Effect of Amino Acid Levels on a Cell-Free System for Protein Synthesis[1] (35014)

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Polyribosomes from the liver of fasted rats which were fed an amino acid mixture deficient in tryptophan show a decrease in the proportion of heavy to light populations and a reduced rate of *in vitro* protein synthesis when compared to hepatic polyribosomes extracted from animals fed a complete mixture of the 20 intraprotein amino acids (1–3). The addition of free amino acids to a cell-free system, which had been incubated without amino acids, markedly increased the proportion of heavy to light polyribosomes and concomitantly stimulated the rate at which the rat liver polysomes incorporated amino acids into protein (4, 5). These changes in both polysome populations and *in vitro* protein synthesis also required the presence of a complete amino acid mixture (4, 5). While the dependence of such cell-free systems on exogenous amino acids was originally suggested by Wettstein *et al.* (6), most investigators have used an equimolar mixture of amino acids in the concentration range of 1 to 100 μmoles/incubation tube for liver (2, 4), brain (7, 8), and skeletal muscle (9, 10) ribosomes. In the studies by Stenzel *et al.* (11) on brain ribosomes and in liver, brain, and skeletal muscle experiments performed in our laboratory (12, 13), an amino acid pattern observed *in vivo* in liver was used.

The present study was undertaken to study the effects of amino acid pool size on the rate of protein synthesis in a cell-free system from rat liver.

Materials and Methods. Livers of decapitated 50–70-day-old male rats of the Wistar strain were perfused *in situ* with ice-cold 0.9% saline until all visible traces of blood had been removed from the tissue; the livers were excised from the animals, chilled in ice-cold 0.9% saline and homogenized in 2 vol of 0.25 *M* sucrose in TKM (0.05 *M* Tris–HCl, pH 7.6, at 25°; 0.065 *M* KCl; and 0.01 *M* $MgCl_2$) (13).

This homogenate was utilized for the preparation of total ribosomes (14) by previously described procedures (13) and cell sap by centrifugation of the postmitochondrial supernatant fluid for 4 hr at 105,000*g* av. Sephadex treatment of the cell sap (14) was performed on a 4 × 24-cm column of Sephadex G-25 (medium),[4] equilibrated with 0.25 *M* sucrose in TKM. Batches of 30–40 ml of cell sap were treated at a time. The protein content of the cell sap fluid was determined by the biuret procedure (15), and aliquots of cell sap were stored at −20° for up to 2 months before use without any change in activity. RNA levels of the ribosomes were measured by modifications (12) of alkaline hydrolysis techniques (16).

The free amino acid level of the cell sap was determined by treating 1 ml of that cellular extract with an equal volume of 9% sulfosalicylic acid; after centrifugation the supernatant fluid was analyzed for amino acid content by ion-exchange chromatography (17). An aliquot of the original homogenate was analyzed for total rRNA by the

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TABLE I. Free Amino Acid Pool Pattern from the Cell Sap of Normal Adult Rat Liver.

Amino acid	mμmoles/μg of rRNA	Amino acid	mμmoles/μg of rRNA
Asp	0.237	Cys	0.010
Thr	0.149	Met	0.016
Ser	0.385	Leu	0.028
Asn	0.009	Ile	0.020
Pro	0.009	Tyr	0.017
Glu	0.492	Phe	0.018
Gln	0.140	Lys	0.072
Gly	0.370	His	0.052
Ala	0.387	Arg	0.003
Val	0.029	Try	0.004

procedure of Blobel and Potter (18). The results were then expressed as millimicromoles of each amino acid per microgram of rRNA.

Results and Discussion. Cell sap which is not treated with Sephadex contains a free amino acid pattern (Table I) which is very similar to that observed in whole homogenate (12). In order to study the effects of various concentrations of the amino acid pattern observed *in vivo* on a cell-free system for the synthesis of protein, it was necessary to remove all traces of endogenous amino acids from the medium. The passage of the cell sap through a Sephadex G-25 column did reduce the concentration of the individual amino acids to less than 2 mμmoles/ml as determined by analysis on an ion-exchange column. If the Sephadex treatment had removed all of the endogenous leucine, then the amount of radioactive amino acid incorporated should have been proportional to the specific activity of the added radioactive amino acid and independent of the amount of radioactivity added, assuming of course, that other cell sap factors were present in excess. The data in Table II, however, indicate that the amount of labeled amino acid incorporated into newly synthesized protein did vary with the amount of radioactivity added. In an attempt to explain this fact, it was concluded that some utilizable (*i.e.*, free and/or bound to tRNA) leucine was present in the Sephadex-treated cell sap, but in amounts not detectable by current ion-exchange chromatographic procedures.

TABLE II. Effect of Added ^{14}C-Leucine.[a]

^{14}C-leucine added (μCi/tube)	Incorporated (dpm/μg of rRNA)
Sephadex-treated cell sap	
0.1	33.7
0.2	39.2
0.5	44.5
1.0	60.8
Sephadex-treated, dialyzed cell sap	
0.2	57.8
1.0	56.0

[a] The cell-free system for protein synthesis contained 0.15 mmoles of sucrose, 50 μmoles of Tris–HCl buffer (pH 7.6 at 25°), 65 μmoles of KCl, 10 μmoles of $MgCl_2$, 5 μmoles of ATP, 0.1 μmole of GTP, 40 μg of ribosomal RNA, 800 μg of cell sap protein, 19 (minus leucine) L-amino acid-^{12}C in the proportions listed in Table I and variable amount of L-leucine-U-^{14}C (New England Nuclear Corp., 4 μmoles/mCi). Incubation conditions and techniques for extraction of nascent protein were previously described (12).

By isotope dilution techniques, it was possible to calculate that Sephadex-treated cell sap added an average of 0.306 mμmoles of leucine/incubation tube, while before Sephadex treatment a similar volume of cell sap contained 1.12 mμmoles of leucine. This decrease to ⅓ the original leucine content resulted in a 3-fold increase in the amount of radioactivity that was incorporated into protein when 0.1 μCi of leucine-^{14}C was added to the cell-free system (unpublished results). If the Sephadex-treated cell sap was dialysed for 24 hr in the presence of 0.005 *M* mercaptoethanol, 0.065 *M* KCl, 0.01 *M* $MgCl_2$, and 0.05 *M* Tris–HCl, pH 7.6 at 25°, all traces of endogenous leucine, as measured by isotope dilution analysis, were removed and the amount of leucine incorporated into nascent protein was similar at both concentrations of added labeled ^{14}C-leucine. In another tube, which contained the Sephadex-treated, dialyzed cell sap, ^{12}C-L-leucine was added so that the total leucine content in an incubation mixture with 0.2 μCi of added ^{14}C-leucine was similar to that when 1.0 μCi was added. Under these conditions the amount incorporated into protein (dpm/μg of ribo-

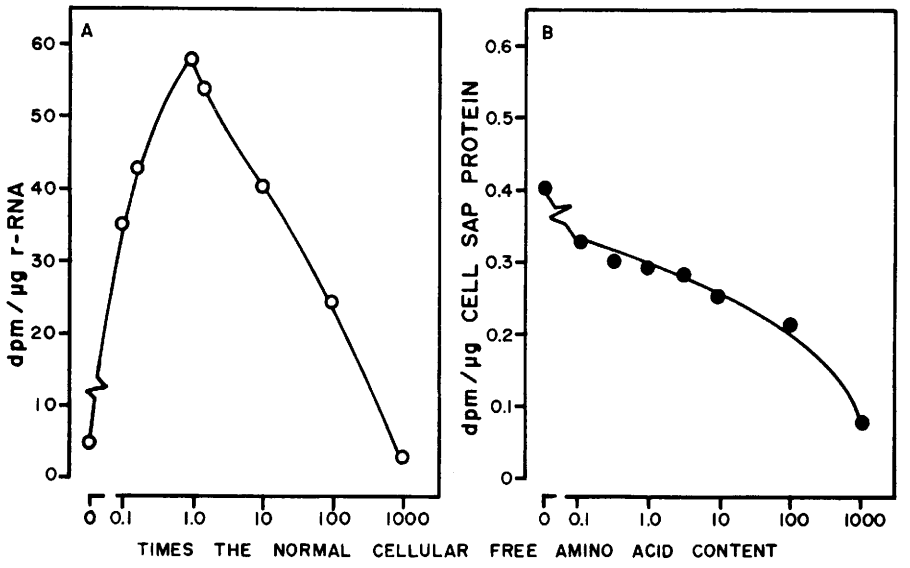

FIG. 1a. Effect of amino acid concentrations on the rate of *in vitro* ribosomal RNA (rRNA)-directed protein synthesis. Each tube contained in 1.0 ml of reaction mixture: 0.15 mmole of sucrose, 50 μmoles of Tris-HCl, pH 7.6 at 25°, 65 μmoles of K^+, 10 μmoles of Mg^{2+}, 5 μmoles of ATP, 0.1 μmole of GTP, 40 μg of rRNA, 800 μg of Sephadex-treated, dialyzed cell sap protein, 0.8 mμmoles of U-^{14}C-L-leucine (0.2 μCi), and either 1000, 100, 10, 2, 1, 0.2, 0.1, or 0.00 times the normal concentrations of 19 (minus leucine) ^{12}C-amino acids/40 μg of rRNA, based on the proportions listed in Table I. Incubation conditions and techniques for extraction of nascent protein were previously described (12). (b) Effect of amino acid concentrations on the rate of *in vitro* nonribosomal-directed protein synthesis. Except for the replacement of the ribosomes by buffer (0.25 *M* sucrose in TKM), the incubation tubes and conditions were the same as in (a).

somal-RNA) was reduced to 14.0 as compared to 57.8 when no carrier leucine was added but the leucine incorporated into nascent protein (mμmoles/μg of ribosomal-RNA), as determined by isotope dilution calculation, was 107 when the incubation medium contained added carrier ^{12}C-leucine as compared to 104 and 101, respectively, when 0.2 or 1.0 μCi of ^{14}C-leucine was present.

When the amino acid mixture (Table I) minus the leucine was added to the cell-free system, which contained the Sephadex-treated dialyzed cell sap, the rate of leucine incorporation into nascent protein was markedly increased when compared to reaction tubes without amino acids (Fig. 1a), with maximal rates of incorporation being achieved when the amino acid levels were those normally present in liver cell sap, *viz,* at 1 times the normal concentration. Since the data confirm previous reports (4, 6) that the cell-free system is almost completely inhibited in the absence of a supply of free amino acids, the amounts of unlabeled amino acids on tRNA of the Sephadex-treated, dialyzed cell sap must have been very small and was unable to support protein synthesis. Therefore, the rate of incorporation does reflect the level of the free amino acid pool. At an amino acid level which was equivalent to 10 times that found in normal liver cells, the rate of leucine incorporation was about only ⅔ of that observed with optimal concentrations, and when the amino acid mixture was increased to 1000 times the normal levels, the synthesis of protein was almost completely inhibited. Munro *et al.* (14) also observed that excess amino acids inhibited the *in vitro* system, but they believed that their results could be explained by leucine contamination of their amino acid mixture. Since ion-exchange chromatography and isotope dilution techniques did not indicate any leucine contamination in our amino acid mix-

ture, the inhibition which was noticed (Fig. 1a) at greater than normal amounts of amino acids must be related to an excess of at least some amino acid(s) in the mixture. At higher amino acid concentration the imbalance in leucine could be a limiting factor which regulated the rate of protein synthesis but in other experiments when leucine was varied from ⅓ to 4 times normal level no changes were noted in the amount of leucine incorporated into nascent protein. These observations may help in understanding why the animal cell is unable to store large amounts of free amino acid (19) and why the feeding of large excesses of certain amino acids can be toxic to the organism (20). The inhibition in leucine incorporation at less than normal concentrations of amino acids was probably due to insufficient saturation of the incorporating system with one or more amino acids.

In the absence of ribosomes, the cell-free system slowly incorporates leucine into protein, including N-terminal groups (9, 21, 22). This sytem differs from ribosomal-directed protein synthesis in that it does not require K^+ ion (8, 22). The data in Fig. 1b would indicate that an exogenous mixture of amino acids is inhibitory to the incorporation of leucine in such a "nonribosomal" incorporating system. Moreover, the presence of exogenous amino acids in the reaction tubes may be merely competing with the radioactive leucine for N-terminal sites.

Summary. Cell sap must be passed through Sephadex G-25 and then dialyzed to remove all of the amino acids. The cell-free system for protein synthesis has an optimal amino acid requirement which is approximately the same level as that observed in normal cell sap and the absence or excess of amino acids can markedly inhibit the rate of protein synthesis in the ribosomal-dependent system. In the nonribosomal system, the incorporation rate as well as the amino acid requirement are low.

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