RESPONSE OF SPECIFIC TRANSFER RIBONUCLEIC ACID LEVELS TO AMINO ACID DEPRIVATION IN A MAMMALIAN CELL LINE

by

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ABBREVIATIONS

FLC	Friend leukemia cells.
TCA	Trichloroacetic acid.
TRIS	Tris(Hydroxymethyl) Aminomethane.
RPC	Reverse phase chromatography.
Isoaccepting tRNAs	A series of chemically different RNA molecules with the capacity to accept the same amino acid.
$tRNA^{His}$	That group of structurally heterogeneous RNA molecules which accept histidine. This designation is common to any amino acid specific tRNA capable of accepting its cognate amino acid.
$His-tRNA^{His}$	Designation for histidine-specific tRNAs esterified with histidine. This kind of designation is used to denote tRNAs acylated with their cognate amino acids.
PPO	2,5-diphenyloxazole.
Me_2POPOP	1,4-bis[2-(4-methyl-5-phenoxazolyl)]-benzene.
ATP	Adenosine 5' triphosphate.
SDS	Sodium dodecyl sulfate.
EDTA	Ethylenediaminetetraacetate.
S	Sedimentation constant at 20°C in H_2O.
A_{260}	Absorbance at 260 nm.
A_{260} unit	That amount of material which in a volume of 1 ml gives an absorbance of 1 in a cuvette with a 1 cm light path.
His	Histidinyl.
Trp	Tryptophanyl.
Leu	Leucyl.
Charging levels of tRNA	Extent of aminoacylation, or the proportion of tRNA amino acid acceptor sites that are occupied by the cognate amino acids.

1X

This designation is used to indicate normal, or control, concentrations of the variable component(s) of the FLC growth media (amino acids). 0.1X, 0.2X, etc., represent equivalent proportions of this control concentration.

RESPONSE OF SPECIFIC TRANSFER RIBONUCLEIC ACID LEVELS TO AMINO ACID DEPRIVATION IN FRIEND LEUKEMIA CELLS

I. INTRODUCTION

Interest in *in vivo* regulation of tRNA concentrations derives both from the role of tRNA in protein synthesis and from the increasing diversity of cellular processes known to be affected by tRNA. The following discussion on the background and characteristics of tRNA is based on references #1-4, which will not be individually cited.

tRNA was first discovered in the 1950's during studies on the activation and incorporation of free amino acids into polypeptide chains *in vivo*. A cytoplasmic RNA component was discovered that could interact in a specific fashion with amino acids and that was necessary for amino acid incorporation into protein. Zamecnik and his colleagues proposed an adaptor role for tRNA in translation of the linear nucleotide sequence of mRNA into the specific amino acid sequence of the polypeptide chain. These proposals were soon verified by kinetic experiments that traced the path from free amino acid to amino acid residue in the polypeptide. The discovery of the genetic code provided the translation dictionary; 64 possible nucleotide triplets, or codons, generated from four bases with each triplet (excepting three of them) assigned to one of the twenty biologically used amino acids.

To carry out its role in decoding the mRNA nucleotide sequence, the tRNA molecule needs three features: specificity in codon recognition and in aminoacylation, and the ability to bind to the ribosome-mRNA complex. The first feature is provided by the anticodon, a nucleotide triplet in which at least the last two members (in the 5'- 3' chain direction) are complimentary bases to those in the first two positions (5'- 3')

of the codon, thus allowing for base-pair hydrogen-bonding. The specific amino acid acceptance of tRNAs is provided by 20 amino acyl tRNA synthetases (E.C.6.1.1..) each of which recognizes but one amino acid and aminoacylates a specific group of tRNA molecules, called an isoaccepting family. These isoaccepting groups are mutually exclusive and the molecular features of a tRNA molecule that allow its recognition by one and only one synthetase are not understood despite years of intensive research in many laboratories. tRNAs contain modified bases not found in other polynucleotides, and different tRNAs differ somewhat in their modified base patterns, which may aid in their differential recognition by synthetases. The aminoacylation site on tRNA is the 2' or 3' hydroxyl group of the adenosine moiety of an invariant C-C-A sequence at the 3' end of the nucleotide chain. Sequence studies of over 80 tRNA molecules reveal other common features summarized in Figure 1. tRNAs appear to have chains of between 75-93 nucleotides, and all can assume the two dimensional "cloverleaf" structure if the chains are folded to allow maximum base pair hydrogen bonding. Extensive nucleotide sequence homology probably lends enough similarity in three dimensional conformation to enable all tRNAs, once aminoacylated, to be recognized by and participate in the protein synthesis machinery.

Besides their roles in protein synthesis, tRNAs also participate in many other cellular processes, including regulation of gene expression (5) and aminoacyl transferase functions (6). It has been suggested that tRNAs may play important roles in cellular differentiation, development and cancer (7) (8), and observed changes in tRNA populations in the course of

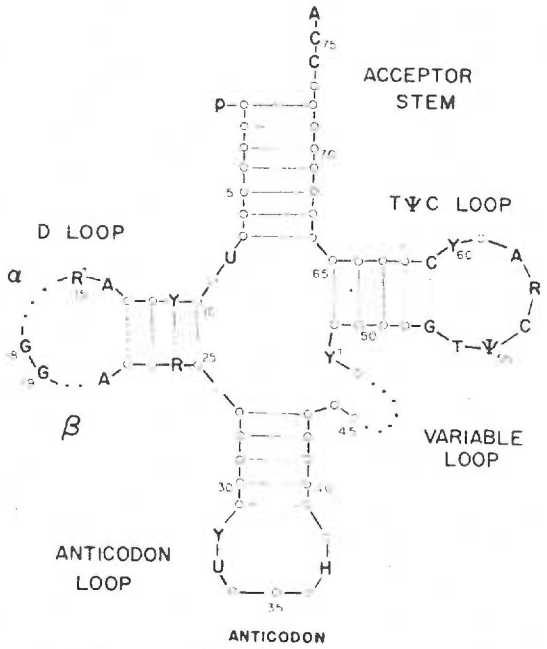


Figure 1. A diagram of all tRNA sequences except for initiator tRNAs. The position of invariant and semiinvariant bases is shown. The numbering system is that of yeast $tRNA^{Phe}$. Y stands for pyrimidine, R for purine, H for a hypermodified purine. R_{15} and Y_{48} are complementary. The dotted regions α and β in the D loop and the variable loop contain different numbers of nucleotides in various tRNA sequences.

Figure 1. A two-dimensional diagram of tRNA. Reproduced from the article by Rich and Schimmel (reference 4 of this thesis) page 385.

these conditions have aroused interest in tRNA metabolism.

In accordance with the economy governing other important metabolites in the cell, intracellular concentrations of tRNAs are closely coupled to the needs of the cell for their cognate amino acids in protein synthesis (9). This is particularly apparent in differentiated cells producing massive amounts of protein with a special amino acid composition. Studies by Garel and his colleagues on the developing silkgland of the silkworm, Bombyx mori, showed that production of fibroin, a major protein product consisting mostly of glycine, alanine and serine, is accompanied by a marked enrichment of their cognate tRNAs which form 2/3 of the tRNA population at the time of maximal fibroin production (9). Litt and Kabat, in studies on hemoglobin synthesis (10) in sheep reticulocytes, found that anemia-induced switchover in hemoglobin production in A/A homozygotes from HbA ($\alpha_2\beta_2^A$) to HbC ($\alpha_2\beta_2^C$) was accompanied by changes in the tRNA population that reflected the amino acid composition of the new hemoglobin synthesized. The chains in HbA contain no isoleucine whereas the chains of HbC have two isoleucine residues, and the shift from β^A to β^C production was accompanied by 2-3 fold elevation in $tRNA^{Ile}$ levels. The B/B homozygote sheep reticulocytes on the other hand produced HbB ($\alpha_2\beta_2^B$) in both normal and anemic states and showed no difference in $tRNA^{Ile}$ proportions. Similar correlations between relative proportions of tRNA species and use of their cognate amino acids in protein synthesis have been found in mouse reticulocytes (11) and in estrogen-treated chick hepatocytes (12). The molecular basis of this coordination between tRNA population composition and protein synthesis needs is not understood.

It is possible that the observed tRNA changes were programmed into

the development pathways of these systems to correspond with the transcription of specific mRNAs (9). The previously discussed findings of Litt and Kabat argue against this; they selected for sheep only on the basis of their globin genes, and which presumably differed randomly with respect to other genes, so that the observed $tRNA^{Ile}$ shift in the A/A homozygote was probably not programmed directly into the erythropoietic pathway (10).

More likely, the observed shifts in tRNA levels were engineered by a feedback mechanism sensing changes in the amino acid composition of proteins being synthesized (10). This feedback could operate on the relative rates of tRNA synthesis, degradation or both to produce the observed changes in tRNA steady state proportions.

One parameter which might play a role in control of proportional tRNA levels is their relative extents of aminoacylation. If an amino acid is not being used for protein syntheses, its cognate tRNAs tend to become 100% charged (13) (14). If, on the other hand, an amino acid is in great demand, either through increased use in protein synthesis or decreased availability to the point of growth and protein synthetic rate limitation, then the steady state level of amino acylation of its cognate tRNAs will tend to decrease (13) (15).

The experiments in this thesis were designed to test the following hypothesis, that reduction in the extent of aminoacylation of a particular tRNA species would produce increased proportion of that species in the tRNA population.

I have attempted to test this prediction using selective amino acid deprivation in Friend virus-infected murine erythroleukemia cells (FLC, line 745) to reduce extents of charging of the tRNA species cognate to

these amino acids. Media concentrations of three different growth essential amino acids, histidine, leucine and tryptophan were independently reduced. In each case, the response in proportional levels of the cognate and a non-cognate tRNA species were compared in amino acid deprived versus control FLC grown to the G_0 or stationary phase of the cell cycle, or approximately 5 days.

In the case of tryptophan deprivation, extent of *in vivo* aminoacylation and response in proportional levels of $tRNA^{Trp}$ were assayed after various periods of tryptophan deprivation. These experiments had a twofold purpose; to document the production of reduced $tRNA^{Trp}$ charging levels through tryptophan deprivation in FLC, and to determine the temporal relationship between the production of reduced charging levels and of increased proportional levels of $tRNA^{Trp}$. The results of this work support the hypothesis that tRNA charging levels act as signals to regulate the metabolism of specific tRNAs.

These studies have been extended to ascertain the effects of amino acid deprivation on individual isoacceptors of the cognate tRNA species, using a reverse-phase chromatography system (19) to fractionate charged isoacceptors. These experiments were performed to contribute to the understanding of the biological basis of the difference between isoaccepting species, and whether this difference is reflected in selective effects of cognate amino acid deprivation on different isoacceptors.

II. MATERIALS AND METHODS

A. Buffers, Media and Reagents

Buffers. RNA extraction buffer: 100 mM NaCl, 10mM Na_2EDTA and 50 mM sodium acetate, pH 5.0. TNM buffer for sucrose gradients: 50 mM TrisCl, pH 7.5, 25mM NaCl and 5mM $MgCl_2$. DEAE buffers for RNA fractionation: 10 mM $MgCl_2$, 1mM Na_2EDTA, and 0.25M or 0.7M NaCl as specified, and 20 mM sodium acetate, pH 4.5. RPC-5 buffers for tRNA fractionation: 10mM $MgCl_2$, 1mM Na_2EDTA, 0.5M or 0.7M NaCl as specified and 10mM sodium acetate, pH 4.5.

Media. MEM (GIBCO), minimum essential medium, Hanks base supplemented with 7mM glutamine, 10% fetal calf serum (GIBCO), and one part per hundred of 100X Penn-Strep (GIBCO).

Reagents: as specified in appropriate procedures in which they are used.

B. Cell Culture

Friend-virus infected erythroleukemia cells [cell line 745 (29), clone 18, obtained from Dr. David Kabat] were used in the experiments described in this thesis. Cells were grown in suspension in MEM supplemented with 10% fetal calf serum (GIBCO) at 37°C in a 5% CO_2 atmosphere. During exponential growth, the doubling time for this cell line is approximately 20-24 hours. The maximal culture density reached approximately 2-3 x 10^6 cells/ml. Cells to be used in experiments were pelleted at half speed for 3 min. in a clinical centrifuge (IEC, model CL) and were resuspended at a density of 3-5 x 10^5 cells/ml in MEM containing 10% fetal calf serum and reduced or normal concentrations of histidine, leucine or tryptophan as specified in the appropriate Results sections.

Cell population growth was monitored by periodic removal of aliquots of cell culture for counting, using an electronic particle counter (Coulter model A). Cells were usually diluted 1:20 to 1:40 for counting. The aliquots to be counted were spritzed 6-8 times with a pasteur pipet to break up cell aggregates prior to dilutions and counting.

C. Preparation of Transfer RNA from Friend Cells

The total cytoplasmic RNA extraction method used in these experiments is essentially that described by T.H. Hamilton (17). The cells were briefly chilled, pelleted at 1000 x g for 5 min., and resuspended in cold fresh medium at a density of 0.5 x 10^8 cells/ml. The cell suspension was then extracted at room temperature for 10 min. with a mixture of 2 volumes of water-saturated phenol and one volume of RNA extraction buffer containing 0.5% SDS. Phenol and aqueous phases were

separated by centrifugation at 6000 x g for 10 min. The aqueous phase was collected, extracted twice at room temperature with an equal volume of chloroform/phenol/isoamyl alcohol (100:100:1, v/v/v) and the phases separated by centrifugation. The final aqueous phase was collected and RNA was precipitated by the addition of cold ethanol to 70% and sodium acetate, pH 4.5 to 0.1M, and by chilling the mixture at least 2 hours at -20°C. The RNA precipitate was then collected by centrifugation at 2000 x g, washed twice with cold 95% ethanol and drained for approximately 1/2 hour in preparation for further fractionation and tRNA collection.

Fractionation of cytoplasmic RNA was usually performed via sucrose density gradient ultracentrifugation essentially as described in the work of Dehlinger, et al. (18). The RNA precipitates to be fractioned were resuspended in TNM buffer and carefully layered on top of 16 ml linear 5-20% gradients, each gradient receiving approximately 40 to 60 A_{260} units in 0.5 ml of sample. The gradients were centrifuged at 25,000 rpm at 4°C for 24 hours in an SW 27 rotor of a Beckman ultracentrifuge. After the 24 hour centrifugation, the gradients were collected and individually pumped, from bottom to top of gradient, through a flow cell in a Gilford spectophotometer and the A_{260} absorbance profile was monitored. These profiles typically showed two major peaks, identified in previous studies (18) as the 18s and 4s fractions, the latter identified as the tRNA fraction through its amino acid acceptor activity (18). The 28s ribosomal RNA pellets under these conditions. Collection of the 4s RNA fraction was begun at the appearance of the 4s peak, and continued to the upper end of the gradient, resulting in a total volume of 4-5 ml collected. 4s RNA was precipitated with 2 vol-

umes ethanol at -20°C (at least 2 hours).

The 16 ml sucrose gradients were constructed from equal volumes (8 ml each) of 5% and 20% sucrose solutions in TNM buffer, which were mixed in a 30 ml plexiglass gradient mixer as they were pumped into 16 ml nitrocellulose centrifuge tubes. All sucrose solutions were made using RNAse-free sucrose (Schwarz-Mann, special density gradient grade) and were boiled before use. After each use the gradient maker was cleaned with 1% SDS, followed by copious rinsing with deionized water.

For the tryptophan-deprivation experiments described in Table 4, adsorption to and elution from DEAE cellulose (19) was used instead of sucrose gradient centrifugation to obtain the 4s (tRNA) fraction from phenol-SDS extracted RNA. The RNA precipitate to be fractioned was resuspended in DEAE starting buffer (DEAE buffer with 0.25M NaCl) and loaded onto a DEAE cellulose column (20-40 A_{260} units per ml of column bed) previously equilibrated at 4°C with DEAE starting buffer. After washing in with 3-4 column volumes of starting buffer, to eliminate residual phenol and other small molecular weight U.V.-absorbing contaminants, the tRNA fraction was eluted with DEAE stripping buffer (DEAE buffer with 0.70M NaCl), using gravity flow. The tRNA peak appeared in the first 3 column volumes of eluate, which was collected in 1.5-2.0 ml fractions. Absorbance at 260 nm was used to locate the tRNA-containing fractions which were then pooled and the RNA precipitated with 2 volumes of ethanol at -20°C for at least 1/2 hour.

The tRNA precipitates collected from either sucrose gradient or DEAE column fractions were resuspended in sterile 1.8M TrisCl, pH 8.0 at 37°C, and incubated at 37°C for 90 min. to strip the tRNA of its

esterified amino acids. This procedure deacylates aminoacyl-tRNAs without destroying amino acid acceptor capability of the tRNA preparation (20). The deacylated tRNA was re-precipitated with ethanol and sodium acetate as previously described, washed twice with ethanol, drained at room temperature for 1/2 hour and finally suspended in 1 mM $MgCl_2$, at a volume calculated to produce a tRNA solution with A_{260} concentrations in the range of 20-80. The A_{260}/A_{280} ratio of these preparations, used as a crude assessment of tRNA purity, was always 2.0 ± 0.2. tRNA samples not immediately assayed were stored at -20°C, in which condition amino acid acceptor capability was stable for at least 2 months.

D. In Vitro Aminoacylation of Transfer RNA

Transfer RNA preparations were assayed for acceptance of [^{14}C] leucine, [^{14}C] histidine, or [^{14}C] tryptophan, and also separately with a mixture of 14 equimolar, equal activity [^{14}C] amino acids (Amersham) to provide an approximate measure of total amino acid acceptor capacity, or "specific activity" of the tRNA preparation. The assay mixtures contained, in a volume of 28μl, .05 - .30 A_{260} units of tRNA, 2 or 5μl of synthetase enzyme (Materials and Methods, section G), 40 mM TrisCl pH 7.5 at 37°C, 10 mMβ-mercaptoethanol and concentrations of amino acid, ATP and $MgCl_2$ as given in Table I. The reaction mixtures were constructed on ice, and the reaction started with the addition of enzyme. After 30 min. of incubation at 37°C, the reaction was stopped with brief chilling followed by addition of 2 ml of cold 5% TCA and brief mixing. After at least 10 min. on ice, the TCA precipitates were collected by filtration on glass-fiber filters (Reeve-Angell, grade 934 AH), rinsed with 95% ethanol and dried for 10 min. under an I.R. lamp. Each

TABLE 1

ATP, $MgCl_2$ and Amino Acid Concentrations Used for Assays of Amino Acid Acceptance

Amino Acid[1]	Amino Acid Concentration µM	ATP mM	$MgCl_2$ mM
Leucine	100	2.5	3.0
Tryptophan	20	4.0	12
Histidine	14	8.0	16
[^{14}C] Amino Acid Mixture	18	4.0	16

[1]The amino acid concentration shown is the total concentration of each amino acid present in the assay mixture.

Each filter was counted by liquid scintillation in 5 ml of a toluene-based cocktail containing 0.3% PPO and 0.03% Me_2POPOP. Counting efficiency of ^{14}C was 75%. All acceptor assays were carried out in duplicate.

Conditions for maximum aminoacylation were determined by assaying a constant amount of tRNA with increasing concentrations of the synthetase enzyme. The point at which addition of extra enzyme produced no further increase in TCA-precipitable ^{14}C counts was used as the condition producing maximum aminoacylation.

To guard against bias introduced by different assay concentrations of tRNA preparations that might contain aminoacylation activating or inhibiting agents, each assay within an individual experiment received the same amount of tRNA in terms of A_{260} units, a value ranging over 0.05-0.30 A_{260} units.

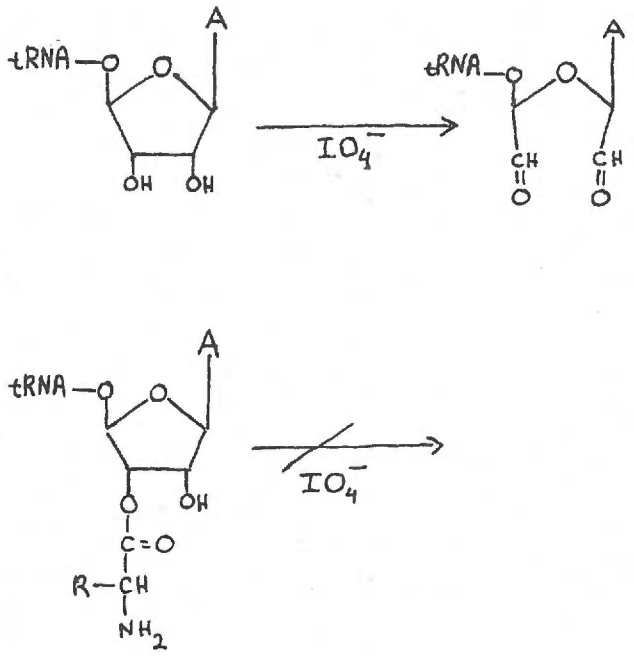
The acceptor capacity for each amino acid is expressed in units of ρmoles of TCA-precipitable amino acid per A_{260} unit of tRNA. The relative acceptance for a single amino acid is defined as the ratio of the acceptance for that amino acid to the acceptance measured with the [^{14}C] amino acid mixture, both acceptances being expressed in units of ρmole/A_{260} unit.

E. Determination of <u>In Vivo</u> Extent of Aminoacylation of tRNA

To halt cellular metabolism abruptly and evenly so as to preserve <u>in vivo</u> steady state levels of aminoacylation, FLC cultures were poured evenly with vigorous stirring into 9 volumes of 20mM sodium acetate, pH 5.0 in 70% ethanol at 37°C[1]. The resulting suspension was chilled

in ice and centrifuged at 1000 x g for 5 min. in the cold. The pellet was resuspended in cold RNA extraction buffer without SDS and extracted as previously described (pg.7) in phenol plus buffer containing 0.5% SDS followed by two extractions with the phenol/ chloroform/ isoamyl alcohol mixture. Cytoplasmic RNA was precipitated from the final aqueous phase with 0.1M sodium acetate pH 4.5/ 70% ethanol (ice-cold), and after 2 hours at -20°C, was collected by centrifugation. The pellet was drained for 30 min. and resuspended in 2 ml. of 0.1M sodium acetate, pH 4.5. Periodate oxidation of uncharged tRNA was performed essentially as described by Vaughn and Hansen (21). This reaction is illustrated on page 14a. Each 2 ml RNA suspension was divided into two one ml aliquots with one of these receiving 0.2 ml. of freshly prepared 12mM sodium periodate, and the other receiving 0.2 ml. of the sodium acetate buffer used as the RNA solvent. Both aliquots were stored in the dark for 15 min. after which 2.5 volumes of cold 95% ethanol was added to precipitate the RNA. After 20-30 min. at -20°C, the RNA precipitates were collected by centrifugation, washed 3X with 0.1M sodium acetate/ 70% ethanol, pH 4.5 at room temperature, resuspended in 1.8M Tris Cl pH 8.0 and incubated at 37°C to de-aminoacylate the remaining charged tRNA (pg.10).

Assay of amino acid acceptance was performed in duplicate as described on page10 , except that 0.75-0.95 A_{260} units of RNA were used per assay. Assay parameters producing maximal aminoacylation were determined as described in Section D of Materials and Methods.



The purpose of this procedure was to destroy the aminoacyl acceptor capacity of uncharged tRNAs by periodate oxidation of the 2'-3' dihydroxyl aminoacyl esterification site on the ribose moiety of the terminal (3' end) adenosine. Aminoacylated tRNAs are protected from such oxidation.

F. Fractionation of Isoacceptor tRNAs by RPC-5 Column Chromatography

Aminoacylated tRNA samples for RPC-5 chromatography were prepared by appropriately scaling up analytical assay reactions described in Section D. In cases where two samples were to be co-chromatographed, one was charged with [^{3}H] labelled amino acid, with the other receiving [^{14}C] amino acid as usual. Aliquots of each reaction mixture were removed for quantitative determination of charged tRNA as described in Section D, except that the [^{3}H] tRNA precipitate was collected by millipore filtration and eluted from the filter with 0.5 ml 1N HCl and heating for 20 min. at 90°C, and the eluate counted at tritium-standard settings in ACS scintillation cocktail.

Charged tRNA was isolated from the bulk of the charging reaction mixtures following their acidification to pH 4.5 with sodium acetate to 0.5M by the addition of 5-6 A_{260} units of *E. coli* tRNA (for bulk carrier purposes), and by phenol extraction and ethanol precipitation.

Reverse-phase co-chromatography of [^{3}H] and [^{14}C] aminoacyl-tRNA preparations was performed essentially as described by Kelmers et al. (19), on an RPC-5 column (0.6 x 10 cm) at 37°C. The aminoacyl-tRNA (approximately 2-3 A_{260} units) was loaded onto the column, washed on with approximately two column volumes of RPC-5 starting buffer and eluted with a linear gradient (total volume of 150 ml) of 0.5 to 0.7M NaCl containing 10mM magnesium acetate , 1mM EDTA, and 10 mM sodium acetate, pH 4.5 at a flow rate of about 45 ml per hour. Fractions of 1.5 ml were collected and mixed with 15 ml of toluene-Triton X-100 scintillation fluid and counted in a Packard Model 3315 scintillation counter at dual-label settings previosly calculated to minimize ^{14}C

spillover into and consequent masking of tritium count values. The tritium counts thus obtained were subsequently corrected for remaining ^{14}C spillover.

G. Preparation of Rat Liver Aminoacyl-tRNA Synthetases

Male Sprague-Dawley rats weighing 300-400 grams were fasted overnight and killed by decapitation. The livers were removed, rinsed with ice-cold saline, minced with scissors and then homogenized on ice in two volumes of 0.1M TrisCl, pH 7.5, containing 0.35M sucrose and 10mM $MgCl_2$. Homogenization was carried out in a Potter glass homogenizer using a teflon pestle driven by a Tri-R-Stir-R motor, model K43 setting 7, for four 15 second intervals. The homogenate was centrifuged at 10,000 x g for 20 min. and the supernate collected for a second centrifugation at 40,000 rpm in a Spinco 40 rotor (Beckman ultracentrifuge) for 1 hour at 4°C. The supernate was then loaded onto a Sephadex G-25 column (0.9 x 28 cm) pre-equilibrated with cold DE-52 loading buffer, washed through with more cold buffer and the blue dextran, or high molecular-weight, fraction was collected. This fraction was directly applied to a DEAE cellulose (Whatman DE-52) column (8 ml total bed volume) pre-equilibrated at 4°C with DE-52 loading buffer (0.005M KCl, 10 mM β-mercaptoethanol, 10 mM $NaHPO_4$ buffer pH 7.4). The sample was washed on with eight volumes of loading buffer and the enzyme-containing protein fraction was eluted with DE-52 stripping buffer (0.25M KCl, 1mM dithiothreitol, 10 mM $NaHPO_4$ buffer, pH 6.5). The crude synthetase-containing fraction, a yellow-colored band, was visually monitored in its elution from the column and was collected, in 2 ml.

fractions, in a total volume of about 4 ml. To this eluate was added glycerol, pre-filtered through a glass-fiber filter to remove traces of charcoal, to a final concentration of 40% (v/v), and the product stored at -20°C until use in tRNA charging assays. Synthetase activity remained stable for at least six months under these conditions. This synthetase preparation procedure is essentially that described by T. Hamilton (17).

III. RESULTS

A. Effects of Histidine Deprivation on Specific tRNA levels in FLC

FLC were incubated in MEM containing normal (1X) or reduced (0.2X) concentrations of histidine. The reduction of histidine to 0.2X normal concentration appears to have no significant effect on initial growth rate of FLC, or on the timing in cessation of growth, but does reduce the final cell density, attained after 4-5 days of growth, to 80% of that attained by control FLC (Fig. 2). tRNA preparations were made from FLC after 5 days of incubation in normal (1X) or deficient (0.2X) histidine-containing media. The relative acceptances of these tRNA preparations for histidine and leucine are shown in Table 2. A significant elevation (approximately twofold) in $tRNA^{His}$ levels in the histidine-deprived versus the control cultures was seen after 5 days in culture. The relative levels of $tRNA^{Leu}$, however, appeared to be unaffected by histidine deprivation.

B. Effect of Leucine Deprivation on Specific tRNA Levels in FLC

FLC were incubated in MEM containing normal (1X) or reduced (0.1X) concentrations of leucine. The growth curves of these cultures (Fig. 3) show that reduction of leucine to 0.1X had no significant effect on initial growth rate of FLC but caused premature cessation of growth with significant decrease in final cell density attained after 4-5 days of growth.

Table 3 shows the relative acceptances for histidine and leucine displayed by tRNA harvested from histidine-deprived and normal cultures

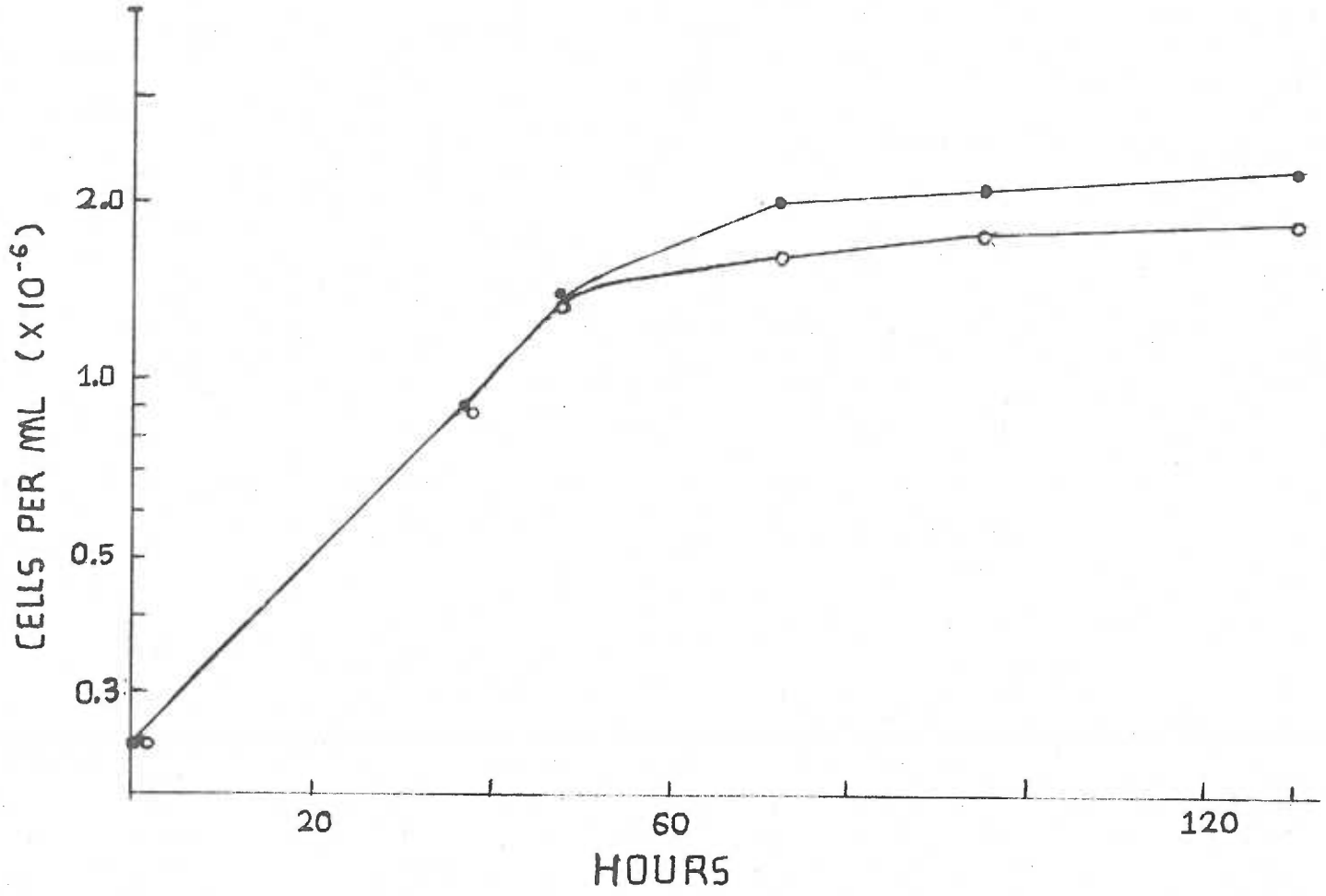


Figure 2. Growth curve of FLC in control and histidine-deficient medium. Solid circles, 1X histidine; open circles, 0.2X histidine. Each point is the average of duplicate determinations on duplicate cultures.

TABLE 2

Relative acceptance for histidine and leucine of tRNA from control and histidine-deprived FLC

Medium	Relative acceptance for[1] Histidine	Leucine	Acceptance for ^{14}C amino acid mixture (pmoles/A_{260} unit)
1X His	0.027±.002	0.099±.002	487± 2
0.1X His	0.051±.003	0.096±.004	571±13

[1]Results shown are the average of duplicate assays of single tRNA preparations ± 1/2 the range.

after 5 days of incubation. The data shows that $tRNA^{Leu}$ levels were increased by about 70% in leucine-deprived versus normal FLC tRNA preparations, but that histidine acceptance was not significantly altered in leucine-deprived versus normal FLC tRNA preparations.

C. Effect of Tryptophan Deprivation on Specific tRNA Levels in FLC

FLC were incubated in MEM containing normal (1X) or reduced (0.1X) concentrations of tryptophan. From the growth curves of these cultures (Fig. 3) it is apparent that reduction of tryptophan to 0.1X normal concentration has little or no effect on initial growth rate of FLC but, as did leucine deprivation, caused premature cessation of growth with significant decrease in the final cell density attained after 4-5 days of growth.

tRNA preparations were made from FLC after 120 hours incubation in MEM containing 1X or 0.1X tryptophan. The relative acceptances of these tRNA preparations for tryptophan and leucine are shown in Table 3 .

These data show that tryptophan deprivation causes the relative levels of $tRNA^{Trp}$ to increase by about threefold in the tryptophan-deprived versus the control cells after 5 days of culture. The relative levels of $tRNA^{Leu}$, however, showed no significant change as a consequence of tryptophan deprivation.

D. Relationship Between FLC Population Growth Phase and Response of $tRNA^{Trp}$ Levels to Tryptophan Deprivation

At what stage in the growth cycle of the FLC cultures do the amino acid-deprivation-induced changes in relative levels of tRNA species first become apparent? To answer this, FLC were incubated in MEM containing either the normal (1X) or reduced (0.1X) concentration of

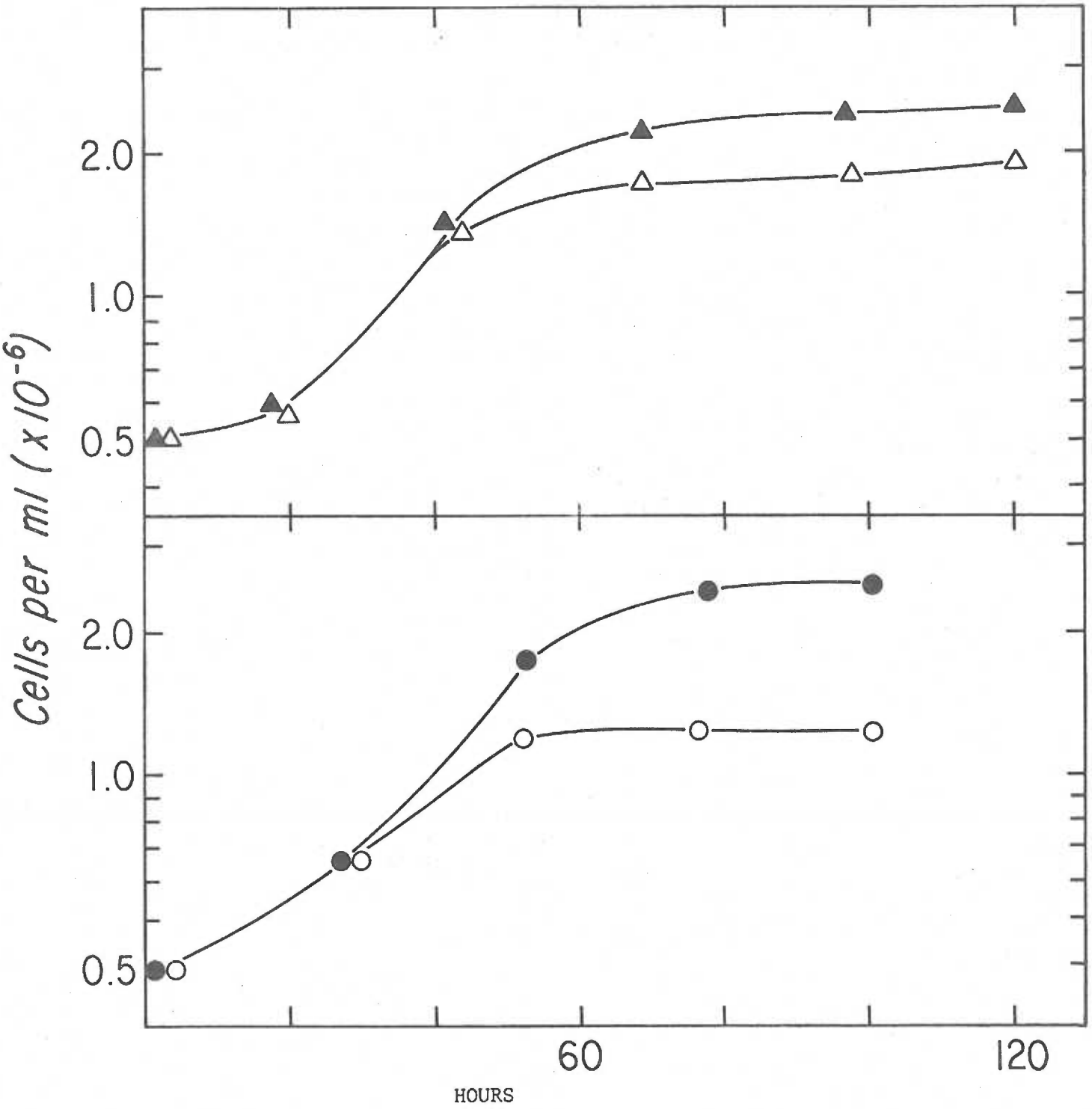


Figure 3. Growth curves of FLC in control and in leucine or tryptophan-deficient media. Solid circles, 1X leucine; open circles, 0.1X leucine. Solid triangles, 1X tryptophan; open triangles, 0.1X tryptophan. Each point is the average of duplicate determinations on duplicate cultures.

TABLE 3

Relative Acceptance for Leucine, Tryptophan and Histidine of tRNA from Control and Amino Acid Deprived FLC

Medium	Relative Acceptance For[1]			Acceptance for [^{14}C] Amino Acid Mixture (pmol/A_{260} unit)
	Leu	Trp	His	
1X Leu	0.089±.008		0.026±.004	470±30
0.1X Leu	0.152±.014		0.028±.002	399±40
1X Trp	0.100±.003	0.028±.003		389±40
0.1X Trp	0.111±.004	0.085±.004		323±40

[1]Results shown are the average of two independent experiments ± 1/2 the range.

tryptophan. Cell growth in both of these cultures was monitered and at various times, aliquots of culture were harvested, tRNA was prepared and assayed for relative tryptophan acceptance.

The results (Table 4) suggest that changes in relative $tRNA^{Trp}$ levels do not become apparent until about the third day of incubation, i.e. when the tryptophan-deprived cells have been in the stationary state of growth for about one day. A two to threefold increase in relative $tRNA^{Trp}$ is seen by the fifth day of culture, or after three days in stationary phase.

E. In Vivo Aminoacylation of $tRNA^{Trp}$ in FLC; Response to Tryptophan Deprivation

The goal of these experiments was to determine the stage in the growth cycle of tryptophan-deprived cells at which the in vivo extent of $tRNA^{Trp}$ aminoacylation begins to decrease, and the extent of that decrease. FLC were incubated in MEM containing 1X or 0.1X tryptophan and at various times aliquots of culture were harvested for RNA isolation and determination of the extent of aminoacylation of $tRNA^{Trp}$ as described in Materials and Methods, section D.

The results, given in Table 5, indicate that $tRNA^{Trp}$ in tryptophan-deprived FLC has undergone extensive deacylation, about 70%, by the second day of incubation in 0.1X tryptophan MEM, whereas $tRNA^{Trp}$ from cells incubated in 1X tryptophan MEM appeared to remain essentially fully aminoacylated.

F. Differential Responses Among Isoaccepting Cognate tRNAs to Amino Acid Deprivation

TABLE 4

Relative acceptance for tryptophan of tRNA from FLC incubated in 1X or 0.1X tryptophan for various periods

	Tryptophan concentration	Incubation time (days)	Cell density (cells/ml x 10^{-6})	Relative acceptance[1] for tryptophan
Experiment 1	1X	2	1.5	0.039±.003
		3	2.2	0.045±.006
		5	2.3	0.032±.003
	0.1X	2	1.4	0.041±.002
		3	1.7	0.042±.010
		5	1.9	0.064±.004
Experiment 2	1X	2	1.8	0.031±.002
		3	2.2	0.034±.005
		5	2.5	0.025±.001
	0.01X	2	1.5	0.032±.002
		3	1.7	0.045±.002
		5	1.8	0.086±.005

[1]Results shown are the average of duplicate assays of single tRNA preparations ± 1/2 the range.

TABLE 5

Extent of in vivo aminoacylation of $tRNA^{Trp}$ as a function of time of incubation in 0.1X or 1X tryptophan

	Tryptophan Concentration	Incubation time (days)	Cell Density (cells/ml x 10^{-6})	Percent in vivo[1] aminoacylation of $tRNA^{Trp}$
Experiment 1	1X	2	---	----
		3	2.2	86±5
		5	2.4	115±16
	0.1X	2	---	----
		3	1.6	29±4
		5	1.7	26±6
Experiments 2-3	1X	2	1.8	79±4
		3	2.3	121±18
		(5)[2]	(2.9)	(80±20)
	0.1X	2	1.6	31±5
		3	1.6	46±6
		(5)[2]	(1.9)	(15±1)

[1]Results shown are the average of duplicate assays of single tRNA preparations ± 1/2 the range.

[2]Results shown are from a third experiment (experiment 3).

Following the observations in FLC of the specific response in tRNA levels to amino acid deprivation, the question was raised on whether different isoacceptors of the respondent tRNA species were affected to the same extent. To answer this question, tRNA preparations from FLC grown to stationary phase in normal 1X, or 0.1X leucine were charged with isotopically-labelled leucine, one preparation (0.1X LEU) receiving [^{3}H]-leucine, and the other (1X LEU) receiving [^{14}C]-leucine (Materials and Methods, section F), and co-chromatographed on an RPC-5 column. The elution profile, given in Figure 4, shows two major species, peaks Ib and peak 3 and putative minor species, peaks Ia, 2, 3a, 4 and 5, all of whose elution positions were not affected by leucine deprivation. The $^{14}C/^{3}H$ ratio of the two major isoacceptor peaks were unchanged. However, minor species 2 was slightly reduced, and minor species 3a and 4 appeared enriched in the preparation made from leucine-deprived cells as compared with the preparation made from control cells. These differences were also seen in an RPC-5 profile of a run performed with the [^{3}H] and [^{14}C] isotope recipients reversed.

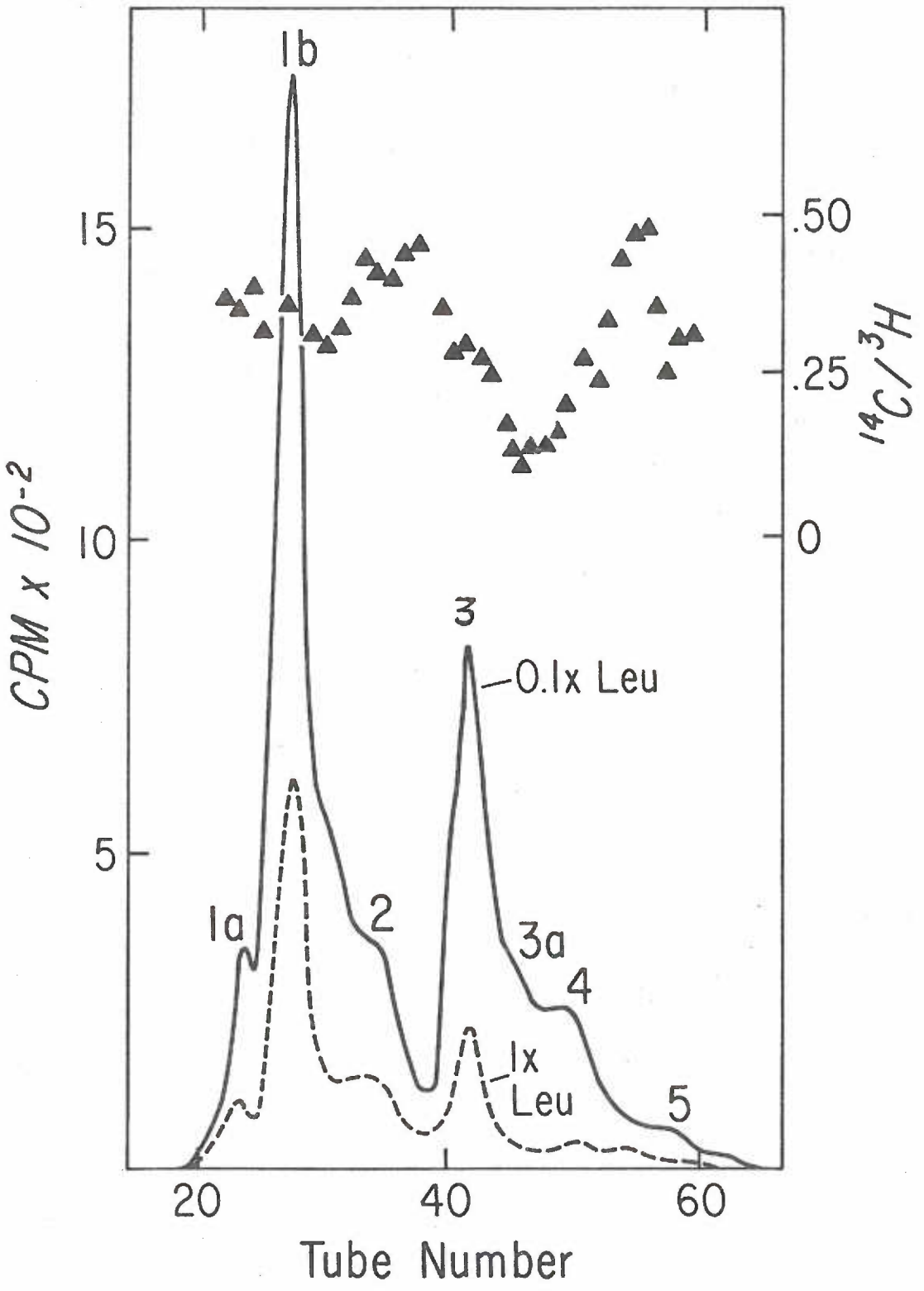
A similar experiment was done with histidyl-charged tRNA preparations from control and histidine-deprived FLC (Fig. 5). The elution profile shows one minor (peak I) and two major peaks, designated 2 and 3. In this experiment, the two major peaks elute later in histidine-deprived than in control tRNA preparations. Also, the ratio of peak 2 to peak 3 is significantly smaller in the tRNA from histidine-deprived cells versus the tRNA from control cells. This can be seen by the increased ^{3}H (1X HIS)/^{14}C (0.1X HIS) ratio of peak 2 relative to the $^{3}H/^{14}C$ ratios for peak 3 and for peak I.

These differences in elution position and relative peak ratios in

histidine-deprived versus control tRNA preparations were reproduced in RPC-5 runs performed with the isotopes reversed.

Figure 4. RPC-5 co-chromatography of ^{3}H leucyl tRNA from FLC grown in 0.1X leucine (solid line) and ^{14}C leucyl tRNA from cells grown in 1X leucine (dashed line). $^{14}C/^{3}H$ ratio is shown by triangles. Column size; 0.6 x 10 cm. tRNAs were chromatographed at a flow rate of about 45 ml per hour at 37°C with a linear gradient (total volume 150 ml) of 0.5 to 0.7 M NaCl containing 10mM magnesium acetate, 1mM EDTA and 10 mM sodium acetate, pH 4.5. 1.5 ml fractions were mixed with 15 ml toluene-Triton X-100 scintillation fluid and counted with a liquid scintillation counter at ^{3}H settings of 90% gain, 50-400 lower and upper discriminator limits, and at ^{14}C settings of 6%, 90-1000. ^{3}H counts were subsequently corrected for spillover of ^{14}C counts into the ^{3}H channel.

Figure 5. RPC-5 co-chromatography of ^{3}H histidyl-tRNA from FLC grown in 1X histidine (solid line) and ^{14}C histidyl-tRNA from cells grown in 0.1X histidine (dashed line). Triangles show the $^{3}H/^{14}C$ ratio. Other details of chromatography were the same as for Figure 4.


Ib
15
10
5
.50
.25
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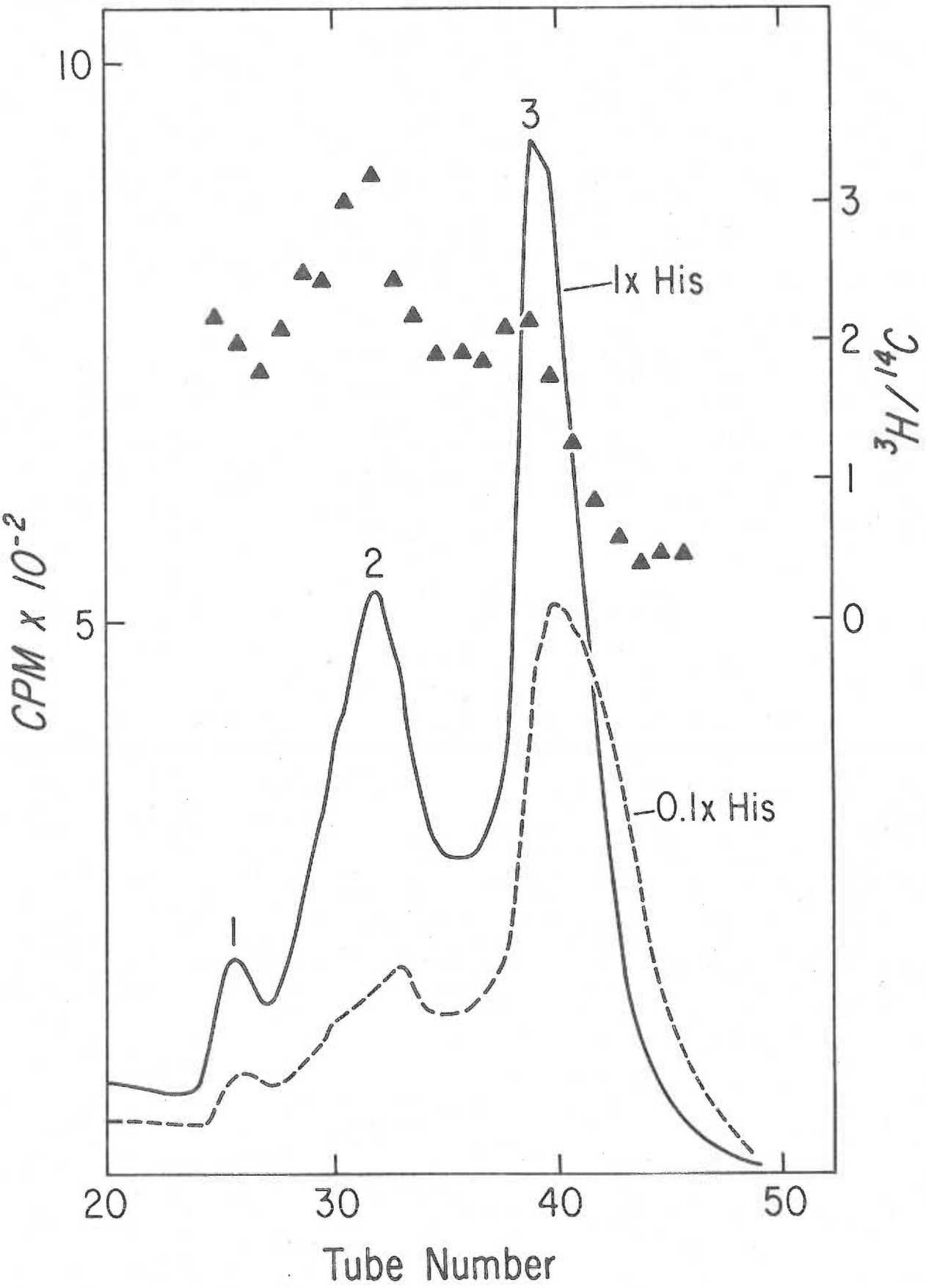
CPM x 10-2
14C/3H
3
0.1x Leu
Ia
2
3a
4
1x
Leu
5
20
40
60
Tube Number


10
5
20
30
40
50
Tube Number
CPM x 10-2
3H/14C
3
2
0
1
1x His
0.1x His
1
2
3

IV. DISCUSSION

The experiments reported in this thesis concern studies on how levels of specific tRNAs may be regulated to correlate with the use of their cognate amino acids in protein synthesis. Feedback control of tRNA levels by their extents of aminoacylation has been proposed. Specifically, increased proportional levels of specific tRNAs were predicted to follow decreased extents of their aminoacylation. Selective amino acid deprivation was employed to reduce the extent of aminoacylation of specific tRNAs in FLC cultures.

The production of selective increases in tRNA levels by deprivation of their cognate amino acids has been confirmed in FLC for three different tRNA species, $tRNA^{His}$, $tRNA^{Leu}$ and $tRNA^{Trp}$ by studies in our laboratory[2].

In the case of tryptophan (the only amino acid tested so far) the increase in $tRNA^{Trp}$ levels was preceded by extensive decline in the extent of aminoacylation of $tRNA^{Trp}$, from about 80-100% in control cultures to approximately 25-35% in tryptophan-deprived FLC. These results are summarized in Figure 6.

Measurements of <u>in vivo</u> aminoacylation must be approached with caution because of the potential for systematic error through failure (23) to rapidly and evenly halt aminoacyl-tRNA metabolism. If protein synthesis is halted before the stopping of tRNA aminoacylation, artificially high tRNA charging levels will result. Conversely, if protein synthesis inhibition lags behind the inhibition of tRNA aminoacylation, tRNA charging levels will be erroneously low. The second type of error is probably not significant in our experiments, as the aminoacylation of

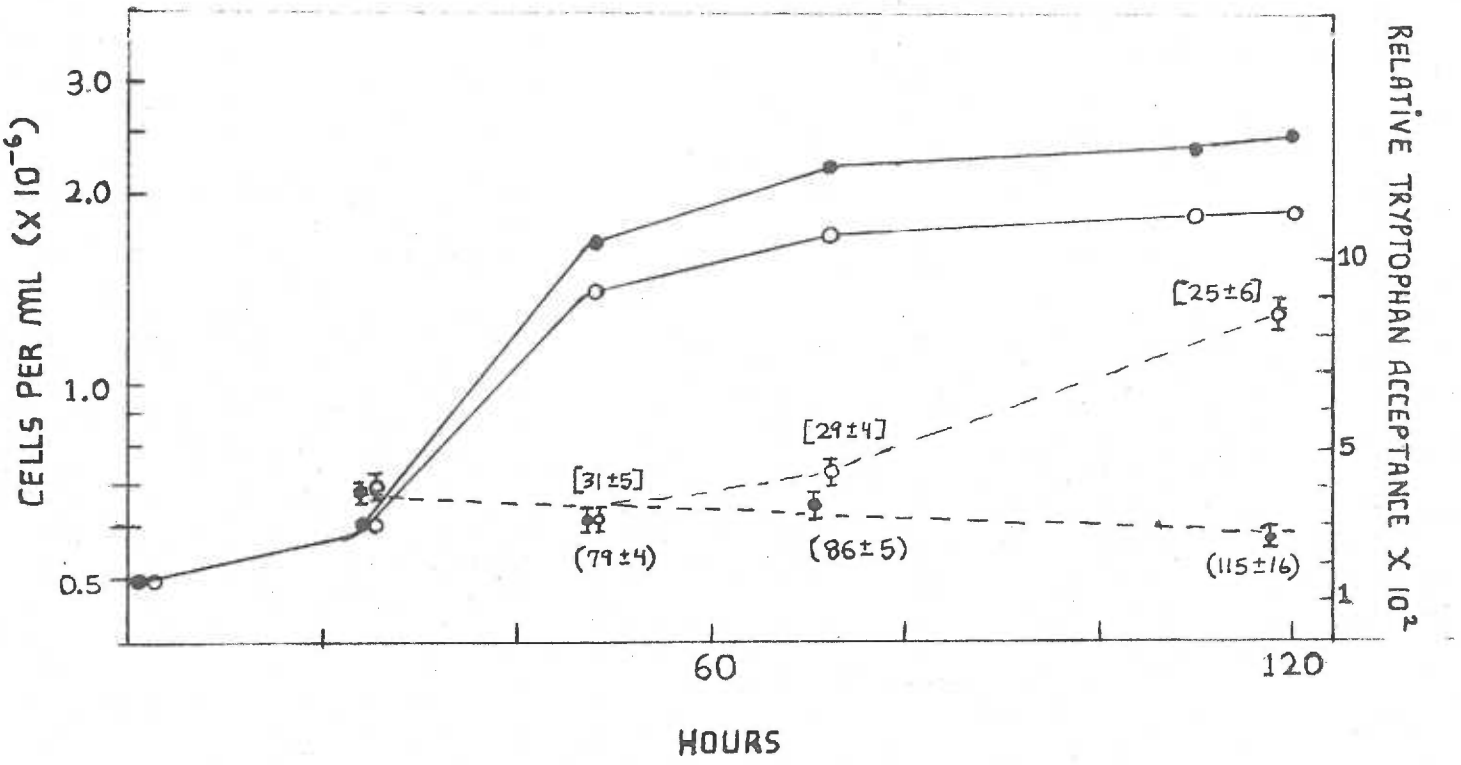


Figure 6. Cell growth curves, in vivo $tRNA^{Trp}$ charging levels and relative tryptophan acceptance of tRNA at various times during tryptophan deprivation. Solid lines, cell growth curves (left ordinate); broken lines, relative tryptophan acceptance curves (right ordinate). Solid circles, 1X tryptophan; open circles, 0.1X tryptophan. In vivo charging levels are indicated by numbers enclosed in parenteses (1X Trp.) or in brackets (0.1X Trp.). Tryptophan acceptance values derive from Table 4. Charging values are the average of duplicate assays of single tRNA preparations ± 1/2 the range. Growth curves are derived from cell density values in Table 4.

$tRNA^{Trp}$ from FLC control cultures appeared essentially complete (80-100%). However, error of the first type is not ruled out and the in vivo aminoacylation values determined in our studies therefore represent maximal values for both control and tryptophan-deprived FLC.

Systematic error potential in periodate oxidation of tRNA in heterogeneous RNA preparations has been discussed (14) (15) (21). Incomplete oxidation of uncharged tRNA could produce erroneously high apparent charging levels. Periodate-labilization of the aminoacyl ester bond in charged tRNA could render these tRNAs open to periodate attack and lower the apparent extent of aminoacylation. These errors cannot be ruled out of our measurements, but if present, would affect the in vivo extent of aminoacylation values for $tRNA^{Trp}$ from both the control and the tryptophan-deprived FLC, and would tend to cancel each other out. The observation of reduced extent of aminoacylation of $tRNA^{Trp}$ preceding the relative elevation of $tRNA^{Trp}$ levels in tryptophan-deprived FLC supports the theory that charging levels of tRNA species may exercise feedback control on their steady-state proportional levels. Whether this control is exercised on their relative rates of biosynthesis, or rates of degradation cannot be determined from the available data.

Control of tRNA levels through their relative rates of biosynthesis is suggested in studies by Fournier et al. on the developing silkworm B. mori. They found that the rates of synthesis of tRNA species correlated with their proportional levels, whereas half-life times were roughly similar, around 100 hours, for all tRNA species examined (27). Experiments are underway in our laboratory to ascertain whether specific amino acid deprivation in FLC produces increased relative rate of bio-

synthesis of its cognate tRNA species along with its proportional increase in the tRNA population. These experiments basically involve pulse-labelling control and amino acid deprived FLC with [^{3}H] uridine, and measuring the [^{3}H] specific activity of purified tRNA species cognate and non-cognate to the deprived amino acid. If increased relative biosynthesis rate is responsible for the observed elevation in cognate tRNA levels in amino acid deprived tRNA preparations, these preparations should show increased [^{3}H] specific activity of the cognate relative to the non-cognate tRNA species, whereas [^{3}H] specific activities should be similar for both cognate and non-cognate tRNA species in the control preparation.

Klyde and Bernfeld (12) reported no relative increase in the rate of $tRNA^{Ser}$ biosynthesis accompanying the observed elevation of $tRNA^{Ser}$ levels in chick hepatocytes synthesizing serine-rich phosphoproteins. They suggested that tRNA labels might be regulated through their relative rates of postranscriptional processing or degradation. These findings conflict with those of Fournier, et al.(27). These disparate reports may represent tissue-specific or tRNA species specific differences in the regulation of tRNA levels. Another possibility is that $tRNA^{Ser}$ in chicken liver may have metabolic roles unrelated to translation and be regulated through mechanisms unapplicable to other tRNA species. Participation of specific tRNAs in a wide variety of cellular processes has been well documented (5)(6)(22)(28).

This discussion now concerns the second objective of this thesis: to determine if amino acid deprivation effects different isoacceptors of the cognate tRNA species to the same extent.

RPC-5 column chromatography was used to fractionate aminoacylated

isoaccepting tRNAs. Elution position of an isoacceptor is determined by its three-dimensional conformation, hydrophobic and hydrophilic properties. These features are conferred by its primary nucleotide sequence, nature and extent of base modification, and the esterified amino acid. Two isoacceptors may differ in their primary nucleotide sequences, in which case they represent separate gene transcripts (9). Or, they may differ solely in their extents of base modification. This is reported to be the case for two isoacceptor fractions of $tRNA^{Asp}$ in Drosophila (24) and a transformed mouse fibroblast cell line (25), which are thought to represent products of the same gene, differing only in possession of a modified guanosine "Q".

The RPC profiles of FLC Leu-$tRNA^{Leu}$ and His-$tRNA^{His}$ showed respectively five and three distinct isoaccepting species. It is not yet known whether the multiplicity in isoacceptors stems from differences in primary nucleotide sequences, or differences in base modification, or both. Thus, the observed amino acid deprivation-induced alterations in leucine (Fig.4) and histidine (Fig. 5) isoacceptor patterns can be interpreted in several ways, providing some assumptions are made on the nature of the difference between the isoacceptors of interest.

Leucyl-tRNAs from FLC grown in 1X or 0.1X leucine, when co-chromatographed on RCP-5 showed changes in proportions of three minor species (Fig.4), with the major species apparently unaffected. Histidyl-tRNA from histidine-deprived versus control FLC showed changes in proportions and shifts in elution positions of the two major isoaccepting species (Fig. 5).

A possible explanation for the observed changes in peak proportions

assumes that the effected isoacceptors have the same primary nucleotide sequence with changes in isoacceptor proportion representing changes in postranscriptional modification in amino acid deprivation. This hypothesis is used to explain the change in proportion of the two $tRNA^{Asp}$ isoacceptors in transformed mouse fibroblasts under different physiological conditions (25) and in Drosophila at different stages in development (24).

Alternatively, the effected isoacceptors could represent different primary sequences. In this case, changes in isoacceptor proportions would represent changes in proportions of different gene products accompanying amino acid deprivation. In both hypotheses, minor shifts in elution position are most likely due to changes in post transcriptional modification of tRNA from cells in different physiological states. Such effects have been well documented (5)(25)(26).

V. SUMMARY

Incubation of Friend virus-infected murine leukemia cells in medium containing reduced amounts of histidine, leucine, or tryptophan produces premature cessation of cell population growth. This cessation of growth is followed by extensive increases in the relative levels of the tRNA species cognate to the amino acid in short supply, with no changes in the relative levels of non-cognate tRNA species. At least in the case of tryptophan, the only amino acid tested so far, tryptophan-induced increase in $tRNA^{Trp}$ proportional levels is preceded by extensive decline in the extent of aminoacylation of that species. These observations support the hypothesis that charging levels of tRNA species may exercise feedback control on their proportional levels in the tRNA population. Whether this control is exercised on their relative rates of biosynthesis or degradation is not indicated from the data. Experiments are underway in our laboratory to approach this question.

FOOTNOTES

1. L. Thompson, personal communication.

2. From data presented in two papers from our laboratory; one in press (Science) and the other submitted for publication.

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