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Preferential utilization of exogenously supplied leucine for protein synthesis in estradiol-induced and uninduced cockerel liver explants

(ribosome transit times/amino acid pools/absolute specific activity of acid-soluble leucine)

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ABSTRACT **A cockerel liver explant system has been used to study protein synthesis and ribosome transit times. After a 2-hr preincubation of explant tissue in the presence of a large concentration of nonradioactive leucine, a small quantity of [^{3}H]leucine was added and the kinetics of uptake of [^{3}H]leucine into the intracellular acid-soluble leucine pool was compared to the incorporation of [^{3}H]leucine into protein. Incorporation of [^{3}H]leucine into protein reaches a linear rate almost immediately after addition of label, whereas the acid-soluble pool does not reach constant specific activity until much later. The length of time needed to reach a linear rate of incorporation of [^{3}H]leucine into protein is approximately equal to the length of time needed to equilibrate nascent polypeptide chains with labeled precursor—that is, one average ribosome transit time. Therefore, it seems that the immediate precursor pool for protein synthesis reaches constant specific activity almost instantly after addition of [^{3}H]leucine. The results indicate that at least part of the supply of leucine for protein synthesis is derived directly from the exogenous incubation medium and not from the intracellular acid-soluble amino acid pool.**

Our analyses of protein synthesis in newt limb regenerates (1, 2) and in sea urchin embryos (3), along with data from other laboratories, have indicated that the intracellular soluble amino acid pool is not the exclusive source of amino acids for protein synthesis. We recently reported a study of protein synthesis and ribosome transit times in cockerel liver explants after an *in vivo* injection of 17β-estradiol (4, 5). During the characterization of the organ explant system, it appeared that incorporation of [^{3}H]leucine into hot (90°C) acid-precipitable protein became linear within minutes of addition of labeled precursor to the incubation medium, whereas the acid-soluble pool did not seem to reach constant specific activity until much later. Similar observations have been published by other investigators, with several different experimental systems (6–10). To further analyze the source of amino acids for protein synthesis in the cockerel liver explants, the absolute specific activity of acid-soluble leucine (dpm of [^{3}H]leucine per nmol of leucine) has been determined and these data are reported here. The intracellular acid-soluble leucine pool does not appear to be the only source of amino acids for protein synthesis in the liver explants; moreover, the amount of time required to reach a linear rate of incorporation into hot acid-precipitable protein after addition of labeled precursor is approximately equal to one average ribosome transit time. Therefore, at least part of the supply of amino acids for protein synthesis appears to be derived directly from the exogenous source in the incubation medium; these amino acids seem to be incorporated directly into protein without entering the intracellular acid-soluble pool.

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MATERIALS AND METHODS

Cockerel liver explants (*Gallus domesticus*, obtained from Squire Valleevue Farm) were prepared and incubated as described (4). Four days after injecting two cockerels with 17β-estradiol the livers were removed, pooled, and prepared as organ explants. The explants were incubated for 2 hr in incubation medium containing 450 μM L-leucine. After preincubation, 500 μCi of [^{3}H]leucine (9.1 nmol; 1 Ci = 3.7×10^{10} Bq) was added and tissue aliquots of ≈200 mg were removed at succeeding time intervals and transferred to tubes containing ice-cold phosphate-buffered saline (0.15 M NaCl/20 mM sodium phosphate, pH 7.0). After all samples were collected, the explant tissue was pelleted in a clinical centrifuge, gently resuspended, washed with ice-cold phosphate-buffered saline, and then repelleted. The washed tissue was sonicated in 2 ml of ice-cold 10% trichloroacetic acid and left to stand on ice for 10 min. The sonicate was centrifuged, and the pellet was washed twice with ice-cold 5% trichloroacetic acid. All supernatants were pooled and extracted three times with 10 vol of ethyl ether to remove the trichloroacetic acid. The acid-insoluble material was solubilized, and RNA was hydrolyzed by incubation in 4.0 ml of 0.3 M KOH at 45°C for 3.5 hr. To precipitate DNA and protein, 2.5 ml of 1.2 M $HClO_4$ was added, and the solution was left to stand 10 min on ice. The acid-insoluble material was collected by centrifugation, and the pellet was washed twice with 0.6 M $HClO_4$. DNA content was estimated by using the indole technique of Cerrioti (11). The concentration of acid-soluble amino acids was estimated by using the fluorescamine assay described by Bohlen *et al.* (12) and Udenfriend *et al.* (13). Fluorescence was measured in an Aminco–Bowman spectrofluorometer with leucine as standard. The acid-soluble leucine concentration in each timed sample was determined by analysis in a Durrum D-500 amino acid analyzer. The lyophilized acid-soluble extracts were solubilized in 20 μl of 0.2 M sodium citrate buffer (pH 2.2) containing a norleucine standard solution and were applied to the analyzer column. Peak areas were automatically integrated by a computer. The eluted amino acids were identified by comparison of their elution times with those of previously determined standards.

To ensure that the radioactivity measured had remained as [^{3}H]leucine during the explant incubation, an aliquot of the acid-soluble fraction from the 120-min time point was analyzed by two-dimensional ascending chromatography. One microgram of standard leucine and 2.0 μl of the solubilized 120-min time

point sample (11,000 cpm) were spotted onto individual washed MN 300 cellulose thin-layer chromatography plates (Brinkmann). The first dimension was developed in butanol/acetic acid/water, 3:1:1 (vol/vol), for approximately 2 hr. After the plate was completely dry, the second dimension was developed in phenol/*m*-cresol/borate buffer, 25 g of phenol:25 g of *m*-cresol:7 ml of borate solution, prepared by combining 50 ml of 0.1 M boric acid and 28.4 ml of 0.1 M NaOH. The second dimension was developed until the front was about 2 cm from the top of the plate. The plates were dried in a fume hood overnight. The leucine standard was visualized by staining the plate in a ninhydrin reagent (14). Radioactive leucine was visualized by fluorography after immersing the chromatography plate in 7% 2.5-diphenyloxazole dissolved in ether (15). The dried plate was placed in contact with x-ray film (Royal X-Omat, Kodak) at −70°C for 2 wk.

The amount of protein synthesized by the explants is proportional to that synthesized by the livers *in vivo* (4). Electron microscopy and radioautography of serial sections obtained from explants after incubation show that all hepatocytes, including those located on the surface of the explants and those located in the center, are equally active in protein synthesis.

RESULTS

It has been demonstrated previously that the cockerel liver explants are very active in protein synthesis and that proteins are synthesized in relative quantities that are very similar to those found *in vivo* (4). The organ explants were preincubated for 2 hr in incubation medium containing a large amount (450 μM) of nonradioactive L-leucine; therefore, addition of [^{3}H]leucine (9.1 nmol) had an insignificant effect upon the total quantity of leucine present. A high concentration of exogenous amino acids also prevents reutilization artefacts because radioactive amino acids that are the products of protein degradation are rapidly diluted out by the high concentration of nonradioactive precursor (9). Hod and Hershko (9) have also shown that washing the tissue in cold buffer before homogenization efficiently removes exogenous amino acids without affecting the specific activity of the intracellular pool. The uptake of [^{3}H]leucine relative to DNA content is shown in Fig. 1 along with the incorporation of [^{3}H]leucine into hot acid-precipitable material.

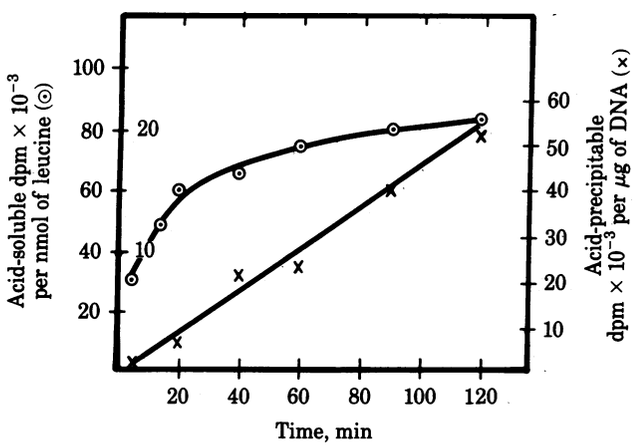

FIG. 1. Comparison of the labeling kinetics of the acid-soluble and acid-precipitable fractions of cockerel liver explants. After a 2-hr preincubation of explant tissue from a 4-day-induced cockerel, [^{3}H]leucine was added, and tissue aliquots were removed at timed intervals and sonicated in trichloroacetic acid. A representative experiment is depicted here; it was repeated >10 times with very similar results. Acid-soluble dpm × 10^{-3} are expressed per nmol of leucine (left ordinate, outside label) or per μg of DNA (left ordinate, inside label).

The equilibration of the acid soluble pool seems to follow the shape of a logarithmic curve, whereas the incorporation of label into acid-precipitable protein is apparently linear from the very earliest time points. The curves presented in Fig. 1 were generated by performing either a logarithmic or linear regression analysis upon the data points for the two curves. The coefficients of determination (r^2) were >0.99 in both cases.

Because of the possibility that the acid-soluble pool was still expanding during the experiment, the absolute specific activity of acid-soluble leucine (dpm of [^{3}H]leucine per nmol of leucine) was determined by quantitating the leucine concentration with an amino acid analyzer. A representative profile of the separation achieved is presented in Fig. 2. To ensure that the radioactivity measured had remained as [^{3}H]leucine, an aliquot of the final time point sample was analyzed by two-dimensional ascending chromatography, and the migration of radioactivity was compared with the migration of a leucine standard (Fig. 3). The data in Fig. 3 demonstrate that all detectable radioactivity remained as [^{3}H]leucine during the incubation. The results of the analysis of the absolute specific activity of acid-soluble leucine are presented in Fig. 1 along with the incorporation of [^{3}H]-leucine into hot acid-precipitable protein. The absolute specific activity of acid-soluble leucine increased logarithmically, whereas the incorporation into protein became linear almost immediately after addition of label. Other experiments in which several additional time points were taken within a 15-min incubation (4) have shown that the incorporation into protein indeed becomes linear almost immediately after addition of [^{3}H]leucine.

The plot describing the absolute specific activity of acid-soluble leucine (Fig. 1), which was generated by performing a regression analysis, has an excellent fit to a logarithmic curve. The coefficient of determination (r^2) value again was >0.99. Attempts to fit the data or parts of the data to other curves were unsuccessful and generated low coefficients of determination (<0.90). Because the data formed a logarithmic curve, a double reciprocal plot enabled us to calculate the specific activity of acid-soluble leucine at the point of equilibration; in addition,

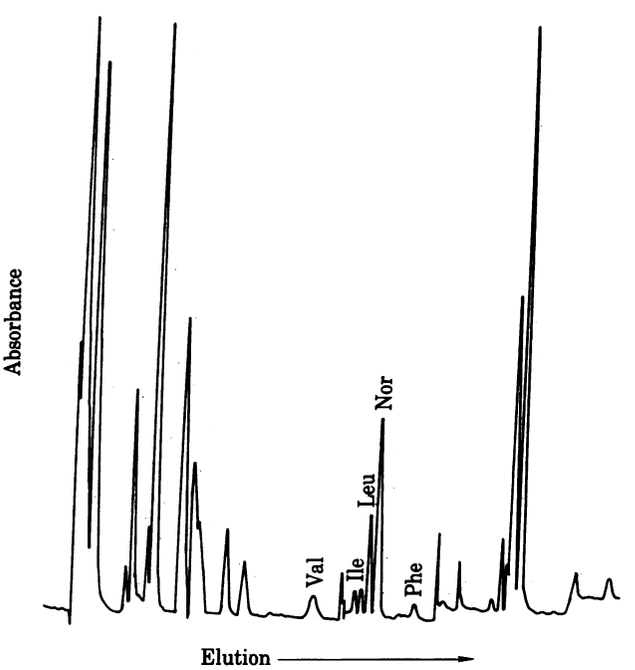

FIG. 2. Automated amino acid analysis of the acid-soluble fraction of the cockerel liver sonicates. Details of the procedures are given in the text. Nor, norleucine.

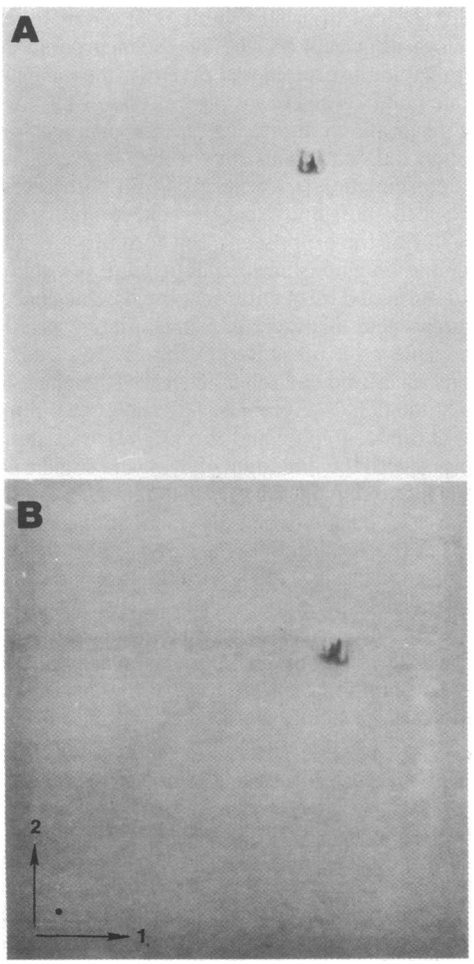

FIG. 3. Analysis by two-dimensional thin-layer chromatography of acid-soluble amino acids. An aliquot of the acid-soluble fraction from the 120-min time point (Fig. 1) was analyzed by two-dimensional thin-layer chromatography. (*A*) Fluorograph of the two-dimensional chromatograph of the 120-min acid-soluble fraction; (*B*) ninhydrin-stained two-dimensional chromatography of standard leucine. Arrows indicate the direction of the first and second dimensions and the black dot indicates the origin.

the time at which equilibration was reached could also be calculated.

Because the quantity of leucine in the incubation medium was known, the theoretical specific activity of 96,500 dpm/nmol of leucine was calculated.‡ The double-reciprocal plot presented in Fig. 4 showed that the acid-soluble leucine pool reached constant specific activity of 83,500 dpm/nmol of leucine at ≈100 min after addition of label. Therefore, the intracellular specific activity in the 120-min time point sample is 86% of the calculated specific activity of leucine in the incubation medium. These data verify the hypothesis that the concentration of exogenous leucine is high enough to cause expansion of the intracellular pool to a level that very closely approximates the specific activity of the outside source. Therefore, changes in the leucine pool that result *in vivo* from treatment with 17β-

‡The theoretical specific activity of acid-soluble leucine in the incubation medium was calculated as follows: the concentration of L-leucine in medium 199 is 450 μM; therefore, 25 ml of medium contains 11.3 μmol of leucine. Five hundred μCi of leucine was added to 25 ml of the incubation medium. Hence, the theoretical specific activity of the acid-soluble leucine in the incubation medium is 9.65×10^4 dpm/nmol of leucine.

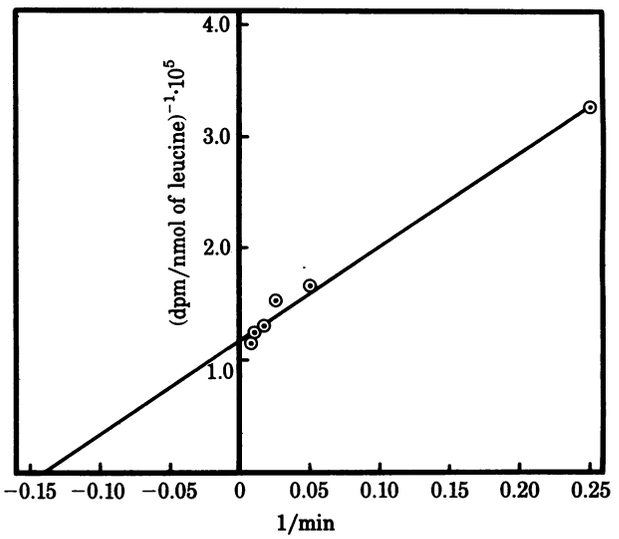

FIG. 4. Determinations of the time needed for the observed specific activity of acid-soluble leucine to reach equilibrium. The graph representing intracellular acid-soluble dpm/nmol of leucine (Fig. 1) has an excellent fit to a logarithmic curve. Therefore, a double-reciprocal plot of the data has been used to calculate the specific activity of acid-soluble leucine at equilibrium. The length of time needed to reach equilibrium is also calculated from the same plot. The equation and coefficient of determination (r^2) of the line presented were determined by a least-squares linear regression analysis. The coefficient of determination (r^2) of the line is 0.99. The inverse of the *y* intercept represents the value of the observed leucine specific activity at equilibrium (83,300 dpm/nmol of leucine). By applying this value in the logarithmic fit equation from Fig. 1 ($y = 9{,}630 + 15{,}800 \ln x$), we calculate that the observed leucine specific activity is reached at about 105 min after addition of label.

estradiol (16) will be obviated by incubation of the tissue in explant incubation medium.

Average ribosome half-transit time refers to one-half the length of time needed for a ribosome to translate an average-sized mRNA and release a completed polypeptide chain (17). Average ribosome half-transit times have been measured in the organ explant system by using estrogen-stimulated and unstimulated cockerel liver tissue (4, 5). An example of the determination of the half-transit parameter in unstimulated cockerel liver explants is shown in Fig. 5, in which the horizontal distance separating the two lines represents the average ribosome half-transit time. The *x* intercept of the line representing incorporation into "total" protein is the point at which incorporation of [^{3}H]leucine into acid-precipitable protein becomes linear. The average ribosome half-transit time (i.e., the horizontal distance separating the two lines) is 0.6 min, thus making the average ribosome transit time 1.2 min.

Our hypothesis is that the immediate precursor pool for protein synthesis in the explants is not exclusively the intracellular acid-soluble pool; instead, it appears that amino acids from the explant incubation medium are utilized directly for protein synthesis without passing through the acid-soluble pool. If the immediate leucine precursor pool for protein synthesis equilibrates with [^{3}H]leucine almost instantaneously, then the length of time needed to reach a linear rate of incorporation into acid-precipitable protein should be approximately equal to one average ribosome transit time.

The data presented in Fig. 5 support the hypothesis by showing that incorporation into acid-precipitable protein becomes linear in 1.25 min, which is slightly more than the average ribosome transit time of 1.2 min. We were not able to perform a similar analysis upon cockerel liver explants after es-

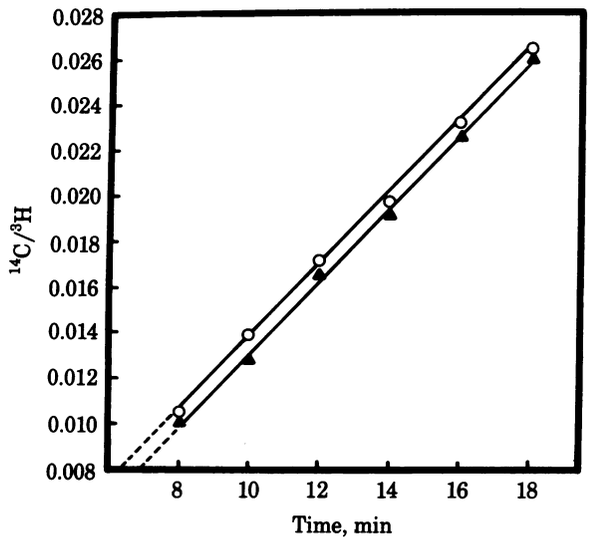

FIG. 5. Analysis of the average ribosome half-transit time in uninduced cockerel liver explants. Details of the determination of average ribosome half-transit times have been published (3). Liver explants were incubated for 2 hr in the presence of [^{3}H]leucine before addition of [^{14}C]leucine. The abscissa indicates minutes after addition of [^{14}C]-leucine. ○, Incorporation into total postmitochondrial supernatant protein; ▲, incorporation into postribosomal supernatant protein. The horizontal distance separating the two lines represents the average ribosome half-transit time. The data presented are the average of two experiments, utilizing four experimental animals.

tradiol stimulation for two reasons. First, after administration of estradiol, the cockerel livers greatly increase synthesis of β-apolipoprotein (subunit M_r, 380,000) and initiate *de novo* synthesis of vitellogenin (subunit M_r, 240,000). Because of the large sizes of the two mRNAs for these proteins, the ribosome transit times are significantly higher than the average value (4, 5). Second, the cockerel liver explants actively secrete proteins, and secretion of labeled proteins becomes linear after ≈15 min (4). Therefore, by the time the polysomes translating mRNAs for β-apolipoprotein and vitellogenin had reached constant specific activity (i.e., after one ribosome transit time for these mRNAs), the liver explants had already "lost" radioactive proteins by secreting them into the incubation medium. Secretion of proteins has no effect upon the determination of ribosome half-transit times because an internal control in the form of a double-label analysis was performed. However, because of the transfer of radioactivity into the incubation medium of the estradiol-stimulated explants, we could only estimate the time at which incorporation into acid-precipitable protein became linear. Because the uninduced explants as described here do not synthesize large quantities of the secreted proteins β-apolipoprotein and vitellogenin, we were able to remove tissue aliquots for half-transit time analysis before significant secretion of labeled proteins began. In this way, the point at which the rate of incorporation of [^{3}H]leucine into acid-precipitable protein became linear was accurately determined.

DISCUSSION

The identification of the true source of amino acids for protein synthesis has been an elusive and controversial experimental problem. To define the mechanisms of amino acid utilization more closely, we have attempted to relate three experimental parameters—namely, average ribosome transit time, the absolute specific activity of acid-soluble leucine, and the level of incorporation of leucine into acid-precipitable protein. Preferential utilization of exogenous amino acids implies that amino acids are taken directly from the explant incubation medium and incorporated into protein without interacting with the intracellular acid-soluble pool. The data in Fig. 1 show that incorporation into protein reaches a linear rate almost immediately after addition of labeled precursor, whereas the intracellular acid-soluble leucine pool does not reach constant specific activity until about 100 min later. These results show that at least part of the supply of amino acids for protein synthesis comes directly from the incubation medium and not from the acid-soluble pool.

To further test the hypothesis of preferential utilization of exogenous amino acids, we have compared the length of time needed to reach a linear rate of incorporation into acid-precipitable protein with the average ribosome transit time. The rationale for this comparison is as follows: if the immediate precursor pool of amino acids for protein synthesis does indeed reach constant specific activity directly after addition of labeled precursor, then the linear rate of incorporation into acid-precipitable protein should begin after one full ribosome transit time. One ribosome transit time is also equal to the amount of time needed to equilibrate the nascent polypeptide chains with radioactive label after constant specific activity of the immediate precursor pool is reached. The data in Fig. 5 suggest that, indeed, the incorporation into protein reaches a linear rate after one average ribosome transit time. This analysis represents an important approach to studying the immediate precursor pool for protein synthesis and, in combination with the determination of absolute specific activity of leucine, the data indicate that at least part of the supply of leucine is incorporated into protein directly after transport from the incubation medium and without equilibration with the acid-soluble pool.

The models that have been proposed to explain the discrepancies between logarithmic equilibration of the acid-soluble pool and linear incorporation into protein have generally involved a "functional heterogeneity" (6) or compartmentalization of the true precursor pool. Hod and Hershko (9) have proposed a two-site activation model, in which there is one intracellular site of aminoacylation, which utilizes amino acids from the intracellular pool, and an external activation site (probably membrane-associated), which activates amino acids as they enter the cell. A similar model has recently been suggested by Reith *et al.* (10). van Venrooij *et al.* (7) also reported that the kinetics of incorporation of amino acids indicate that aminoacylation of tRNA occurs near or on the plasma membrane in close association with the amino acid transport system. Tscherne *et al.* (18) described aminoacyl-tRNA synthetases that were organized, in a complex fashion, in large lipid-containing aggregates which may have been membrane-associated. Our data support the model proposed by Hod and Hershko, in which amino acids from the explant medium are activated as they enter the liver cells, and at least some of these activated amino acids are directly utilized for protein synthesis without entering the acid-soluble amino acid pool.

The biological significance of preferential utilization of amino acids for protein synthesis is not clear. However, it has been suggested that at least part of the amino acids are activated for protein synthesis on the plasma membrane (7–10, 19). The association of amino acid activation and the plasma membrane is energy dependent and implies the use of ATP for amino acid transport. The first reaction in the activation is elevation of the group potential of the amino acid as follows:

$$\text{amino acid} + \text{ATP} \rightleftharpoons \text{aminoacyl AMP} + \text{PP}_i$$

This is a fully reversible reaction and is often utilized for the determination of amino acid-dependent ATP exchange reac-

tion. It follows that the activated amino acid is either attached to tRNA without further energy utilization or released to the acid-soluble pool by the reverse of the above reaction. In the reverse reaction, the ATP used for activation would be regenerated. This mechanism might allow the cell to make a more efficient use of energy, because the supply of amino acids is linked to feeding, which is periodic, whereas protein synthesis must be continuous. For such a mechanism, the rate of amino acid transport and activation must be greater than the rate of protein synthesis; moreover, the affinity of the activating enzyme should be higher for exogenously supplied amino acids. An abundant supply of exogenous amino acids could then be coupled with expansion of the intracellular pool and increased amino acid storage. Indeed, in sea urchin embryos, the pool can expand 7-fold in 1 min when an exogenous supply is available (20). The presence of an exogenous source of amino acids then blocks almost completely the apparent use of the endogenous pool (3), which is the natural supply of amino acids at that stage of development.

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1. Ilan, J. & Singer, M. (1975) *J. Mol. Biol.* **91**, 39–51.
2. Singer, M. & Ilan, J. (1977) *Dev. Biol.* **57**, 174–187.
3. Ilan, J. & Ilan, J. (1981) *J. Biol. Chem.* **256**, 2830–2834.
4. Gehrke, L., Bast, R. E. & Ilan, J. (1981) *J. Biol. Chem.* **256**, 2514–2521.
5. Gehrke, L., Bast, R. E. & Ilan, J. (1981) *J. Biol. Chem.* **256**, 2522–2530.
6. Kipnis, D. M., Reis, E. & Helmreich, E. (1961) *Biochim. Biophys. Acta* **51**, 519–524.
7. van Venrooij, W., Moonen, H. & van Loon-Klassen, L. (1974) *Eur. J. Biochem.* **50**, 297–304.
8. Airhart, J., Vidrich, A. & Khairallah, E. (1974) *Biochem. J.* **140**, 539–548.
9. Hod, Y. & Hershko, A. (1976) *J. Biol. Chem.* **251**, 4458–4467.
10. Reith, M. E., Schotman, P., van Zwiten, B. J. & Gispen, W. H. (1979) *J. Neurochem.* **32**, 413–420.
11. Cerrioti, G. (1952) *J. Biol. Chem.* **198**, 297–303.
12. Bohlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220.
13. Udenfriend, S., Stein, S., Bohlen, P., Dairman, W., Leimsruber, W. & Weigele, M. (1972) *Science* **178**, 871–872.
14. Stepka, W. (1957) *Methods Enzymol.* **3**, 504–528.
15. Storti, R. & Rich, A. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 2346–2350.
16. Dierks-Ventlins, C. & Jost, J.-P. (1974) *Eur. J. Biochem.* **50**, 33–40.
17. Fan, H. & Penman, S. (1970) *J. Mol. Biol.* **50**, 655–670.
18. Tscherne, J. S., Weinstein, I. B., Lanks, K. W., Gersten, N. B. & Cantor, C. R. (1973) *Biochemistry* **12**, 3859–3865.
19. Martin, A. F., Rabinovitch, M., Blough, R., Prior, G. & Zak, R. (1977) *J. Biol. Chem.* **252**, 3422–3429.
20. Fry, B. J. & Gross, P. R. (1970) *Dev. Biol.* **21**, 125–146.