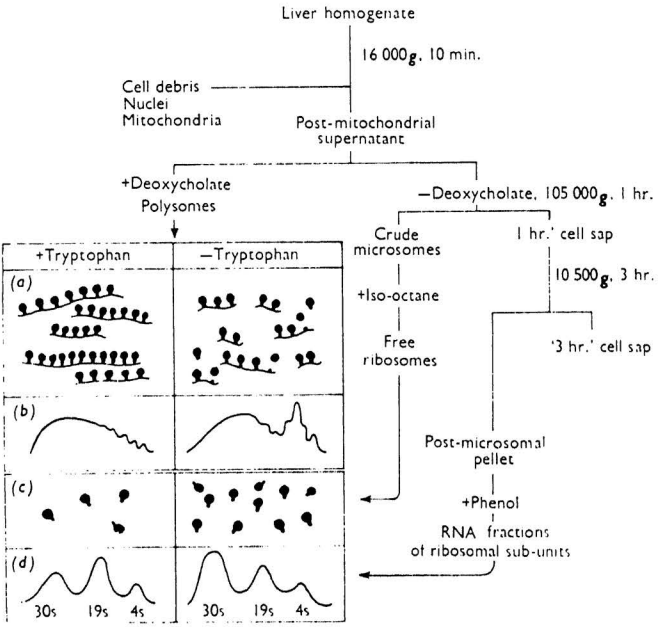

Fig. 3. Summary of changes comparing responses in fractions to giving the tryptophan-free amino acid mixture and the complete amino acid mixture to starved rats. The flow-diagram describes the procedure for preparing particulate fractions, whereas the insert illustrates: (*a*) the increased amount of small ribosomal aggregates in polysomes of tryptophan-deficient animals, (*b*) the corresponding polysome patterns which support the illustrations in (*a*) by showing the increase in oligosomes relative to the total polysome population under the tryptophan-deficient conditions, (*c*) the increased amount of free ribosomes after feeding with the amino acid mixture lacking tryptophan and (*d*) the difference in ribosomal sub-unit RNA patterns on sucrose gradients showing an increased proportion of 30s RNA in the post-microsomal pellet of tryptophan-deficient rats. (from Wunner *et. al. [10]*)

mitochondrial supernatant is first treated with deoxycholate to remove these membranes, and then the polysomes are concentrated by centrifuging them into dense sucrose. When the polysome pellet is subsequently spun through a gradient of increasing density of sucrose, the different sizes of ribosome aggregate attached to messenger RNA remained near the top of the gradient (monosomes), and the heavier aggregates passed further down the tube (Fig. 4). The process of concentrating the polysomes through heavy sucrose results in some loss of the smaller aggregates (monosomes, disomes, etc.). We, therefore, took the supernatant remaining after removal of the polysomes and harvested centrifugally from it these

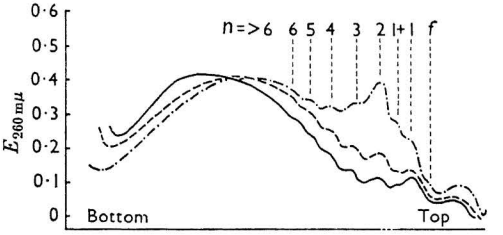

Fig. 4. Polysome profiles from rat liver obtained after feeding rats with a complete amino acid mixture (——) or one deficient in tryptophan (–·–·–) or simply fasted (– – –). (From Wunner *et al. [10]*)

smaller aggregates which we have collectively called "oligosomes." When the oligosome fraction was spun through a sucrose gradient (Fig. 5), it revealed three or four major peaks of UV absorption, representing ferritin, monosomes, disomes, and trisomes. The identity of the peaks was established by various procedures. For example, incubation of the oligosomes with a trace of ribonuclease broke down the messenger RNA between the aggregate and they become ribosomes, thus identifying the site of the monosome peak. The next fraction to be isolated were free ribosomes independent of membrane attachment (11). This we obtained by a procedure in which the microsome-polysome complex was first removed centrifugally. Then the mixture of free ribosomes and smooth endoplasmic reticulum membranes remaining in the supernatant fluid was treated with iso-octane and centrifuged; this resulted in flotation of the membranes to the interface between the isooctane and water, whereas the ribosomes came down as a pellet. Electron microscopy and study on sucrose gradients confirmed that this material was consistent with free ribosomes or monosomes.

Finally, the cell sap prepared in the traditional manner was fractionated by further centrifugation to provide a post-microsomal pellet and final cell sap (12). When the post-microsomal pellet was run on a sucrose gradient, we were able to identify peak of UV absorption corresponding to 55S and 32S. These were consistent with the two subunits making up the ribosomal particle. This was confirmed by extracting the RNA from the post-microsomal fraction and examining its components. When the RNA was separated on a sucrose gradient, it showed the 28S and 18S RNA species characteristic of the two ribosome subunits (Fig. 6). However, the proportions of the 28S to 18S differed from that of whole ribosomes and, moreover, varied from preparation to preparation depending on the diet fed. This suggested that the ribosomal subunits were present in dissociated form in this cell fraction. These studies on the RNA of the post-microsomal fraction were confirmed using an agarose gel electrophoresis technique which we have recently devised for fine discrimination between different species of RNA (13). Fig. 7 shows that intact ribosomes show a larger 28S band than an 18S band of RNA on the gel,

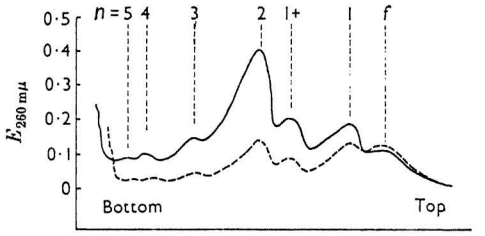

Fig. 5. Oligosome pattern obtained from livers of rats after feeding a complete amino acid mixture (----) or one devoid of tryptophan (——). (From Wunner *et al. [10]*)

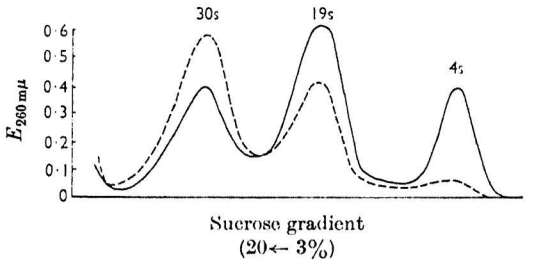

Fig. 6. Comparison of distribution of 30S, 19S, and 4S RNA in ribosomes and in the post-microsomal fraction. (From Wunner *et al. [10]*)

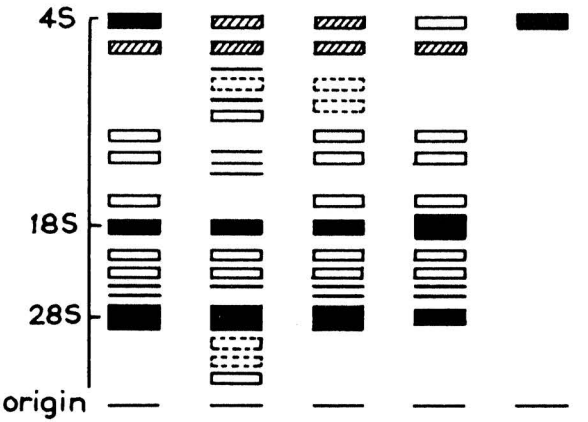

Fig. 7. Electrophoretic separation of RNA species on an agarose gel. From left to right, the RNA was extracted from (1) whole liver cells. (2) liver nuclei, (3) liver polysomes, (4) liver post-microsomal fraction, and (5)liver cell sap. (From McIndoe and Munro [13])

whereas the post-microsomal fraction has the relative concentrations of these RNA species reversed. This fraction appears to accumulate in amount when the liver cell is losing RNA. Table 3 shows the relative amounts of RNA in the post-microsomal fraction and in the final cell sap obtained from rats receiving a protein-free diet, or fasting for 18 hours, or after a meal of protein. The fasted rats show a much larger amount of RNA in the post-microsomal fraction, which coincides with the period of rapid loss of RNA from the cell (Fig. 1). In contrast, similar animals absorbing amino acids after a meal of protein have a much smaller amount of RNA in the post-microsomal fraction, at a time when other fractions of the cells are gaining RNA.

TABLE 3--EFFECT OF PROTEIN INTAKE ON THE AMOUNT OF RNA IN DIFFERENT FRACTIONS OF LIVER CELL-SAP AFTER CENTRIFUGING FOR A FURTHER 3 HOURS AT 100,000 g

(Reproduced from data of Munro, McLean, and Hird, 1964)

Dietary Group	Amount of Liver RNA/100 g. Body Weight		
	In Supernatant	In Pellet	Pellet RNA / Supernatant RNA
Protein-depleted	3.19	0.88	28%
Adequate protein diet (fasting 18 hr)	3.17	1.31	41%
Adequate protein diet (fed protein 2 hr)	3.65	1.23	33%

This subcellular fractionation scheme was applied to the livers of rats that had been fed by stomach tube two hours before killing with the complete or tryptophan-deficient amino acid mixture (10). Figure 4 shows the polysome profiles for each group of rats. The absence of tryptophan from the mixture caused a reduction in heavy aggregates and an increase in the lighter ones, particularly the disome and other smaller particles. The area of the polysome profile was measured by planimetry and the contribution of each peak calculated. Removal of tryptophan from the mixture resulted in a redistribution of about 15 percent of the UV absorbing material from the heavier to the lighter end of the polysome profile. The large change in the disome peak provided a useful sensitive indicator of amino acid deficiency, and an experiment was set up in which rats were killed at various times after being fed the amino acid mixtures and the area occupied by the disome peak was plotted (Fig. 8). Even within one hour of feeding the two amino acid mixtures, there was a difference in the proportions of disomes recovered. This was much larger

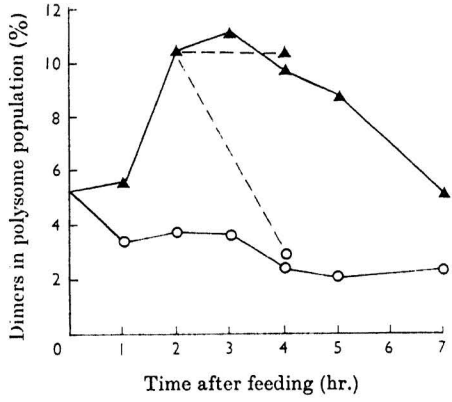

Fig. 8. Percentage of dimers among rat-liver polysomes after feeding a tryptophan deficient amino mixture (Δ—Δ) or a complete amino acid mixture (0—0). At two hours after feeding, some animals were re-fed the deficient mixture or the complete mixture (- - -). (From Wunner *et al*, *[10]*)

at two hours and persisted for several hours. Some of these rats receiving the tryptophan-deficient mixture were aspirated and refed the complete amino acid mixture at two hours after the start of the experiment. Figure 8 shows that this caused an immediate reduction in the proportion of disomes present. This demonstrates the considerable sensitivity of the mechanisms to amino acid supply.

We also harvested the oligosome material not included in the polysome profile. As shown in Figure 5, animals killed two hours after receiving the tryptophan-deficient mixture showed a greatly increased disome peak with some increase in the other peaks as well. Indeed, the total oligosome fraction increased to three times the amount found in the group receiving the complete amino acid mixture. When we turn to the free ribosome fraction prepared by the iso-octane procedure, we find a similar phenomenon. Table 4 shows that omission of tryptophan from the amino acid mixture resulted in an increase in the RNA of the cell fraction by some 50 percent. Finally, tryptophan deficiency also increased the amount of RNA recovered from the post-microsomal fraction but not from the cell sap proper (Table 4). It will be remembered that the post-microsomal fraction consists of free ribosome subunits. It is noteworthy that the larger subunit increased more than the smaller subunit, as shown by a change in the profile of RNA extracted from the post-microsomal fraction. It will not have escaped attention that the RNA of the cell sap, mainly the transport RNA (sRNA), was not affected by manipulation of the composition of the amino acid mixture. This is contrary to the views of some investigators (14) who have observed changes in amino acid activation and

TABLE 4--EFFECT OF FEEDING WITH COMPLETE AND TRYPTOPHAN-FREE AMINO ACID MIXTURES ON THE RNA CONTENT OF LIVER FREE RIBOSOMES, THE POST-MICROSOMAL FRACTION AND THE RESIDUAL CELL SAP FRACTION

(Reproduced from data of Wunner, Bell, and Munro, 1966)

Fraction Containing RNA	RNA (mg/g. liver)		
	Complete Amino Acids Fed	Tryptophan-free Amino Acids Fed	Change in RNA Content
Free Ribosome Fraction	371	852	+48%
Post-microsomal Fraction	166	223	+34%
Residual Cell Sap	681	709	+ 4%

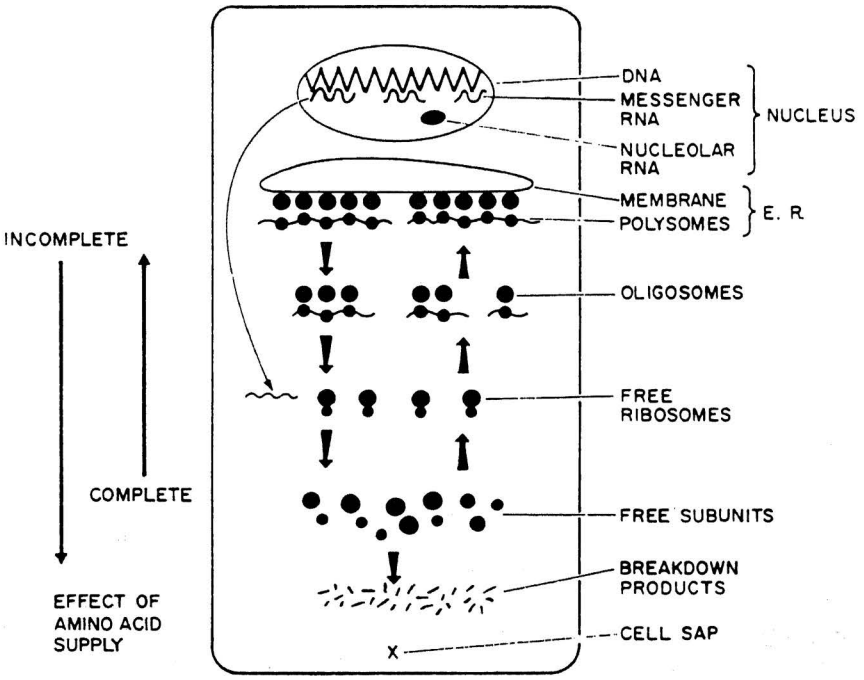

Fig. 9. Subcellular structures of liver to show action of amino acid supply.

attachment to sRNA by the cell sap prepared from the livers of rats subjected to protein depletion. Neither our present studies nor previous ones dealing with protein-depleted animals support this view, however.

We are now in a position to review the overall picture provided by these studies on rats fed amino acid mixtures. As shown in Figure 9, the deficient mixture results in a loss of heavier polysomes, with accumulation of smaller aggregates (oligosomes) and free ribosomes and also of ribosome subunits (post-microsomal fractions). On the other hand, the complete mixture causes the opposite effects. It is tempting to conclude that these various forms of ribosomes are in equilibrium and that general lack of amino acids produced by feeding a protein-free diet, or specific lack of one essential amino acid such as tryptophan, can shift the equilibrium in favor of the smaller units and away from the large polysome aggregates. In fact we have evidence (15) that the free ribosomes and the microsome-attached polysomes are in a state of continuous exchange. Rats were injected with ^{32}P and the RNA was isolated from the ribosomes of the microsomal part of the liver cell and also from the free ribosomes. As shown in Figure 10, the free ribosomes acquired the

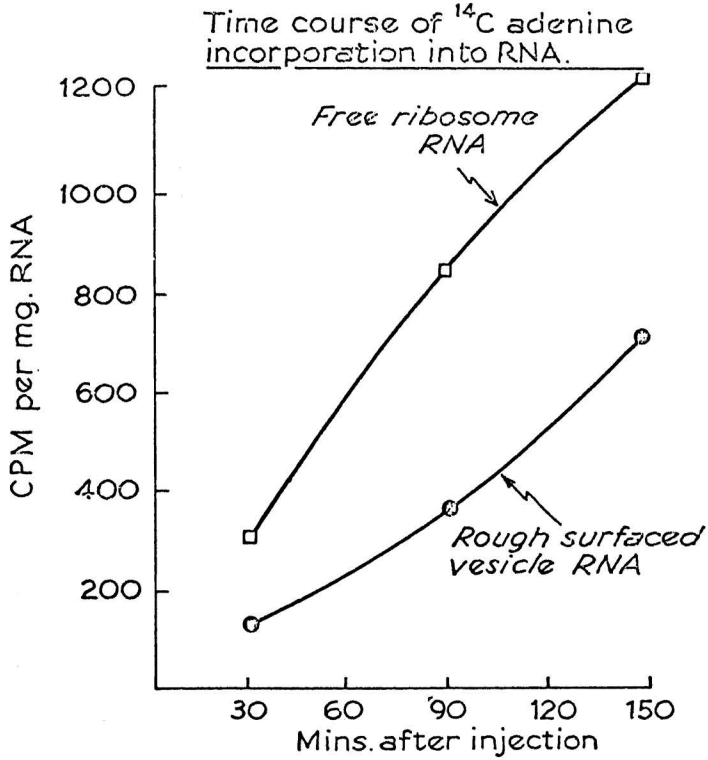

Fig. 10. Uptake of ^{14}C-adenine into free ribosome fraction and membrane-attached ribosome fraction of rat liver. (From Hallinan and Munro [15])

label first, followed by the attached ribosomes. Work published by Moule and her colleagues (16) suggests that the labelling pattern of the two species of ribosome eventually coincide—that there is a continuous exchange from the free to the attached form of ribosome. To complete our picture, we have one final feature to demonstrate. Lack of protein in the diet or deletion of an essential amino acid eventually causes a net loss of RNA from the liver. In some way, therefore, the disintegration of the polysomes and the accumulation of free ribosomes must lead to an accelerated rate of RNA breakdown. This has yet to be demonstrated, but it is to be expected that the RNA of the free subunits (post-microsomal fraction) would be susceptible to attack, since it is known that separation of ribosomes into their subunits activates the latent ribonuclease contained in the intact particles.

Having described the effects of amino acid deficiency on the protein synthesizing machinery of the liver cell, we must now consider the consequences in terms of the amount of protein manufactured. For this purpose, we prepared microsomes from the livers of rats either fed a complete amino acid mixture or the mixture deficient in tryptophan one hour before killing (17). These microsomes were then incubated with cell sap from a common source, an energy generator, and ^{14}C-leucine. Table 5 shows that the origin of the microsomes determined their activity in this incorporation system. The amount of ^{14}C-leucine bound in the form of protein was about 30 percent less in the case of microsomes prepared from animals fed the tryptophan-deficient mixture, as compared with incorporation into microsomes obtained from rats given the complete mixture. This is comparable with what is known about the relative capacities of different sizes of polysome aggregates to incorporate amino acids into proteins. As shown by Wettstein and his colleagues (18), polysomes with less than five ribosomes on the messenger strand have a reduced capacity for amino acid incorporation. Thus, accumulation of oligosomes in the livers of the tryptophan-deficient animals would lead to less efficient protein synthesis. Consequently, the distribution of ribosomes between polysome aggregates and oligosome forms would appear to provide a means of controlling rate of protein synthesis in relation to amino acid supply.

We must now try to identify the mechanism by which this liver cell response to amino acid supply is achieved. The factors involved are shown in Fig. 9. Both messenger RNA and ribosomes are synthesized in the cell nucleus and are secreted into the cytoplasm. The synthesis of RNA necessary for this process can be inhibited with actinomycin D at appropriate dose levels, and consequent release of new RNA into the cytoplasm can be prevented. It is possible with appropriate dosage of actinomycin D to suppress the messenger needed for production of adaptive liver enzymes such as tryptophan pyrrolase. We have used doses of actinomycin known to have this effect on messenger synthesis, and then have fed rats on the complete or tryptophan-deficient amino acid mixtures. Table 5 shows that microsomes prepared from the livers of these animals and incubated with cell sap and ^{14}C-leucine are less able to incorporate the labelled amino acid into protein than are microsomes made from the livers of rats not treated with the drug.

TABLE 5--IN VITRO UPTAKE OF ^{14}C-LEUCINE BY MICROSOMES FROM RATS FED ONE HOUR BEFORE DEATH WITH EITHER A COMPLETE OR TRYPTOPHAN-DEFICIENT AMINO ACID MIXTURE. SOME ANIMALS WERE PRETREATED WITH ACTINOMYCIN D,

(Reproduced from Fleck et al., 1965)

		Counts/min/mg. Microsome Protein		
Actino-mycin	Incubation time (min.)	Incomplete Amino Acids	Complete Amino Acids	Difference
None	10	3690	4630	+940
	40	4600	5580	+980
Pre-treated	10	2020	2540	+520
	40	2520	3550	+1030

However, even after actinomycin administration, the type of amino acid mixture fed still affects the capacity of the microsomes to incorporate amino acids. This picture is complemented by the liver polysome profiles of the same animals. Fig. 11 shows that actinomycin administration reduced the heavy aggregates and increased the light ones, accounting for the reduced incorporating activity of the microsomes from the same livers. However, the animals fed the two amino acid mixtures still demonstrated differences in polysome profile in spite of having received actinomycin before being fed. This implies that formation of new RNA by the nucleus is not a necessary part of the mechanism involved in the response to amino

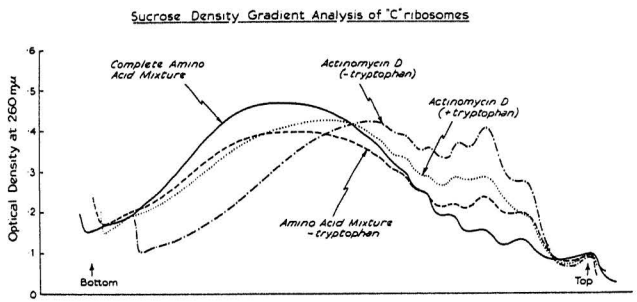

Fig. 11. Polysome profiles from the livers of rats fed amino acid mixtures either lacking tryptophan or nutritionally complete. Some animals also received actinomycin D before feeding. (from Fleck *et al.* [17])

acid supply. It can be concluded that we are dealing with a cytoplasmic phenomenon, possibly related to the mechanism of ribosome assembly on the messenger strand.

Having reached this conclusion, we thought it profitable to see whether we could replicate the events occurring in the liver cell (19). We prepared polysomes from rat liver and added to them an energy source, cell sap, that had been dialyzed to remove free amino acids, sRNA stripped of amino acids, and ^{14}C-labelled amino acids. This system incorporated the labelled amino acid into protein for 20 minutes and then ceased (Fig. 12). When a mixture of amino acids lacking tryptophan was added to the system, uptake was slightly better but was much improved by addition

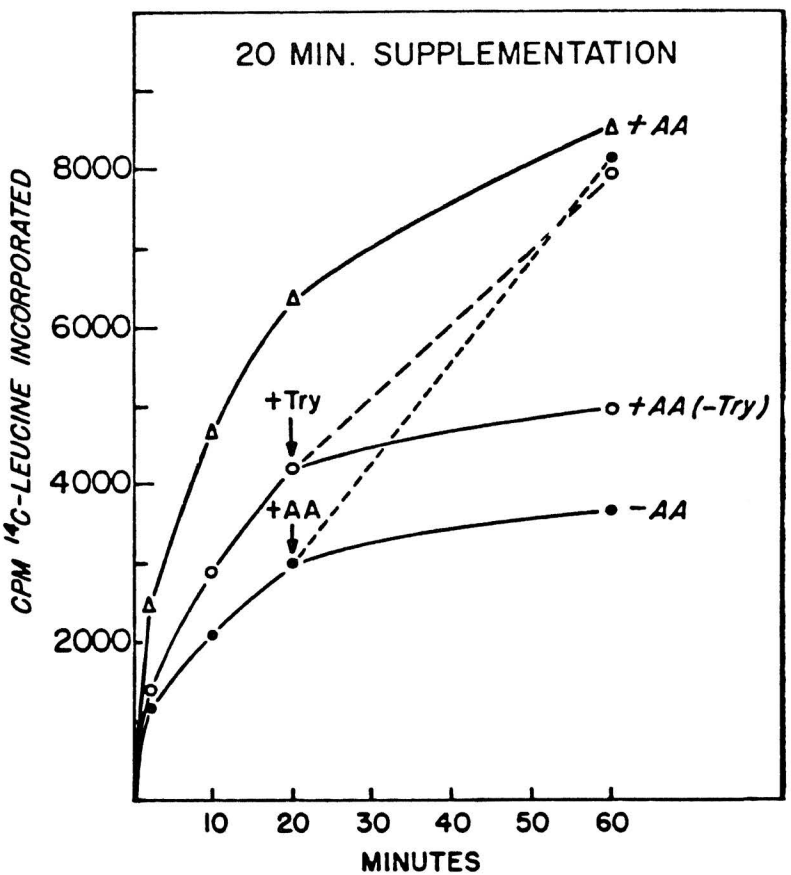

Fig. 12. Uptake of ^{14}C-leucine into protein by a cell-free system consisting of liver polysomes, amino acid activating and transfering enzymes, sRNA, and an energy source. The system was depleted of free amino acids which were then restored either initially or after 20 minutes of incubation. (Unpublished data of Baliga, Pronczuk and Munro.)

of a complete amino acid mixture to the incubation medium. Furthermore, incorporation continued to be brisk for a longer period of incubation. If we took the amino acid deficient system after it had attained a plateau some 20 minutes after the start of incubation and added to the medium the missing amino acids, incorporation became rejuvenated again and finally reached the same level as that observed with the incubation mixture containing all amino acids from the start of the experiment. This implies that the messenger RNA and ribosomes must be reasonably well preserved in spite of the cessation of protein synthesis caused by lack of free amino acids. One might conclude that the addition of amino acids at this point had resulted in re-assembly of polysomes and, as a consequence, renewal of protein synthesis. This, in fact, was found to be the case. A polysome profile made after 20 minutes of incubation in the amino acid deficient medium showed mainly monosomes and other oligosomes to be present. On adding amino acids to the medium for a further two minute period, the monosome and disome peaks were

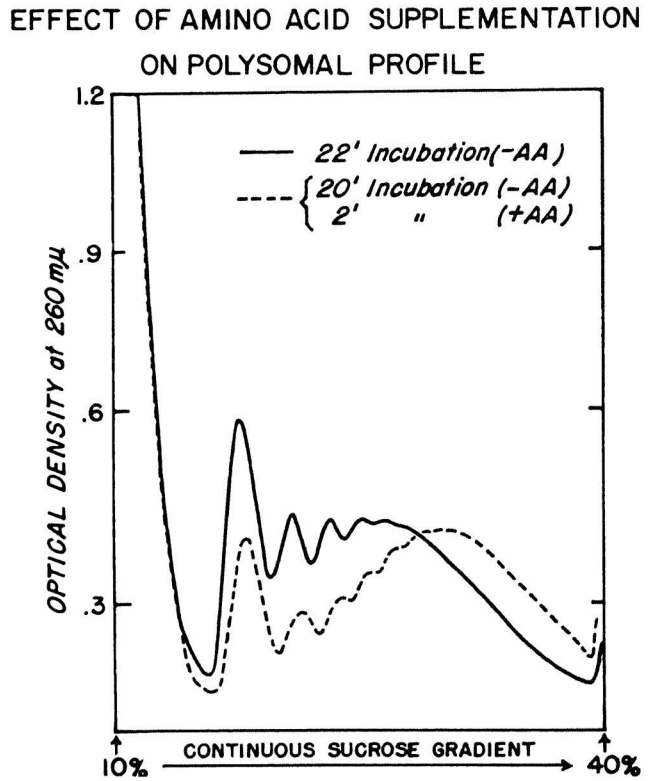

Fig. 13. Reconstruction of polysomes in a cell-free system incubated for 20 minutes without amino acids and then with amino acids added for two minutes before harvesting the polysomes on a gradient. (Unpublished data of Baliga, Pronczuk, and Munro.)

appreciably reduced and the heavy polysome part of the gradient showed a distinct increase in amount (Fig. 13). This indicates that the amino acids had effected a reconstitution of the polysomes.

Much remains to be done to establish whether these findings have any significance in the general mechanism by which tissues respond to changes in dietary protein intake. It has to be admitted that our experiments have concentrated almost exclusively on the effects of deleting tryptophan from an otherwise nutritionally complete amino acid mixture. We have since examined the effects of deleting other essential amino acids. The liver polysome profile did not seem to be adversely affected by removal of any amino acid other than tryptophan. However, it must be noted that each amino acid is required by the cell-free system for reconstitution of polysomes. There is, therefore, a difference between the response of the whole animal and that of the reconstructed cell-free system.

It must also be admitted that the cytoplasmic response to amino acid supply described above is probably not the only result produced by amino acids in the liver cell. Although there does not appear to be any indication of intervention by the nucleus in the polysome changes described above, we have good evidence that liver cell nuclei do undergo changes in response to variations in amino acid supply. A series of histochemical studies by Lagerstedt (20) and our own chemical studies of liver nuclei (21) demonstrate that the nucleolus and its contained RNA are influenced shortly after feeding a meal containing protein or amino acids. The significance of this change remains obscure.

Finally, we have already noted that the responses of various tissues to dietary protein depletion seems to be varied and we need not expect our observations with the liver to be universally applicable. Nevertheless, it is important to try to explore further these intimate mechanisms of control. Through such studies it will someday be possible to explain why the rate of protein synthesis of large mammals is retarded in comparison with small mammals, and to amplify and extend the evidence of mammalian control mechanisms so brillantly displayed by Samuel Brody in his "Bioenergetics and Growth."

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