A STUDY OF THE POSSIBLE ROLE OF TRANSFER RNA IN THE REGULATION OF ENZYME SYNTHESIS IN NEUROSPORA

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ABSTRACT

In several organisms, developmental transitions are accompanied by transfer RNA (tRNA) alterations. These alterations are usually observed in the chromatographic profile of amino acyl-tRNA specific for one or more amino acids and are of interest because of their possible significance in the regulation, at the translation level, of specific protein synthesis and cell differentiation. I have investigated whether such alterations accompany the biochemical differentiation of vegetative cultures of Neurospora crassa which occurs in response to "hard-times," e.g., starvation or inhibition by amino acid analogs or cycloheximide. The synthesis of tyrosinase is a well-known characteristic of this developmental transition.

After determining the conditions required for the complete charging of all 20 amino acids to Neurospora tRNA, I compared the chromatographic profile on methylated albumin-Kieselguhr columns of amino acyl-tRNA's from vegetative cultures to those of cultures which were derepressed for tyrosinase with ethionine, a methionine analog. No qualitative tRNA alterations were observed; the same number of components for each amino acid were found in cultures of both developmental states and they had the same chromatographic mobilities. However, quantitative changes of acceptor activity were observed for several amino acids. The time course of the pattern of quantitative alteration suggests that the observed changes result from partial ribonuclease digestion of the tRNA complement. I believe this ribonuclease

is synthesized in response to the deprived environment and its function is to hydrolyze the RNA which is present in excess, in order that the catabolic products may be used as building blocks for the synthesis of other kinds of molecules.

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INTRODUCTION

Transfer RNA Alterations and Differentiation

Alterations in the chromatographic profile of amino acyl-transfer RNA (tRNA) accompanying certain developmental changes have been observed recently in several organisms, ranging from bacteria to mammals (1-12). The usual technique used to make such observations is co-chromatography on methylated-albumin-Kieselguhr (MAK) columns of a C^{14}-labeled amino acid attached to tRNA taken from one physiological or developmental state of an organism and tRNA taken from some other state attached to the same amino acid labeled with tritium (H^3). In the most intensively investigated case, Sueoka and his co-workers found that the chromatographic pattern of E. coli tRNA specific for 1 of 17 amino acids tested, leucine, is altered after T_2 infection (8). In the other systems investigated the amino acyl-tRNA affected is not necessarily leucyl-tRNA.

In their original report (13), Sueoka and Kano-Sueoka proposed a theory, the "adaptor-modification" hypothesis, in which tRNA alterations control major metabolic transitions at the translational level. According to this theory, changes within the set of tRNA adaptors specific for a single amino acid will affect the relative efficiency of translation of the degenerate messenger RNA (mRNA) codons for that amino acid and therefore regulate protein synthesis. By eliminating, for example, the activity of a tRNA species for decoding one (or a few)

codewords for a particular amino acid, all messengers containing that codeword would be untranslatable and whole classes of proteins could not be synthesized.

It has become customary in reports of tRNA alterations during differentiation to suggest such a regulatory role for these alterations and to refer to Sueoka's theory. However, finding an alteration in the chromatographic profile of a particular species of tRNA accompanying developmental changes does not, by itself, establish a regulatory role for this alteration. In order to establish such a role it is necessary to show that this alteration significantly changes the relative efficiency of translation of the degenerate codons which specify the particular amino acid. This is so because it seems probable that chemical modification of tRNA molecules which do not affect the enzyme recognition site or the codon binding site can alter the chromatographic mobility of the molecule without changing its translating efficiency or specificity.

Only the Lee and Ingram group and the Sueoka group have made any attempt to test whether their observed tRNA changes might have any regulatory significance. Lee and Ingram have reported that the coding behavior of the two methionyl-tRNA's present in embryonic chick erythrocytes, one of which is present in adult reticulocytes only as a trace component, is identical (14). The relative loss of one methionyl-tRNA component during differentiation does not appear to affect the translation capability of avian red blood cells. The report, if confirmed,

tends to disprove any regulatory role for this tRNA alteration in this system.

By investigating the time course of the leucyl-tRNA alteration during phage infection, Sueoka and Kano-Sueoka determined that it occurred too early (2 minutes after infection) to play a role in regulating the transition from early to late functions but could act in the "shutting off" of host protein synthesis (8). The coding behavior of each of the leucyl-tRNA components before and after infection was examined by Sueoka *et al.* in collaboration with Nirenberg using the ribosome binding assay. Based on the available data Sueoka *et al.* claim that "...there is a distinct difference in the codon response pattern (of leucyl-tRNA fractions) before and after infection" (15).

In summary, developmental changes in several organisms are accompanied by alterations in the chromatographic profile of amino acyl-tRNA; a regulatory role for these alterations has been proposed but in no case has such a role been firmly established.

In *principle* a limiting concentration of a single tRNA component could be involved in regulating protein synthesis. It has been reported (16) that a limiting concentration of the arginine-tRNA acceptor activity which responds to the codons AGA and AGG can regulate *in vitro* the rate and amount of polypeptides synthesized under the direction of the synthetic messenger poly AG in an *E. coli* system.

In addition to the possible regulatory significance of tRNA alterations, there is the problem of the mechanism which produces them. Two divergent viewpoints have been expressed and some evidence has

been presented to support each of them. Sueoka and Kano-Sueoka believe that tRNA changes result from chemical modification of pre-existing tRNA molecules because continued protein synthesis is required for the alteration of E. coli leucyl-tRNA following T_2 infection and because the relative amount of total leucine acceptor activity remains constant (8). Weiss and his co-workers (17) investigating the same system report that they have detected, by hybridization, the synthesis of phage specific tRNA. Subak-Sharpe et al. (18) have, in a mammalian virus system, also found evidence after infection for tRNA which preferentially hybridizes to viral DNA. This evidence supports the model, suggested by Doi et al. (2) of selective transcription of different tRNA genes as the mechanism of altered amino acyl-tRNA profiles.

It is certainly the case that several mechanisms are known for the chemical modification of tRNA. These include methylation (19), thiolation (20), oxidation (21), deamination (22), and partial ribonuclease digestion (23). Both of these modifications is capable of affecting the chromatographic profile of at least one amino acyl-tRNA (19-23).

The functional significance even of methylation, the most thoroughly examined mechanism of tRNA modification, is still not clear. The results of studies of the coding of certain tRNA's as a function of the degree of methylation are not entirely clear but do suggest, for example, that the fidelity of translation of normally methylated tRNA is better than that of undermethylated tRNA (19, 24, 25). Finally,

Borek and his group have shown changes in the methylating capacity and specificity of phage-infected bacteria and in mammalian tumors (26).

Sexual Differentiation in Neurospora and the Synthesis of Tyrosinase

In the fall of 1964, shortly after Sueoka and Kano-Sueoka's original report of the modification of E. coli leucyl-tRNA upon phage infection (13) I decided to investigate whether a similar alteration was involved in the differentiation of the eukaryote, Neurospora crassa. The developmental phenomenon to be examined was the response of vegetative mycelium to "hard-times," i.e., starvation in buffer, addition of amino acid analogs or inhibition of protein synthesis with cycloheximide. Starvation on solid medium results in sexual differentiation, the formation of protoperithecia (27). Protoperithecia do not form in liquid culture, and biochemical operations using cultures growing on solid medium are not convenient. However, since certain of the enzymes characteristic of sexually differentiated cultures, e.g., tyrosinase (28), are made in liquid culture in response to "hard-times" (29) it is presumed that the same mechanisms are operating but that the entire process cannot be completed in liquid culture. This view is supported by the fact that two mutants ty-1 and ty-2 which in liquid culture do not respond to starvation by synthesizing tyrosinase, when tested on solid media are female-sterile; they cannot form protoperithecia (29).

Transfer RNA Alterations in Neurospora During Differentiation

I thought it possible that the regulation of this system occurs

at the translational level because a major shift in the metabolic pattern of the culture is involved and because the known ways of stimulating this shift all involve reducing the rate of functional protein synthesis.

After initially determining the conditions required for the complete charging of Neurospora tRNA with the 20 amino acids, chromatographic comparisons were made between amino acyl-tRNA from vegetative, control mycelium and amino acyl-tRNA from cultures which were stimulated to synthesize tyrosinase by treatment with ethionine, a methionine analog which is incorporated into Neurospora protein (30).

Alterations in the chromatographic profile of tRNA of the type reported for other systems, i.e., changes in the number or mobility of components, were not found in this system for any of the 20 amino acids of protein. However, significant loss of total tRNA was observed following ethionine treatment as were changes in the specific acceptor activity for several amino acids. The decline of total tRNA is correlated with a decrease in the rate of tRNA synthesis and an abundance in differentiated cultures of ribonuclease activity. The observed quantitative tRNA changes are probably not directly involved in regulation.

MATERIALS AND METHODS

Neurospora Strains

Wild-type strains of Neurospora crassa, 69-1113a and 74A, are used exclusively in these experiments. Strain 69-1113a is used as the source for tRNA and is able to produce high levels of tyrosinase (31); amino acyl-tRNA synthetases are extracted from 74A because, when grown exponentially, it is virtually free of ribonuclease. (I do not know whether this property is a general feature of exponential cultures of Neurospora, or whether it is peculiar to this strain.)

Chemicals

In Table 1 I have summarized the source and grade of the critical chemicals used in this work. All other chemicals used were reagent grade or the best grade available.

The liquefied phenol (88%) is redistilled before use and stored as the water-saturated solution in sealed brown bottles at -20°C.

The scintillation fluid I used contains 1 part Triton x-100 to 2 parts of a toluene solution containing 2,5-diphenyloxazole (PPO) as the primary fluor and 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) as a secondary fluor.

Table 2 is a summary of the radioactively-labeled amino acids and nucleosides used, including the supplier and specific activity of each.

TABLE 1. SOURCE AND GRADE OF CRITICAL CHEMICALS

Chemicals	Source	Grade
Dipotassium adenosine 5'-triphosphate (ATP)	Calbiochem	A
Bovine serum albumin (BSA)	Armour Pharmaceutical Company	Fraction V (Reagent for microbiological use)
Kieselguhr	Johns-Mansville	Hyflo-Supercel
Phenol	Mallinckrodt	Analytical Reagent
Trisodium 2-phosphoenolpyruvic acid (PEP)	Calbiochem	A
2,5-Diphenyloxazole (PPO)	New England Nuclear	Scintillation
1,4-Bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP)	Packard Instrument Company	Scintillation
Triton x-100	Rohm and Haas	Commercial
Sucrose	C and H	Table Sugar

TABLE 2. SUPPLIERS AND SPECIFIC ACTIVITIES OF RADIOACTIVE COMPOUNDS

Compound	Position of label	Supplier	Specific activity (mC/m mole)
L-alanine	C^{14} (U)	S	50
"	3-H^3	S	4800
L-arginine	C^{14} (U)	S	50
"	H^3 (U)	S	600
L-asparagine	C^{14} (U)	NC	102
"	H^3 (U)	NC	155
L-aspartic acid	C^{14} (U)	S	50
"	2,3-H^3	NS	70.8
DL-cysteine	3-C^{14}	NC	33.8
L-ethionine	ethyl-1-C^{14}	NEN	3.66
"	ethyl-1-H^3	NEN	70.7
DL-p-fluorophenylalanine	3-C^{14}	C	4.9
L-glutamic acid	C^{14} (U)	S	50
DL-glutamic acid	3-H^3	NEN	27×10^3
L-glutamine	C^{14} (U)	NEN	52.3
Glycine	C^{14} (U)	S	50
"	2-H^3	S	1300
L-histidine	C^{14} (U)	S	50
"	H^3 (U)	S	4700
L-isoleucine	C^{14} (U)	S	50
"	H^3 (U)	S	385
L-leucine	C^{14} (U)	S	50
"	4,5-H^3	S	2000
L-lysine	C^{14} (U)	S	50
"	H^3 (U)	S	480
L-methionine	methyl-C^{14}	NC	27.6
"	methyl-H^3	NC	1370

TABLE 2 (continued)

Compound	Position of label	Supplier	Specific activity (mC/m mole)
L-phenylalanine	C^{14} (U)	NEN	366
"	H^3 (U)	S	2500
L-proline	C^{14} (U)	S	50
"	H^3 (U)	S	2000
L-serine	C^{14} (U)	S	50
"	H^3 (U)	S	330
L-threonine	C^{14} (U)	S	50
"	H^3 (U)	S	675
L-tryptophan	side chain 3-C^{14}	NEN	21.8
"	H^3 (U)	NC	500
L-tyrosine	C^{14} (U)	S	50
"	H^3 (U)	S	500
L-valine	C^{14} (U)	S	50
"	H^3 (U)	S	870
Guanosine	H^3 (U)	S	1500

The symbol (U) following isotope indicates uniformly labeled material. Abbreviations of suppliers: S is Schwartz Bioresearch; NC is Nuclear-Chicago; NS is Nuclear Supplies; NEN is New England Nuclear; and C is Calbiochem.

Neurospora Culture

The 2 wild-type strains are maintained on slants of Horowitz complete medium (32). For extraction of tRNA, strain 69-1113a is cultured according to the "short term procedure" developed by Pall (33). A conidial suspension is prepared by adding 10-15 ml sterile distilled water to a large slant (150 mm x 18 mm) of a conidiating culture. Five to seven drops of this suspension are used to inoculate each 125 ml Erlenmeyer flask containing 20 ml of "subminimal" medium [0.5X Vogel's salts, medium N plus biotin (34) and 0.5% (w/v) sucrose]. The flasks are incubated at 25 ± 1°C for 48 hours and a mycelial pad forms in each flask. The flasks are then placed on a reciprocal shaker (60-70 excursions per minute). After 24 hours of shaking, 2 mg of DL-ethionine dissolved in 1 ml sterile distilled H_2O is added to each flask.

Strain 74A is grown exponentially according to a modification of the methods of Luck (35) and Davis and Harold (36). Large amounts of conidia are produced by growing cultures on 40 ml of solid medium in 250 ml Erlenmeyer flasks (1.0X Vogel's salts + biotin, 2% sucrose, 2% Bacto-Agar) in constant light. The total yield of conidia from 3-250 ml flasks is suspended in 50 ml sterile distilled water, filtered through glass wool and immediately used to inoculate a 2500 ml low-form culture flask containing 750 ml of minimal medium (1X Vogel's salts plus biotin and 2% sucrose). These flasks are placed on a reciprocal shaker (approximately 150 excursions per minute) and the mycelium is harvested after 18 hours, near the end of the exponential phase of growth.

Extraction Procedures

Tyrosinase and Total Soluble Protein

Crude extracts are used for the assay of tyrosinase and total soluble protein. Individual mycelial pads are washed with distilled water, pressed dry between paper towels and ground in a mortar and pestle with sea sand. The ground mycelium is extracted with 0.1 M sodium phosphate buffer pH 6.0 (10 ml/g wet weight) and centrifuged at 12,000 x g for 10 minutes at 4°C. The pellet is discarded and the supernatant taken as the crude extract.

Amino Acyl-tRNA Synthetase

The synthetase enzymes are prepared from freshly harvested exponential mycelium according to the method developed for *Neurospora* by Barnett (37) except that the dialyzed preparation is stored as a 50% solution in glycerol solution at -20° C.

Soluble RNA

I developed a procedure for extracting the soluble RNA (sRNA) from *Neurospora* combining aspects of the *E. coli* procedure of von Ehrenstein and Lipman (38) and the Holley *et al.* procedure for yeast (39). The proportions indicated are those used for the extraction of mycelium from one basket of 125 ml flasks (45 flasks, approximately 6 g wet weight). Mycelium is collected on filter paper in a large Buchner funnel in the cold (4°C), washed with ice-cold distilled water, pressed dry and stored at -80°C. The frozen material is ground in a cold

mortar and pestle with sea sand in the 4°C cold room. To the ground mycelium 25 ml of water-saturated redistilled phenol and 25 ml of ice-cold buffer are added with vigorous stirring. The buffer used is 0.001 M Tris-HCl, pH 7.3 containing 0.01 M magnesium acetate [$Mg(Ac)_2$]. The homogenate is transferred to a 125 ml screw-cap flask, and placed on a rotary shaker (top speed) for 1 hour at 25°C. The mixture is transferred to 2 centrifuge tubes and spun at 10,000 x g for 30 minutes. (All centrifugation steps in this procedure are performed in a Sorvall RC-2B at 4°C using the SS-34 rotor.) The upper, aqueous phase is removed by pipette and kept at 4°C. The lower, phenol layer is re-extracted with a small volume of buffer (5 ml/tube) and spun at 10,000 x g for 15 minutes. The aqueous phase is again removed and added to the first aqueous phase. To the pooled aqueous phase, 0.1 volume of 20% (about 2 M) potassium acetate (KAc), pH 5.1 is added and the RNA is precipitated with 2 volumes of 100% ethanol at -20°C. After two hours the precipitate is collected by centrifugation (10,000 x g for 15 minutes), washed with cold 67% ethanol and resuspended in 10 ml of 0.5 M Tris-HCl, pH 8.8 - 0.1 M $Mg(Ac)_2$. The RNA is incubated for 45 minutes at 37°C to remove attached amino acids by saponification and is again precipitated by the addition of 1/10 volume 20% KAc, pH 5.1 and 2 volumes of ethanol. The precipitate is left overnight at -20°C, collected by centrifugation and suspended in saline buffer [1.0 M NaCl, 0.2 M Tris-HCl, pH 7.3, 0.1 M $Mg(Ac)_2$]. This operation defines soluble RNA; high molecular weight RNA is insoluble in 1 M NaCl. Insoluble (ribosomal and other high molecular weight) RNA is pelleted by

centrifuging the saline suspension at 48,000 x g for 30 minutes. The supernatant containing the soluble RNA is carefully decanted and extracted in a separatory funnel twice with an equal volume of anhydrous ether to remove any remaining phenol. Residual ether is removed by bubbling N_2 through the solution. The RNA is again precipitated with KAc and ethanol for 2 hours as above. The precipitate is washed by suspension and centrifugation twice with 80% ethanol (20 ml), twice with 95% ethanol (20 ml), and dried in a vacuum dessicator over KOH pellets. The dried material is then suspended in 5-10 ml of appropriate buffer and the concentration measured by the ultraviolet absorption at 260 mμ of a 1/100 dilution. The RNA solution, stored at -20°C, does not appear to be affected adversely by freezing and thawing.

Roberts' Procedure for Fractionating Mycelium

A modification of the fractionation procedure designed for *E. coli* by Roberts (40) is used for the analysis of amino acid and nucleoside incorporation experiments and for quantitative measurements of total nucleic acids. This procedure separates 4 main fractions from the mycelium: cold acid soluble, hot acid soluble, ethanol-ether soluble, and alkali soluble compounds. The mycelium of a single flask is collected in a Buchner funnel and the culture medium filtrate is collected and saved. The mycelial pad is washed with cold distilled water and dried by suction. The following steps extract these fractions of interest from the mycelium without grinding the entire pad. In a centrifuge tube (Nalgene - 102 mm x 30 mm) the dried pad is

extracted with 4 ml of iced 5% perchloric acid (PCA) for 60 minutes. The pad is then pelleted by a brief centrifugation and the supernatant is collected. This cold acid extraction is performed twice more but with 2 ml each time and for 15 minutes. The three cold acid extracts are pooled. The pad is extracted for 30 minutes with 4 ml 75% ethanol at 50°-60°C and then for another 30 minutes with 4 ml of a 1:1 mixture of 75% ethanol and ether, also at 50°-60°C. The ethanol and ethanol-ether fractions are pooled and the pad is dried for 45 minutes at 60°C. The nucleic acids are extracted by incubating the mycelium with hot (90°-100°C) 5% PCA, first with 4 ml for 75 minutes followed by 2 ml for 15 minutes. The pad is then washed with a 1:1 mixture of ethanol: ether (to remove residual PCA) and dried at 60°C. Finally the mycelium is extracted overnight at room temperature with 4 ml of 3% NaOH. As a result of this procedure the culture is divided into five fractions. The cold acid extract contains small molecules, e.g., amino acid and nucleoside pools. The ethanol-ether fraction contains lipids. Nucleic acids including amino acyl- and peptidyl-tRNA are in the hot acid extraction, and the proteins are extracted by the NaOH treatment. The fifth fraction is the culture medium. The volume of each fraction is measured and an aliquot of each is examined for incorporation of radioactivity. Since PCA does not absorb in the 260 mμ region the amount of nucleic acid can be measured directly by the absorption at 260 mμ of the hot acid fraction.

Assays

Tyrosinase is measured in crude extracts by the method of Horowitz et al. (41). Soluble protein is measured by the biuret method reported by Gornall et al. (42).

Amino Acyl-tRNA Formation

The reaction mixture for the assay of amino acid acceptor activity includes in Tris buffer, generally, sRNA, an amino acid (labeled with H^3 or C^{14}), ATP, $Mg(Ac)_2$, dithiothreitol (DTT, a sulfhydryl reducing reagent), and an enzyme preparation. The proportions of these components are, for some amino acids, quite critical and will be discussed in RESULTS. Assays designed to measure total acceptor activity are performed with tRNA rate limiting and amino acid, ATP and enzyme in excess. Reactants, without enzyme, are mixed in the cold and then equilibrated at 30°C. The reaction is initiated by the addition of enzyme and is completed in 10-20 minutes. For analytical purposes, the formation of amino acyl-tRNA is measured by the amount of amino acid radioactivity rendered acid-insoluble using a filter paper procedure developed by Chambers (43). An aliquot of the reaction mixture is applied to a filter paper disc (Whatman 3MM, 2.3 cm diameter) and allowed to dry for a minute. The disc is then submerged in a beaker of cold 10% (w/v) trichloroacetic acid (TCA), which precipitates the tRNA within the fibers of the disc. The remainder of the procedure consists of a series of washings which remove from the disc, in order, free amino acid, TCA, and finally water. The disc is dried, placed in

a vial and counted with 5 ml of solution A scintillation fluid (described below). The control used is an identical reaction mixture without added tRNA; its radioactivity does not exceed 5% of the experimental value. In order to achieve this low background level for 2 amino acids, tryptophan and cysteine, it was necessary to replace the filter paper discs with glass fiber discs (Whatman GF/A).

The acid-insoluble radioactivity is expressed either as μμmoles of amino acid or as counts per minute (cpm). The molar value is calculated by multiplying the observed radioactivity (cpm) by a conversion factor (μμM/cpm) which is determined for each isotope, counted under each condition and in each machine used. Table 3 gives the calibration data for C^{14} amino acids on filter paper and glass fiber discs, using the Beckman LS 200 B and CPM 200 liquid scintillation counters. Tritiated amino acids are not used in experiments which require counting on discs because the efficiency of tritium counting, under these circumstances, is too low.

From such calibration data, an efficiency of counting can be calculated for C^{14} counted under different conditions and used to calculate a conversion factor for other C^{14} amino acids of different specific activity.

To prepare amino acyl-tRNA for chromatography the reaction is terminated by the addition of an equal volume of water-saturated phenol and the capped reaction vial is shaken for 7 minutes at 4°C. The mixture is then centrifuged at 5000 x g for 15 minutes, the aqueous phase is carefully removed with a disposable pipette and the charged tRNA is

TABLE 3. CALIBRATION DATA FOR CONVERSION OF CPM TO μμMOLES

A = μμmoles/disc	B = net cpm/disc	C = B/A = cpm/μμmole
5*	227	45.4
10	426	42.6
20	934	46.7
25	1151	46.04
50	2126	42.52
100	4346	43.46
	average =	44.5
5≠	311	62.2
10	628	62.8
20	1289	64.45
25	1598	63.92
50	3167	63.34
100	6508	65.08
	average =	63.6

* = C^{14} tryptophan on GF/A discs, counted in CPM 200

≠ = C^{14} glycine on 3MM discs, counted in LS 200 B

Radioactive amino acid, diluted to 1 μμM/μl, was pipetted onto discs and allowed to dry. To each disc 100 μl of diluted enzyme (1:10) was then added. The dried discs were counted in solution A. Each value in the table is the average of duplicate samples. The specific activity of the tryp was 48.4 dpm/μμM and the gly was 111 dpm/μμM. So, efficiency of counting on GF/A is 44.5/48.4 or 92.0% and for counting on 3MM is 63.6/111 or 57.3%.

precipitated by the addition of 1/10 volume 20% KAc, pH 5.1 and 2 volumes of cold absolute ethanol. After 2 hours at -20°C the precipitate is collected by centrifugation and dissolved in 50 ml of starting column buffer. It may be stored in this buffer indefinitely at -20°C.

MAK Chromatography

Chromatography of amino acyl-tRNA on methylated albumin Kieselguhr (MAK), described first by Sueoka and Yamane (44), is performed on columns prepared by a modification of the technique of Mandel and Hershey (45). Serum albumin is methylated for 5 days at 35° instead of 3 days at room temperature and is stored as a dried powder over KOH in a dessicator.

The column is prepared in a 22 mm diameter chromatography tube fitted at one end with a 19/22 outer glass joint. The inner joint has a sealed-in sintered glass disc and a variable bore stopcock (Kontes).

To prepare methylated albumin-coated Kieselguhr, 16 g of Kieselguhr is suspended in 80 ml of starting column buffer (0.05 M sodium phosphate, pH 6.3, 0.4 M sodium chloride). In early columns the initial buffer was 0.2 M NaCl. The suspension is boiled, to expel air, and then cooled. Another suspension, using 1 g Kieselguhr and 10 ml buffer is similarly boiled, cooled and used for the top layer of the column. To the cooled 80 ml suspension, 4.5 ml of a 1% solution (in water) of methylated albumin is added slowly with stirring. This precipitates the protein onto the Kieselguhr.

Before pouring the MAK a pad of powdered cellulose is formed on the sintered glass disc by suspending powdered cellulose in starting

buffer in the chromatography tube and then packing it by applying nitrogen pressure at 3 lbs/in^2 to drive the excess buffer down to the level of the packed material. The tube is then filled with MAK using a wide-bore pipette and similarly packed down with pressure. This process is continued until all of the MAK is poured. The 10 ml Kieselguhr suspension is then poured as an upper protective layer. The succeeding steps including washing, sample application and elution are all performed with an applied N_2 pressure of 3 lbs/in^2.

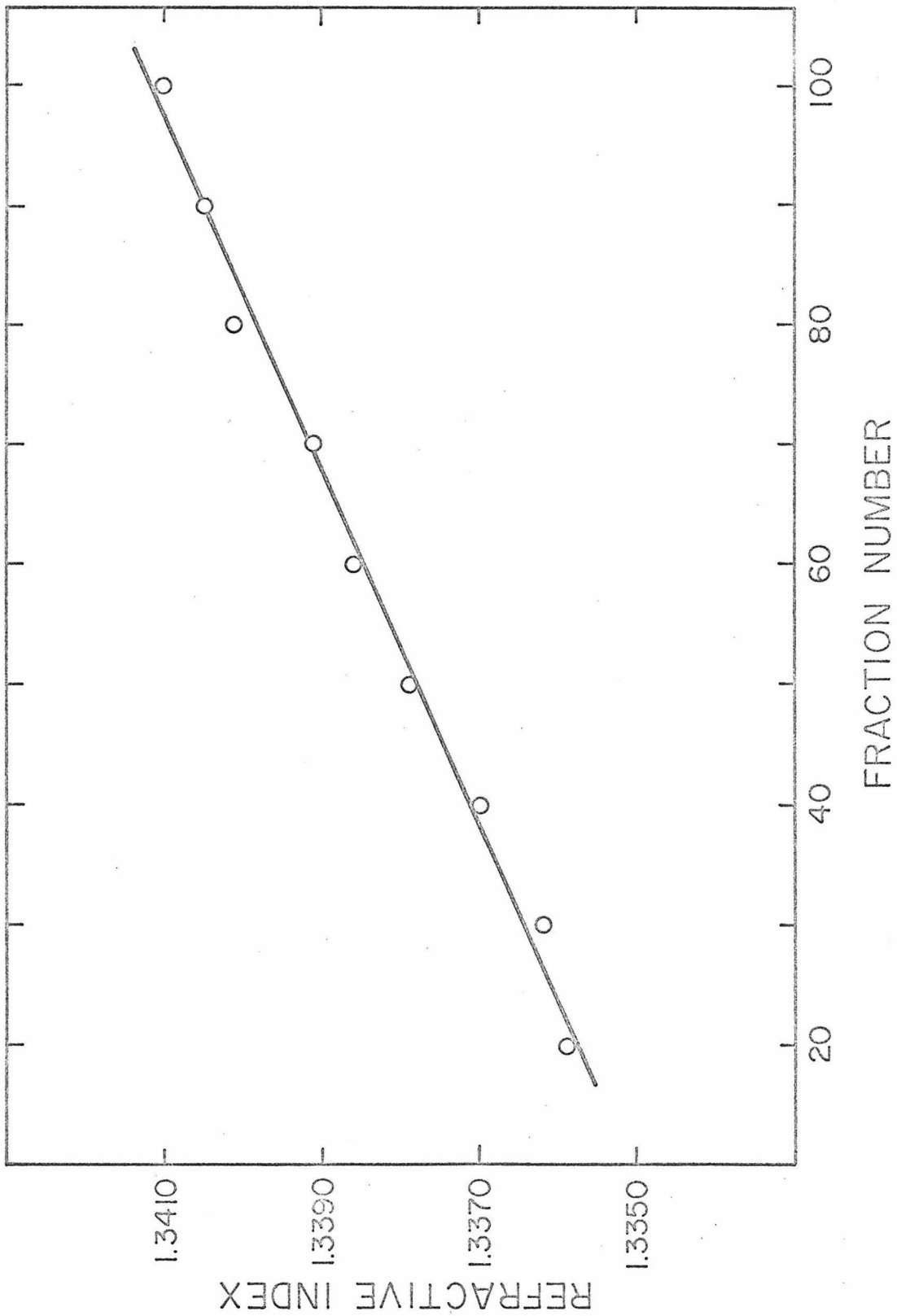
The prepared column is washed with 150 ml of starting buffer to remove excess protein. The sample (up to 2.5 mg tRNA) is applied in 50 ml starting buffer and the column is again washed with 150 ml of starting buffer to remove free amino acid, ATP and residual phenol. The tRNA is then eluted with a linear salt gradient from 0.2 M NaCl or 0.4 M NaCl to 1.0 M NaCl. The linearity of the gradient for a representative column was confirmed by measuring the refractive index of every tenth fraction (Figure 1).

The per cent transmission at 265 mμ of the effluent from the column is continuously monitored with a Gilson ultraviolet (UV) absorption meter and recorded on a Texas Instruments rectilinear recorder. The value for each fraction is converted to optical density [O.D. = $-\log_{10}$ (% T/100)].

Two ml fractions are collected at the rate of 30 fractions/hour. The entire chromatography procedure is performed at 18°C.

FIGURE 1. LINEARITY OF SALT GRADIENT

The refractive index of every tenth fraction of an MAK chromatogram was measured with a Zeiss refractometer. The initial buffer was 0.05 M sodium phosphate, pH 6.3, 0.2 M NaCl. The NaCl concentration of the final buffer was 1.0 M.


REFRACTIVE INDEX
1.3410
1.3390
1.3370
1.3350
20
40
60
80
100
FRACTION NUMBER

Scintillation Counting

All radioactivity measurements were made with a Beckman liquid scintillation counter, either model CPM 200 or LS 200 B. Filter paper or glass-fiber discs were counted in 5 ml of solution A [0.01% (w/v) dimethyl POPOP, 0.4% PPO (w/v) in toluene].

Doubly-labeled (C^{14} and H^3) samples from MAK chromatography were counted directly in a mixture of solution A and Triton x-100, a non-ionic detergent (46). Each 2 ml fraction was transferred to a vial containing 15 ml of a 2:1 mixture of solution A and Triton x-100 and 1 ml of distilled water. The aqueous phase enters a stable solution after vigorous shaking with a Vortex jr. mixer, and will remain in solution indefinitely as long as the temperature remains between 20° and 30°C. There are two main advantages to this "cocktail": 1) high counting efficiency and 2) ease of sample preparation. It is possible to count samples directly with this mixture because the increasing concentration of salt throughout the gradient has no effect on the counting efficiency, i.e., it does not increase the degree of quenching (Table 4). The actual proportions of the mixture were dictated by the large sample size and the high ionic strength.

The gain can be set such that equal amounts of C^{14} are recorded in channel A and channel B, but less than 0.1% of the H^3 counts in channel A are recorded in channel B. Thus the channel B counts are taken to be the C^{14} counts and the tritium counts are the total channel A counts minus the channel B counts.

TABLE 4. EFFECT OF NaCl ON COUNTING EFFICIENCY

NaCl molarity	Efficiency (in %)	
	H^3	C^{14}
0.2	9.28	29.3
0.3	9.22	29.7
0.4	9.33	30.6
0.5	9.66	31.7
0.6	9.57	31.3
0.7	9.40	29.4
0.8	9.38	30.1
0.9	9.66	31.5
1.0	9.39	30.5

Known amounts of C^{14} or H^3 glycine were counted in the presence of an increasing concentration of NaCl. Each vial contained 15 ml Triton x-100 scintillation fluid, 2 ml of NaCl solution at the indicated concentration and 1 ml of diluted radioactive amino acid solution.

Calculation of H/C, the Plotting Ratio

In order to minimize problems arising from differential counting of doubly-labeled samples, each chromatographic comparison was made twice, on the same day, each time with a given isotope in the opposite sample. For example in a chromatographic comparison of $tRNA_{Leu}$ extracted from control (C) and ethionine-treated (E) cultures, one column would compare C^{14} Leu-$tRNA_C$ with H^3 Leu-$tRNA_E$ and the other H^3 Leu-$tRNA_C$ with C^{14} Leu-$tRNA_E$. By using the same amount of unfractionated tRNA from each culture on each column, it is possible to calculate a "plotting ratio" of H^3 counts to C^{14} counts, without requiring an independent determination of the counting efficiency or specific activity of isotope. This enables semiquantitative comparisons to be made by simple inspection of the chromatographic profile. The plotting ratio is a number which converts H^3 cpm/μμM to C^{14} cpm/μμM and is calculated in the following way.

let: $H = H^3$ cpm/μμM and $C = C^{14}$ cpm/μμM

a, b, x, y are sums of cpm recovered from columns.

$$a = \Sigma\ C^{14}\text{-}tRNA_C \text{ and } b = \Sigma\ H^3\text{-}tRNA_E$$

$$x = \Sigma\ H^3\text{-}tRNA_C \text{ and } y = \Sigma\ C^{14}\text{-}tRNA_E$$

The amount of whole tRNA of both types applied to each column is equal, thus:

$$O.D._a = O.D._b \text{ and } O.D._x = O.D._y$$

Since specific acceptor activity does not depend on the isotope used to measure it:

$$\frac{a}{O.D._a} \cdot \frac{1}{C} = \frac{x}{O.D._x} \cdot \frac{1}{H} \quad \text{and} \quad \frac{b}{O.D._b} \cdot \frac{1}{H} = \frac{y}{O.D._y} \cdot \frac{1}{C}$$

rearranging terms,

$$\frac{aH}{Cx} = \frac{O.D._a}{O.D._x} \quad \text{which equals} \quad \frac{O.D._b}{O.D._y} = \frac{bC}{Hy}$$

$$\text{then } \frac{H^2}{C^2} = \frac{bx}{ya} \quad \text{and} \quad \frac{H}{C} = \left(\frac{bx}{ya}\right)^{\frac{1}{2}}$$

All chromatographic profiles are presented using this method except for glun-tRNA and cys-tRNA. For these amino acids tritium labeling is not commercially available.

RESULTS

Protein and RNA Metabolism in Cultures Synthesizing Tyrosinase

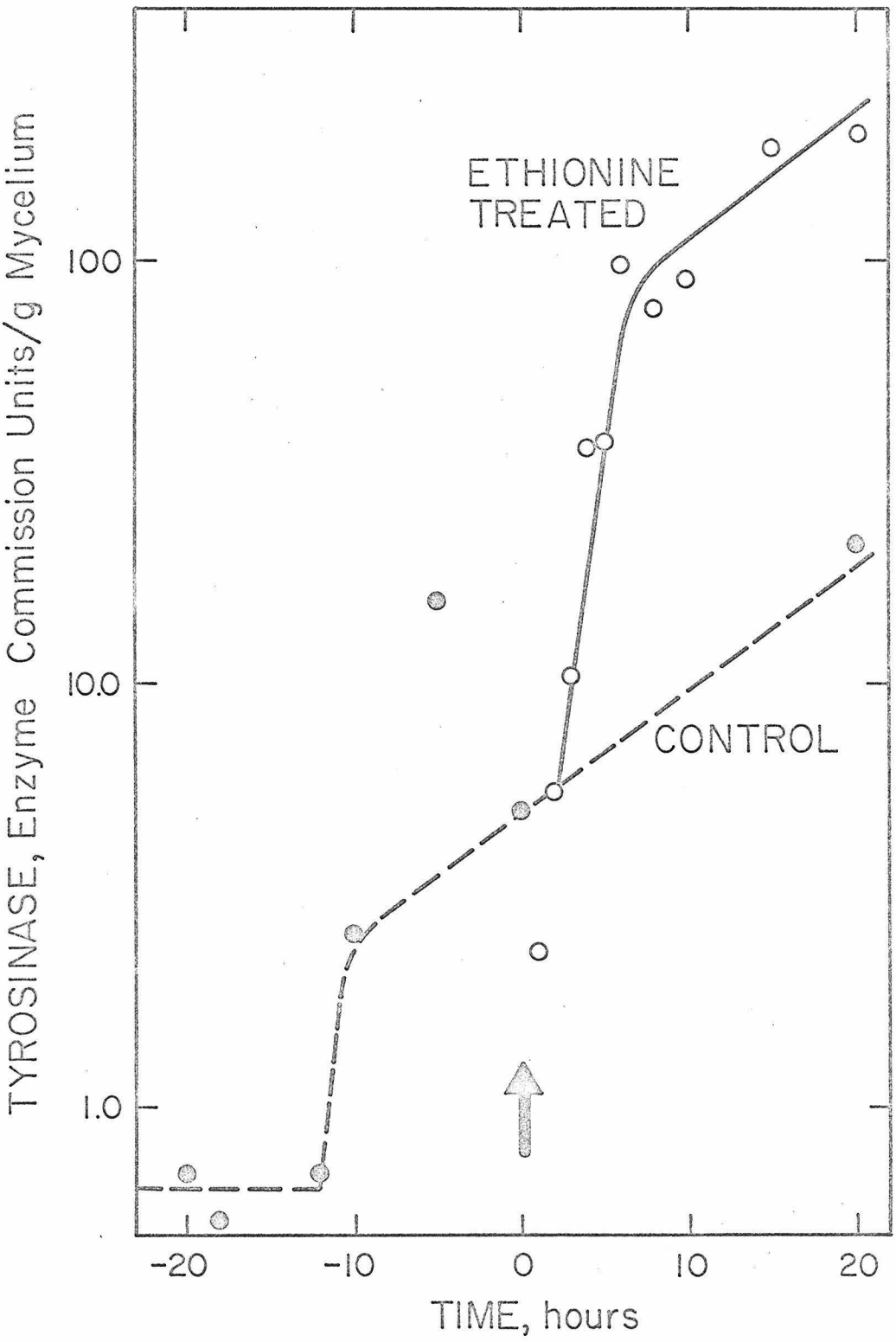
Time Course of Tyrosinase Synthesis

After 60 hours of growth (48 stationary and 12 shaking) in medium containing 25% of the amount of sucrose and 50% of the concentration of Vogel's salts used in ordinary minimal medium, strain 69-1113a begins to synthesize a small amount of tyrosinase. The rate of synthesis quickly declines, so that for the next 24 hours there is only a slow accumulation of enzyme (Figure 2). If ethionine is added at 72 hours after inoculation (indicated by the arrow in Figure 2 and defined as "0" time), then after a two-hour lag an additional period of rapid synthesis occurs. If no ethionine is added at "0" time then 20 hours later there is an accumulation of 21.5 Enzyme Commission units (e.c.u.)/g wet weight of mycelium; if ethionine is added at 0 time the level after 20 hours is increased to 201.5 e.c.u./g. In many strains of Neurospora no enzyme appears without the addition of inhibitor; however strain 69-1113a was selected by Horowitz as a high producer of tyrosinase by virtue of its sensitivity to starvation (31). The important point is that the addition of the amino acid analog stimulates a rapid net synthesis of tyrosinase. Similar conditions have been shown to also stimulate, specifically, the synthesis of at least two other enzymes (47, 48).

Under the conditions used the addition of ethionine leads to a slight decline in the dry mass of the mycelium. The apparent increase

FIGURE 2. TIME COURSE OF TYROSINASE SYNTHESIS

The time scale of the ordinate is relative to the time of ethionine addition (arrow). At 0 time, according to the figure, a culture is 72 hours old. The first 48 hours are stationary, followed by reciprocal shaking. The tyrosinase values at each point, indicated in Enzyme Commission units (e.c.u.) per gram of mycelium (wet weight), are averages of several cultures from different experiments. The dashed line and solid circles represent control cultures, never exposed to ethionine. The solid line and open circles represent ethionine-treated cultures.


TYROSINASE, Enzyme Commission Units/g Mycelium
100
10.0
1.0
ETHIONINE
TREATED
CONTROL
-20
-10
0
10
20
TIME, hours

in the wet weight of such pads is perhaps due to progressive cell wall changes which affect water retention (Figure 3).

Protein Metabolism

During the period of maximal rate of tyrosinase synthesis, the rate of general protein synthesis, as measured by the incorporation of tritiated phenylalanine into TCA insoluble material, decreases slightly (Table 5). This rate calculation depends on the hypothesis that the mole fraction of phe in protein remains constant after ethionine treatment (50).

After the addition of ethionine the total accumulation of mycelial soluble protein declines sharply and then after 10 hours levels off (Figure 4). The decline in the rate of protein synthesis is not nearly large enough to account for this change in protein content.

Nucleic Acid Metabolism

Ethionine treatment of mycelium results in a 38% average loss of total RNA within 5 hours relative to zero hour control cultures (Table 6). This is a much larger loss than would be expected from 5-hour untreated controls. For comparison, Minigawa et al. (51) have reported that Neurospora conidia contain about 65 $O.D._{260}$ units of RNA for an equivalent mass (0.125 g wet weight).

I have measured the sRNA content of mycelium at several times both before and after the addition of ethionine and the resulting stimulation of tyrosinase synthesis (Figure 5). There is considerable

FIGURE 3. MYCELIAL PAD WEIGHTS AS A FUNCTION OF TIME AFTER ETHIONINE ADDITION

Pads, blotted between sheets of paper towels, were used for wet weight determinations (circles) and then assayed for tyrosinase (Figure 2). For determination of dry weights (squares) blotted pads were dried in an oven at 97°C for 90 minutes. The number of cultures contributing to each average value is given in parentheses. Controls are solid symbols, dashed line; ethionine-treated cultures are open symbols, solid line. Arrow indicates time of ethionine addition to treated cultures.

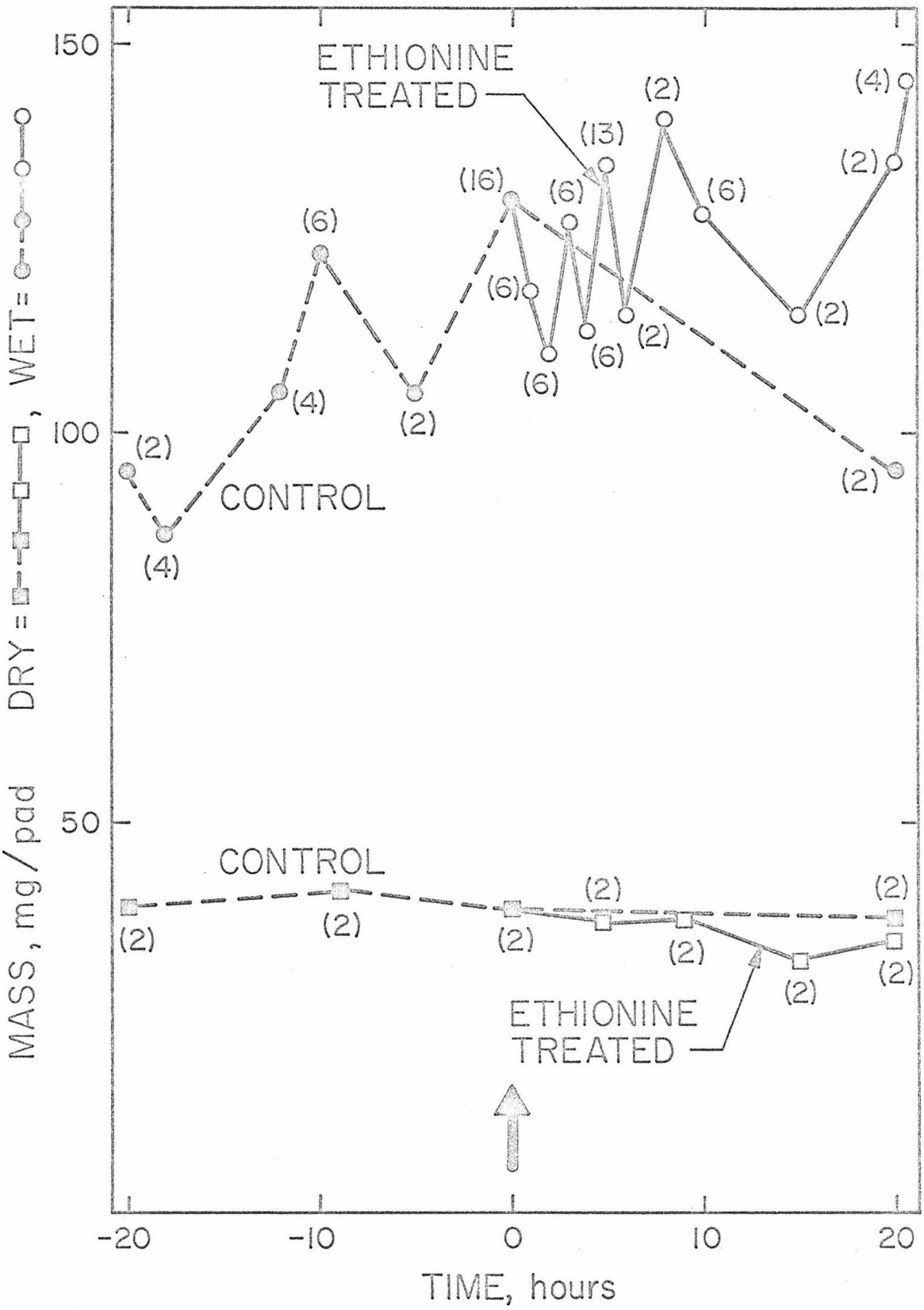

150
100
50
MASS, mg/pad DRY = □—□, WET = ○—○
ETHIONINE TREATED
CONTROL
CONTROL
ETHIONINE TREATED
(2)
(4)
(6)
(4)
(2)
(16)
(6)
(6)
(13)
(6)
(6)
(2)
(2)
(6)
(2)
(2)
(4)
(2)
(2)
(2)
(2)
(2)
(2)
(2)
(2)
(2)
-20
-10
0
10
20
TIME, hours

TABLE 5. EFFECT OF ETHIONINE TREATMENT ON RATE OF PROTEIN SYNTHESIS

	Parameter	Units	Hours in ethionine 0	5
A.	free phe pool	mμM	35.64	33.0
B.	uptake of H^3 phe in 15 minutes*	cpm x 10^{-5}	16.8	10.7
	B' uptake of H^3 phe	mμM	2.13	1.35
C.	specific activity of H^3 phe in pool†	$\frac{cpm}{\mu M}$ x 10^{-5}	444.8	311.5
D.	H^3 phe incorporated into protein in 15 minutes*	cpm x 10^{-5}	5.78	3.52
E.	rate of phe incorporation‡	μM/hour	.0520	.0452
F.	mole fraction of phe in Neurospora protein§		0.036	0.036
G.	rate of amino acid incorporation‖	μM/hour	1.44	1.26
H.	rate of protein synthesis¶	mg/hour	0.157	0.137

* values measured for B and D are each the average of two determinations

† $C = B/(A+B')$

‡ $E = 4(D/C)$

§ data from: DeBusk, B. G., and A. G. DeBusk, Neurospora Newsletter, 11, 3 (1967)

‖ $G = E/F$

¶ $H = 0.109 \left(\frac{mg}{\mu M}\right) G$

Four 72-hour cultures were divided into pairs. One pair of cultures was pulsed immediately with 12.5 μCuries of H^3 phenylalanine for 15 minutes and fractionated according to the Roberts procedure. The other two cultures were first treated with ethionine for five hours and then pulsed and fractionated. A, the phenylalanine pool size, was measured in identically treated pads, using a quantitative ninhydrin procedure

TABLE 5 (continued)

after high voltage electrophoresis of cold TCA extracts (33). B and D are also measured quantities. C, E, G and H are calculated values; F is a published value. For the conversion of amino acid incorporation from μmoles to mg, H, I calculated the number-average molecular weight of amino acid residues in Neurospora protein (108.72) based on the published composition (49).

FIGURE 4. MYCELIAL PROTEIN CONTENT IN RELATION TO TIME OF ETHIONINE ADDITION

Total soluble protein content was measured in crude extracts by the biuret method. Solid circles are control cultures; open circles are ethionine-treated cultures. Limit bars indicate range of duplicate samples.

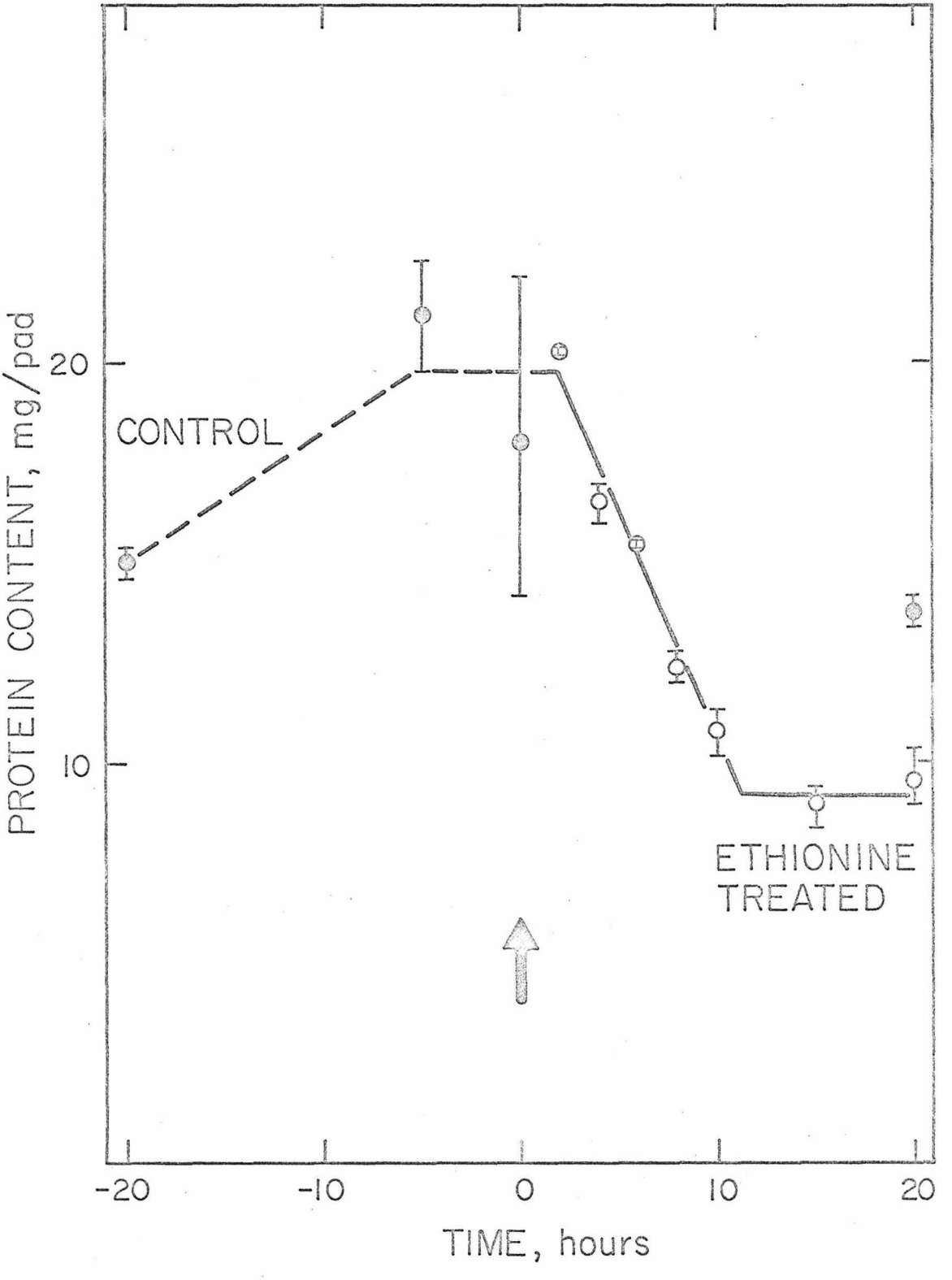

PROTEIN CONTENT, mg/pad
20
10
CONTROL
ETHIONINE
TREATED
-20
-10
0
10
20
TIME, hours

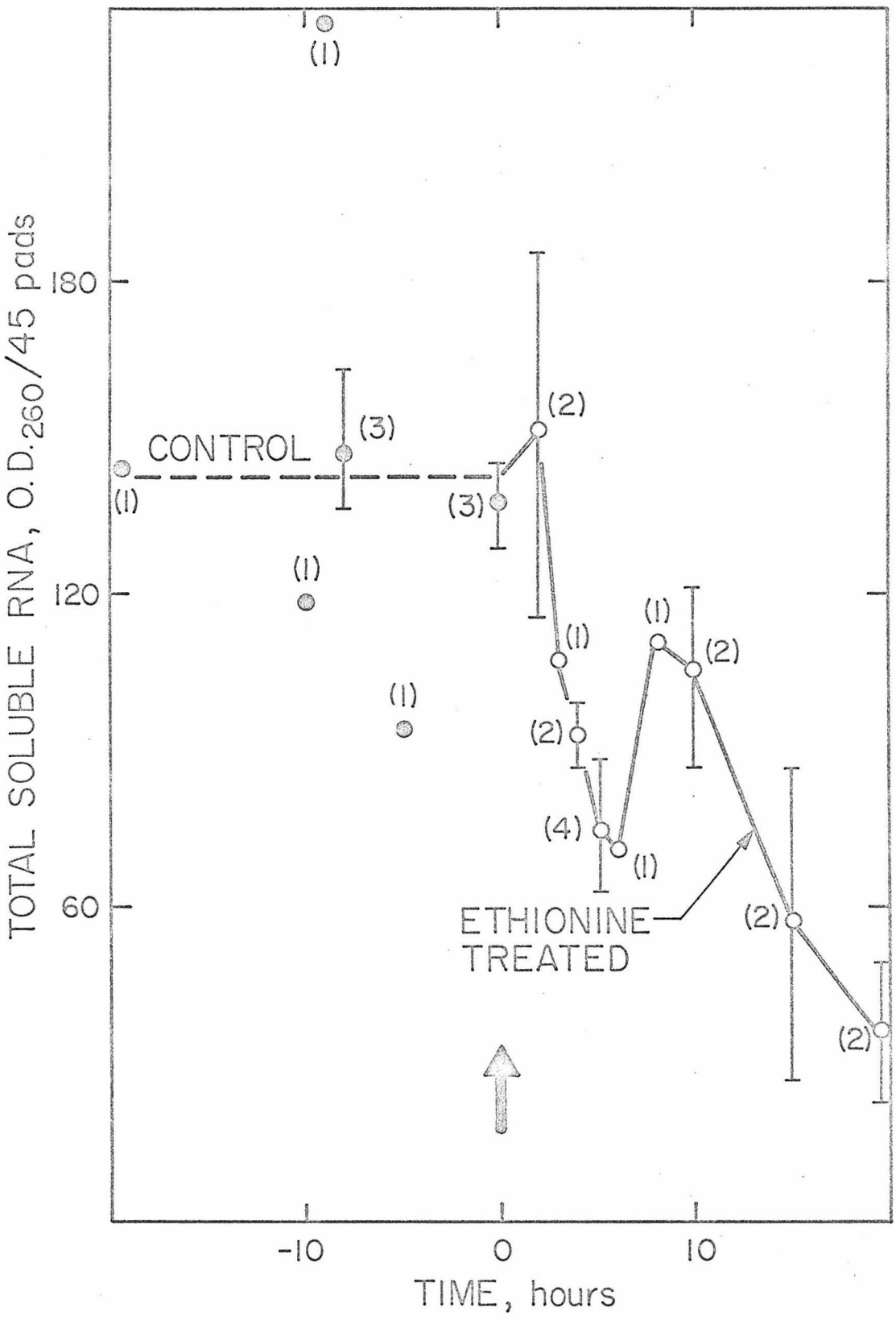
TABLE 6. EFFECT OF ETHIONINE ON MYCELIAL RNA CONTENT

Length of ethionine treatment (hours)	Sample	Total RNA ($O.D._{260}$/pad)
0	a	77.8
	b	70.2
	average	74.0
5	a	35.1
	b	56.7
	average	45.9

Mycelial pads were grown according to standard procedure, incubated for the indicated time in ethionine and fractionated by the Roberts procedure. Total RNA is defined as all of the ultraviolet absorbing material in the hot PCA-soluble fraction.

FIGURE 5. SOLUBLE RNA CONTENT AS A FUNCTION OF TIME AFTER ADDITION OF ETHIONINE

At indicated times, sRNA was extracted from 45 pads by phenol method. Arrow indicates time of ethionine addition. Limit bars indicate range of multiple determinations. Number of determinations is within parentheses. Dashed line is average value of all control cultures (solid circles). Solid line is ethionine-treated cultures (indicated by open circles).


TOTAL SOLUBLE RNA, O.D.260/45 pads
180
120
60
-10
0
10
TIME, hours
CONTROL
ETHIONINE
TREATED
(1)
(2)
(3)
(4)

scatter in the data, probably because the average efficiency of extraction is only 25%. However, the general picture is clear. The response of the mycelium to the addition of ethionine includes a rapid loss of sRNA. Within 5 hours 40% has been lost; this loss increases to 75% by 20 hours.

The rate of synthesis of sRNA as measured by the incorporation of tritiated guanosine is decreased, after the addition of ethionine to roughly 30% of the pretreatment rate (Table 7). The chromatographic profile of the material newly synthesized at the two times is quite similar qualitatively (Figure 6).

Methylation, Ethylation

I have investigated the rate of methylation of sRNA *in vivo* in zero-hour control and 5-hour ethionine-treated cultures by measuring the incorporation of pH 8.8 stable methyl groups into sRNA during a 15 minute pulse of methionine isotopically labeled in the methyl group, and have compared the specificity of methylation by MAK chromatography of the pulse-labeled material (Figure 7). There is no striking qualitative difference in the material extracted from the two cultures although the extent of methylation is reduced in the ethionine-treated cultures by 20-25%.

A rough estimate, based on the amount, in μmoles, of methyl groups incorporated per optical density unit of sRNA in untreated cultures, indicates that on the average in a 15-minute pulse 2 of every 3 sRNA molecules receive one methyl group. In comparison, an estimate of the

TABLE 7. EFFECT OF ETHIONINE ON SOLUBLE RNA SYNTHESIS

Length of ethionine treatment	Incorporation		Soluble RNA	
	cpm/pad	%	$O.D._{260}$/pad	%
0	8,068	100	3.11	100
5	2,353	29	1.78	57

After indicated treatment 5 cultures were each pulse-labeled with 10 μC of tritiated guanosine for 15 minutes. Each group of 5 was washed, pooled with 40 unlabeled, but similarly treated pads and sRNA was extracted by phenol method.

FIGURE 6. MAK CHROMATOGRAPHIC PROFILE OF PULSE-LABELED SOLUBLE RNA

sRNA, pulse-labeled with H^3 guanosine, from the experiment described in Table 7, was chromatographed on MAK. Each sample was eluted from a separate column. The patterns were lined up using O.D. profile as the reference marker. Dashed line, zero hour control culture; solid line, 5 hour ethionine treatment.

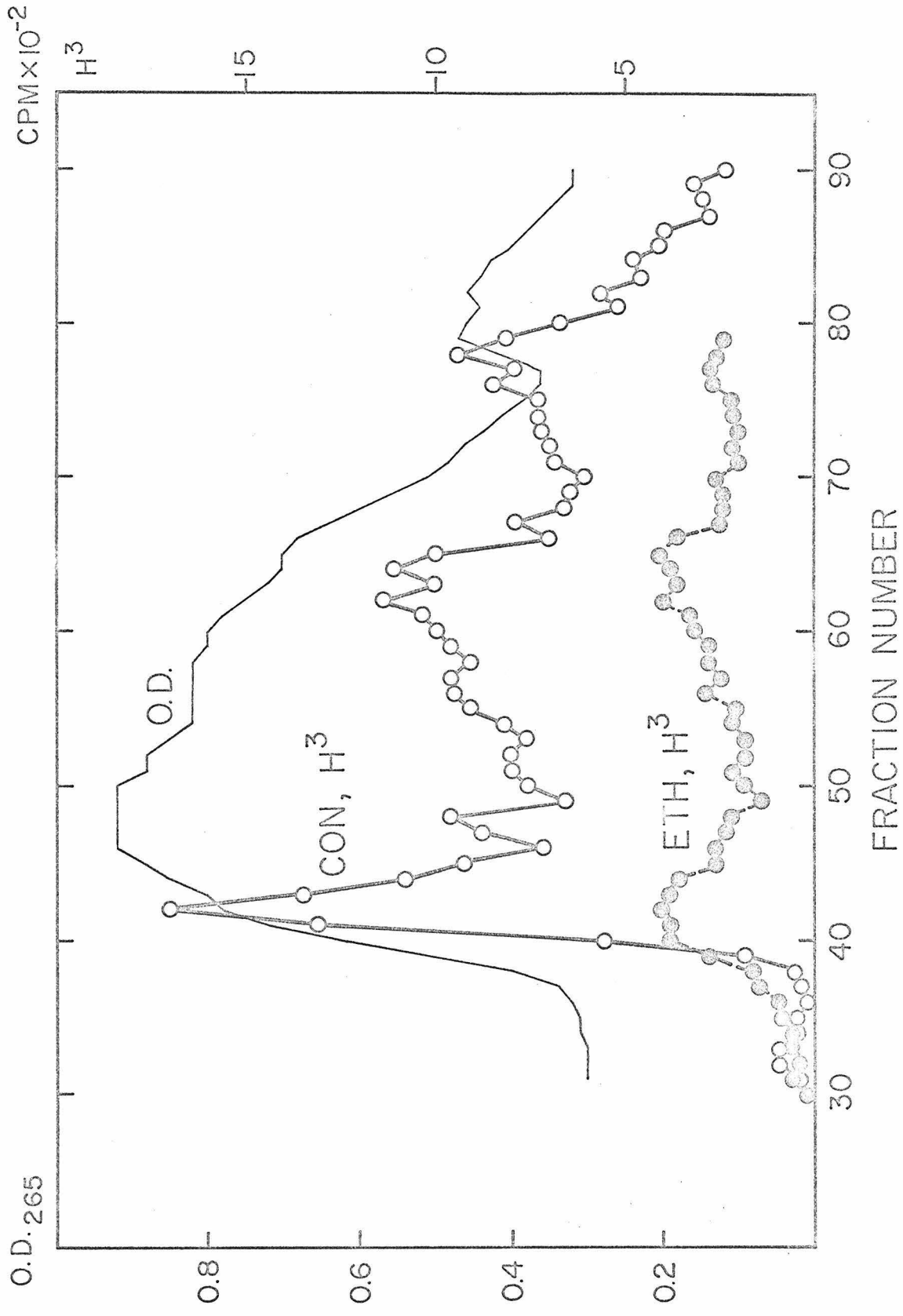
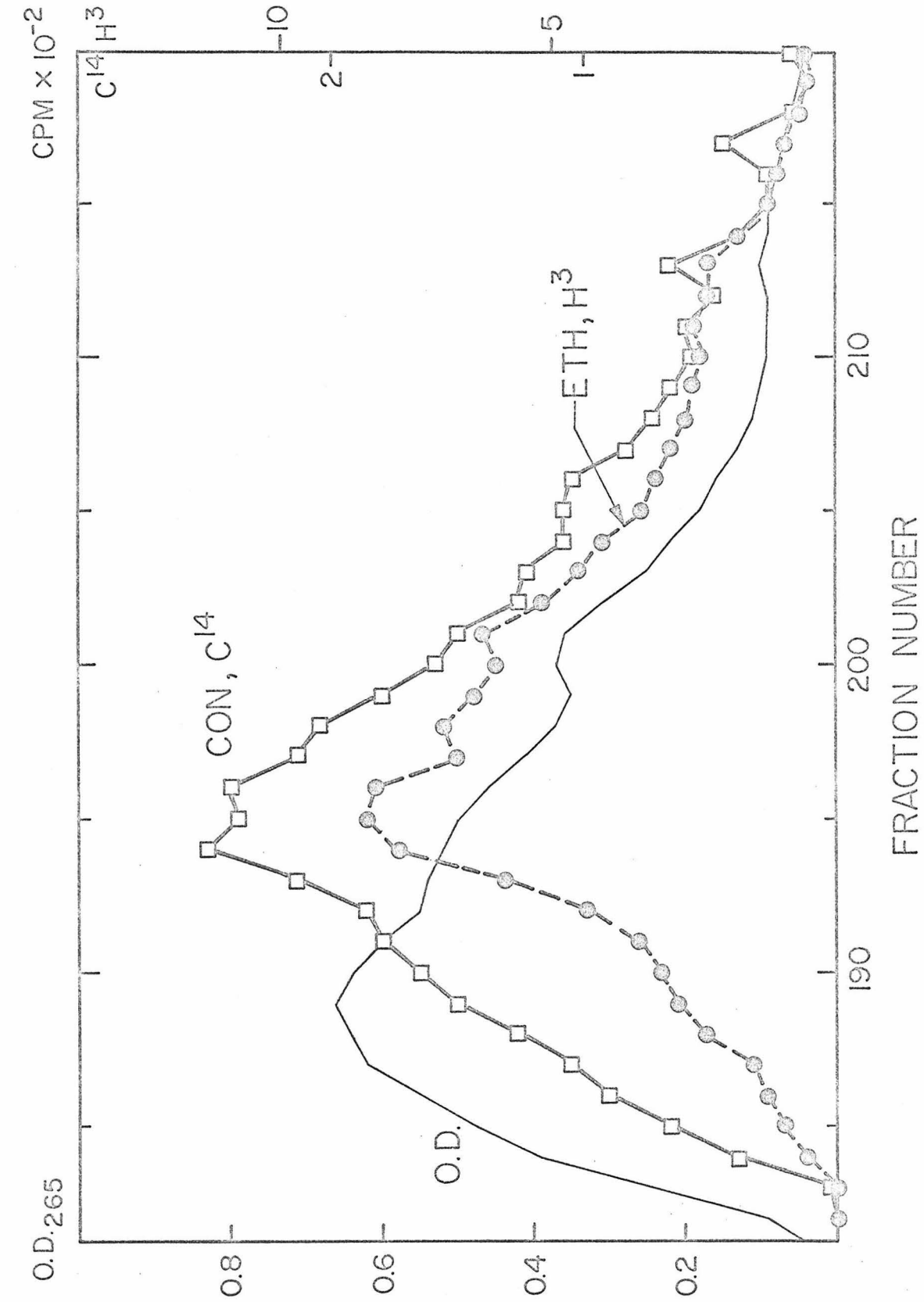

CPM×10⁻²
H³
15
10
5
O.D.
CON, H³
ETH, H³
O.D.265
0.8
0.6
0.4
0.2
30
40
50
60
70
80
90
FRACTION NUMBER

FIGURE 7. MAK CHROMATOGRAPHIC PROFILE OF METHYLATED sRNA

Ethionine-treated (5 hours) and zero-hour control cultures were pulse-labeled with methyl-labeled methionine. The sRNA, from the 2 sources, was phenol-extracted, pooled and co-chromatographed on MAK. Incubation of the RNA preparations at 37°C, pH 8.8 eliminated the possibility that any of the counts were in the form of methionyl-tRNA. Filled squares and dashed line, C^{14} methyl groups from control culture; open circles and solid line, H^3 methyl groups from treated cultures.


CPM x 10⁻²
C¹⁴ H³
10
5
2
1
CON, C¹⁴
ETH, H³
O.D.
O.D.265
0.8
0.6
0.4
0.2
190
200
210
FRACTION NUMBER

number of ethyl groups transferred *in vivo* from ethyl-labeled ethionine to sRNA indicates a maximum of 2 ethyl groups for every 54 sRNA chains. Thus in this system ethylation of tRNA proceeds at less than 6% of the rate of methylation. Even this number may be an exaggeration, since the actual number of counts recovered was only a small increase over background.

Ribonuclease Activity

In preliminary experiments I attempted to extract amino acyl-tRNA synthetases from cultures similar to those I was using for both sRNA extraction and measurement of tyrosinase production. Such preparations showed enzymatic activity when measured by the conversion of radioactive amino acid (methionine or leucine) to a form insoluble in ethanol or cold acid, in the presence of sRNA. However, if the reaction mixture was deproteinized with phenol and chromatographed on MAK, then virtually none of the counts and little of the optical density was found in the position expected for whole tRNA.

These results suggested that the enzyme preparation was contaminated with ribonuclease. The presence of ribonuclease was directly confirmed by measuring the enzyme preparation's ability to stimulate the loss of acid-precipitable radioactivity from unfractionated RNA labeled with tritiated uridine (Table 8). More than 75% of the counts were rendered acid-soluble in 20 minutes.

The rate of degradation is somewhat less with sRNA as substrate. In this regard the activity is similar to the *Neurospora* endonuclease

TABLE 8. RIBONUCLEASE ACTIVITY OF AMINO ACYL-tRNA SYNTHETASE PREPARATION FROM ETHIONINE-TREATED MYCELIUM

Reaction mixture	cpm recovered after treatment				
	Time (minutes)				
	0	5	10	15	20
No enzyme added	2160	2085	1974	1966	2183
Enzyme added	2000	1410	1224	892	462

Reaction mixture and conditions were like those used to assay charging of amino acid to tRNA, except that sRNA was replaced by unfractionated RNA (prepared by Martin Pall) labeled with tritiated uridine and radioactive amino acid was omitted. Enzyme was prepared from ethionine-treated 69-1113a. At indicated times aliquot was removed from reaction mixture and assayed for TCA-precipitable radioactivity.

characterized by Linn and Lehman (52), which is most active with nucleic acids lacking an ordered structure.

Measurement of Total Amino Acid Acceptor Activity

In vivo Charging

In order to circumvent the problems caused by the contamination of the amino acyl-tRNA synthetase preparation with ribonuclease, I tried several times to charge tRNA *in vivo*. The tRNA extracted from cultures pulsed with radioactive leucine, phenylalanine, or methionine contained no detectable labeled amino acyl-tRNA. Methionine did, however, serve as a methyl donor for tRNA as described above.

Several factors are involved, I believe, in this failure to detect *in vivo*-charged tRNA. Strain 69-1113a is prototrophic and consequently contains relatively high endogenous amino acid pools which dilute any radioactive amino acid taken up. The tRNA extraction procedure involves many steps over a three-day period and, even without the incubation in dilute base, is not designed to protect the amino acyl bond. Finally, many tRNA molecules *in vivo* are in the form of peptidyl-tRNA which is even less stable than amino acyl-tRNA.* This approach was abandoned when I discovered that amino acyl-tRNA synthetase preparations from exponential cultures of 74A are free of interfering ribonuclease and have charging activity for all 20 amino acids.

---

*A discussion with Dr. Noboru Sueoka in December of 1966 at Princeton University contributed to my interpretation of these experiments.

Standard Reaction Conditions

The reaction resulting in the attachment of an amino acid to tRNA is given by the equation (53):

$$ATP + RCHNH_2COOH + RNA - OH \underset{\text{Enzyme}}{\overset{Mg^{++}}{\rightleftharpoons}} RNA - OCOCHNH_2R + AMP + PP_i + H_2O$$

The reaction requires ATP, amino acid, acceptor RNA, enzyme and magnesium ions. The magnesium is most likely required for the maintenance of RNA secondary structure (54). Generally a sulfhydryl reducing reagent is also added because many of the synthetase enzymes contain sulfhydryl groups.

There are two criteria used to determine the saturation or maximal acceptor activity of tRNA for a particular amino acid. A plot of the amount of amino acyl-tRNA formed as a function of time should plateau (Figure 8, curve I); addition of more enzyme, ATP or amino acid after the plateau is reached should not cause any increase in the amount formed. The interpretation of such evidence is that every tRNA molecule capable of accepting the amino acid has reacted with it by that time.

Using a standard reaction mixture (Table 9) both criteria were satisfied for the attachment of 14 amino acids to tRNA; the reactions went to completion in 10-20 minutes at 30°C. Quite different kinetics are observed if the same reaction mixture is used to assay the six other amino acids - serine, glycine, methionine, glutamic acid, glutamine, and alanine. For serine the result is similar to curve II in

FIGURE 8. KINETICS OF CHARGING REACTION

Four observed patterns of the amount of amino acyl-tRNA (in arbitrary units) recovered from reaction mixture as a function of time. Explanation of each pattern is discussed in the text.

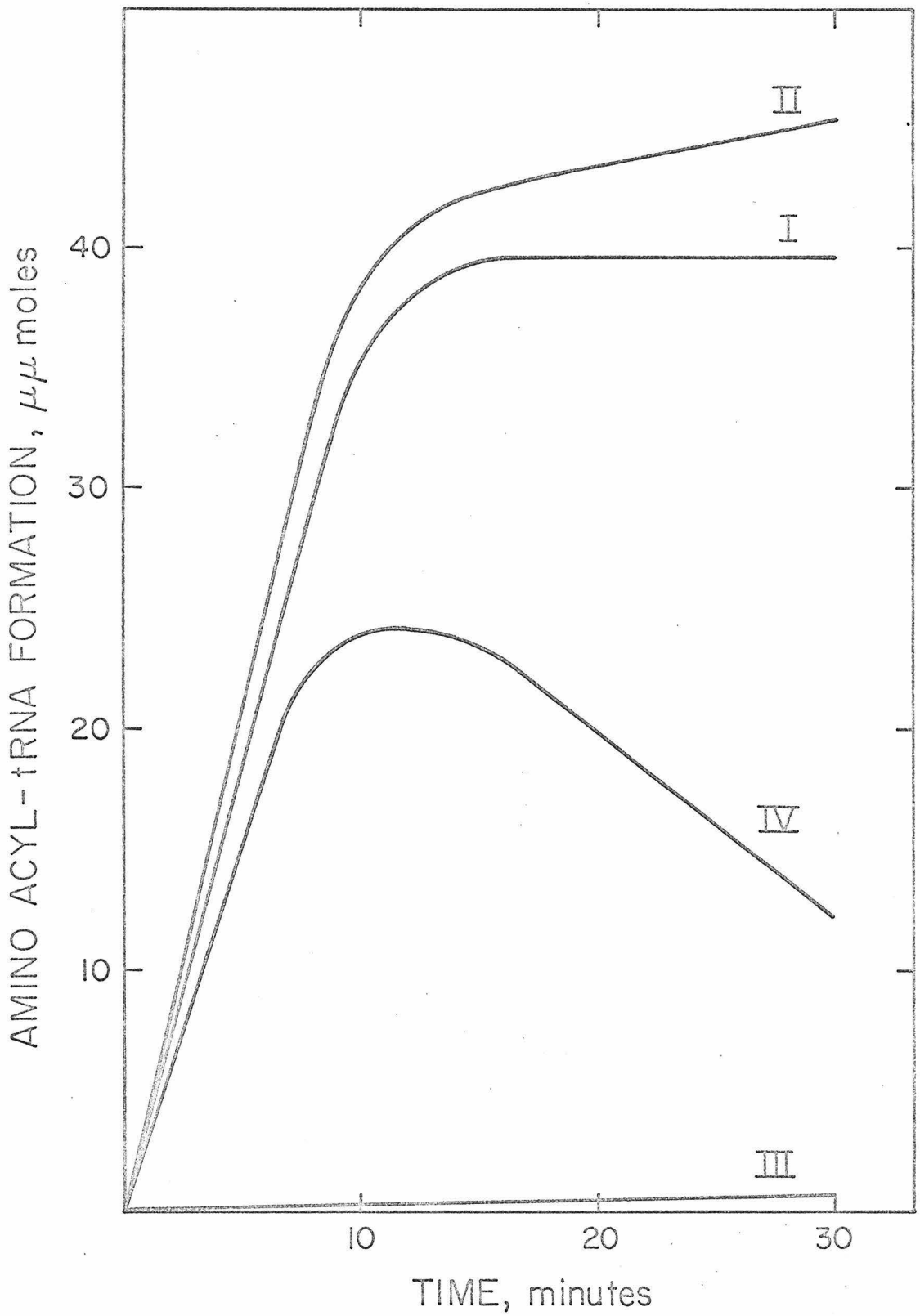

AMINO ACYL-tRNA FORMATION, μμ moles
40
30
20
10
II
I
IV
III
10
20
30
TIME, minutes

TABLE 9. COMPOSITION OF STANDARD REACTION MIXTURE

Component	Amount per ml reaction
Tris-HCl, pH 7.3-7.5	100 μmoles
$Mg(Ac)_2$	50 μmoles
ATP	2.5 μmoles
Glutathione (reduced)	5.0 μmoles
C^{14}-amino acid	0.02 μmoles
sRNA	10 $O.D._{260}$ units ~ 0.4 mg
Enzyme preparation (in 50% glycerol)	1 mg

Figure 8. Like curve I, the reaction begins with a high initial rate but instead of reaching a plateau after a short time, the reaction continues at a reduced rate. No reaction at all occurs with glutamic acid (curve III, Figure 8). The kinetics are quite unusual for the other four amino acids. The amount of amino acyl-tRNA formed reaches a peak within 5-10 minutes and then declines (curve IV, Figure 8).

Serine

Saturation kinetics for the attachment of serine to tRNA are observed if the concentration of tRNA in the reaction mixture is lowered by a factor of five (Figure 9). This implies that in the original mixture tRNA was in excess. However, the kinetics are not those expected for a reaction with excess substrate. In Neurospora there are 4 chromatographically separable tRNA components present in different amounts which accept serine (Figure 12). The kinetics observed at high tRNA concentration could be explained if the four components were charged at different rates. This explanation is supported by comparison of the K_M and V_{Max} for each of the 4 partially separated components (Table 10).

These data indicate that $tRNA^{I}_{Ser}$ and $tRNA^{IV}_{Ser}$, which are present in unfractionated tRNA as minor components (19% and 10% respectively) have less affinity for the synthetase enzyme than do the major components. Since the kinetics of charging serine to unfractionated tRNA is the sum of these 4 reactions proceeding at different rates, the slow

FIGURE 9. SERYL-tRNA FORMATION AT DIFFERENT CONCENTRATIONS OF tRNA

Attachment of C^{14} serine to unfractionated tRNA was assayed at 3 different tRNA concentrations. Squares, 5.5 $O.D._{260}$ units per reaction; triangles, 2.2 units; circles, 1.1 units.

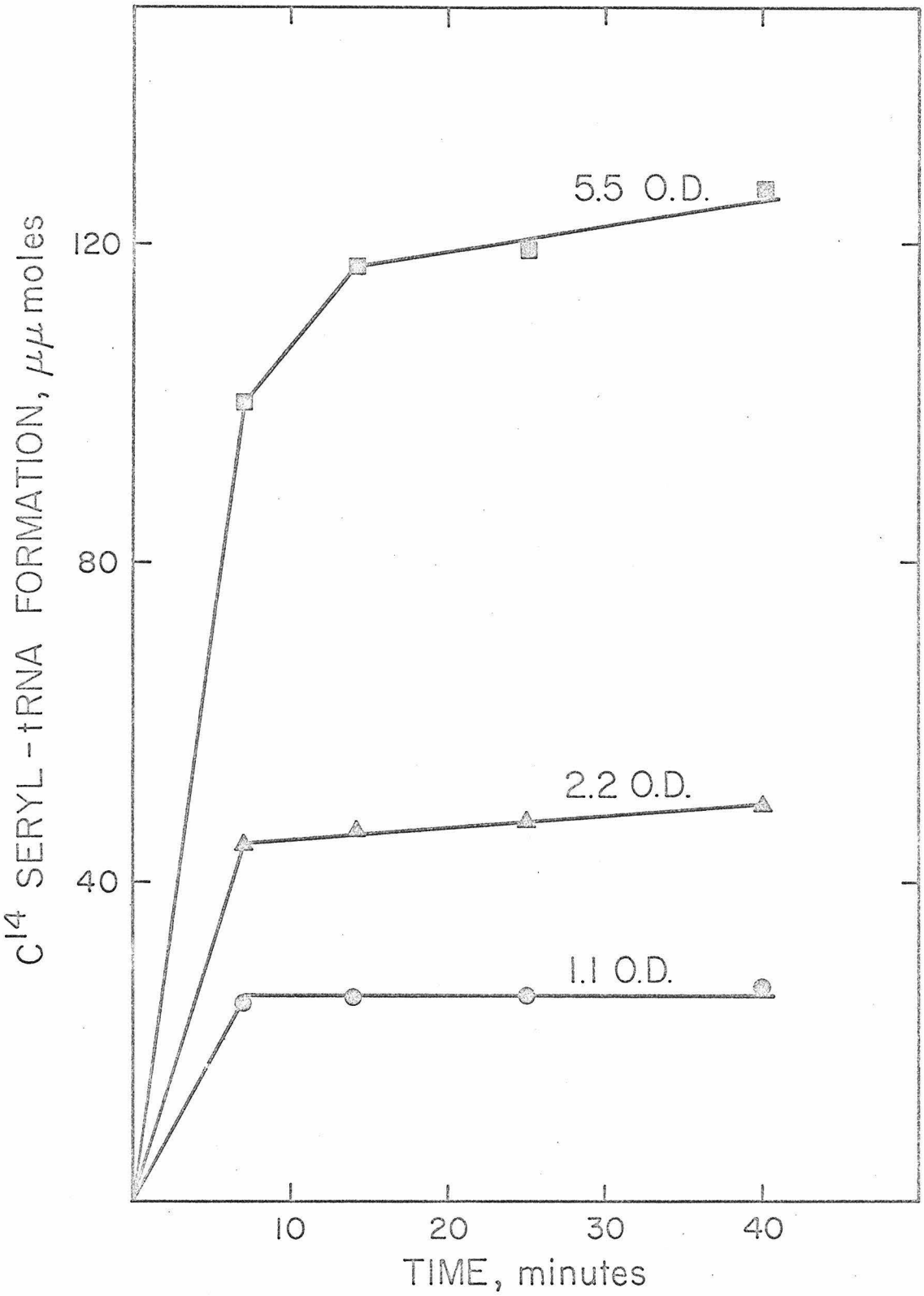

5.5 O.D.
2.2 O.D.
1.1 O.D.
120
80
40
10
20
30
40
TIME, minutes
C14 SERYL - tRNA FORMATION, μμ moles

TABLE 10. KINETIC PROPERTIES OF ISOLATED SERINE tRNA COMPONENTS

Fraction	Purity (in %)				K_M ($M \times 10^7$)	V_{Max} ($\mu\mu M$/min)
	I	II	III	IV		
A	86.4	10.5	3.1	-	1.70	9.75
B	17.1	75.8	7.1	-	0.613	13.5
C	-	18.4	69.1	12.5	<B	20.0
D	1.7	9.6	33.2	55.5	1.22	16.1

Uncharged sRNA was chromatographed on MAK. The eluted fractions were pooled into 4 groups (A, B, C, D) based on the expected mobility of the 4 serine tRNA components. The purity of each pooled fraction was determined by charging it with either C^{14} or H^3 serine and re-chromatographing it. In unfractionated tRNA the proportions of the 4 components are: I = 18.6%, II = 37.3%, III = 34.2% and IV = 9.9%. Fraction C is believed to have a lower K_M than B because the rate of charging was maximal at the lowest concentration of C tried.

rate of reaction at late times may just be the residual charging of components I and/or IV after II and III are saturated.

Glutamic Acid, Glutamine, Methionine, and Alanine

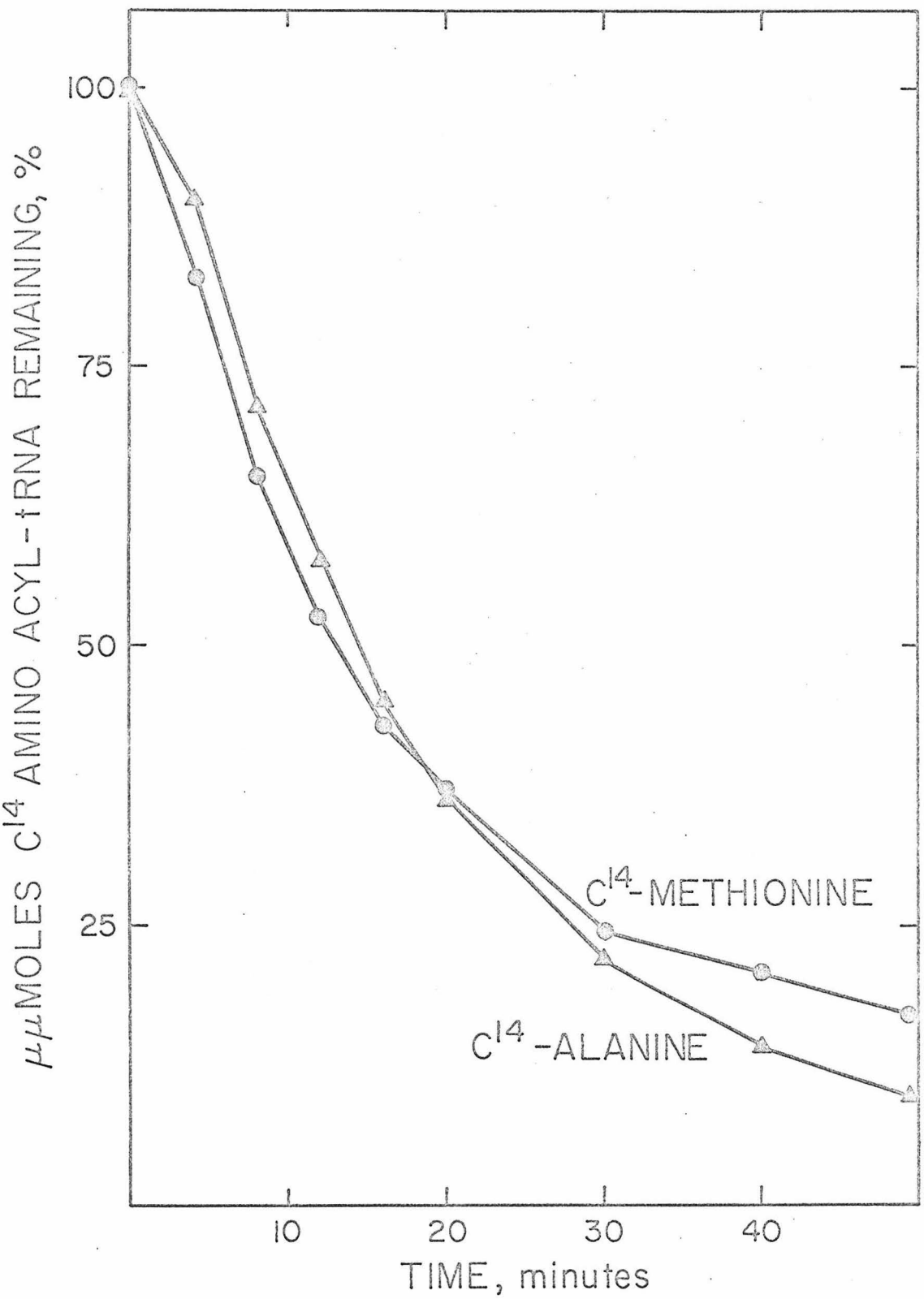
The high Mg^{++} concentration in the standard reaction mixture (0.05 M) is responsible for the apparent lack of acceptor activity for glutamic acid. At a lower concentration (0.01 M Mg^{++}) the initial rate of reaction is maximal, but the kinetics are still not normal; they are now of type IV (Figure 8). The acid precipitable counts reach a peak and then decline, thus grouping glutamic acid with glutamine, alanine, methionine and glycine.

The loss of counts suggests either that the RNA molecule is being degraded or the amino acyl bond once formed is unstable. The former explanation was ruled out by extracting the tRNA late in the reaction (after the peak had been reached) and re-assaying it. The same peak acceptor activity was observed. The instability of the amino acyl-tRNA was confirmed for alanine and methionine by extracting the peak material and reincubating it in buffer (Figure 10). A loss of TCA precipitable counts was observed.

The instability of the amino acyl-tRNA is, however, not sufficient to explain the observed type IV kinetics. Since as long as all the components required for the formation of the product are present, the reaction should still reach a plateau even though at a value lower than that expected for maximal acceptance. This contradiction can be resolved by postulating that one of the components is

FIGURE 10. INSTABILITY OF METHIONYL-tRNA AND ALANYL-tRNA IN BUFFER

C^{14} amino acyl-tRNA was extracted with phenol from charging mixture after a 10-minute reaction at 30°C. The ethanol-precipitable material was resuspended in charging buffer and incubated at 30°C. The loss of TCA insoluble radioactivity was determined by assaying aliquots at several intervals by the filter paper disc method.


100
75
50
25
μμMOLES C14 AMINO ACYL-tRNA REMAINING, %
C14-METHIONINE
C14-ALANINE
10
20
30
40
TIME, minutes

no longer present late in the reaction, that it is destroyed in the early part. It was stated above that the tRNA is not destroyed; therefore some other component must be involved.

For the charging reaction with glutamine, glutamic acid, and methionine, the supply of ATP is the critical factor. The addition of ATP during the course of the reaction results in a burst of charging; a low initial ATP concentration leads to a large reduction in the initial rate of the reaction. These results indicate that in the initial phase of the incubation, in addition to the amino acid being attached to tRNA at the expense of an equivalent amount of ATP, the bulk of the excess ATP present (> 1000-fold) is hydrolyzed. As the relatively unstable amino acyl bond is cleaved, the amino acid cannot be recharged because the enzyme requires a high concentration of ATP which is no longer present in the mixture. The problem is solved by including in the original mixture a means of regenerating ATP. Only PEP is necessary since the synthetase enzyme preparation apparently contains pyruvate kinase activity.

The cause of type IV kinetics for the charging of alanine appears to involve an alkali-sensitive enzyme, although I have investigated this case less extensively. Both the initial rate and the extent of the reaction are reduced as the pH is increased (Table 11). It appears that alanyl-tRNA synthetase is inactivated even at only slightly alkaline pH and that at late times in the standard reaction, little active enzyme is present to recharge the discharged alanyl-tRNA.

TABLE 11. EFFECT OF pH ON THE CHARGING OF ALANINE TO tRNA

pH	Initial rate (μμmoles/min)	Extent of reaction (μμmoles)
8.0	1.30	5.22
7.5	3.76	18.9
7.0	8.65	67.2

Three reaction mixtures, differing only in pH of buffer, were assayed for amino acyl-tRNA at several intervals over a 40-minute period. The initial rate is defined here as the average rate for the first 2 minutes. For the pH 8.0 and pH 7.5 reactions, which never reach a plateau, the extent is the value at the peak.

Normal saturation kinetics are obtained if the reaction mixture is buffered at pH 7.0.

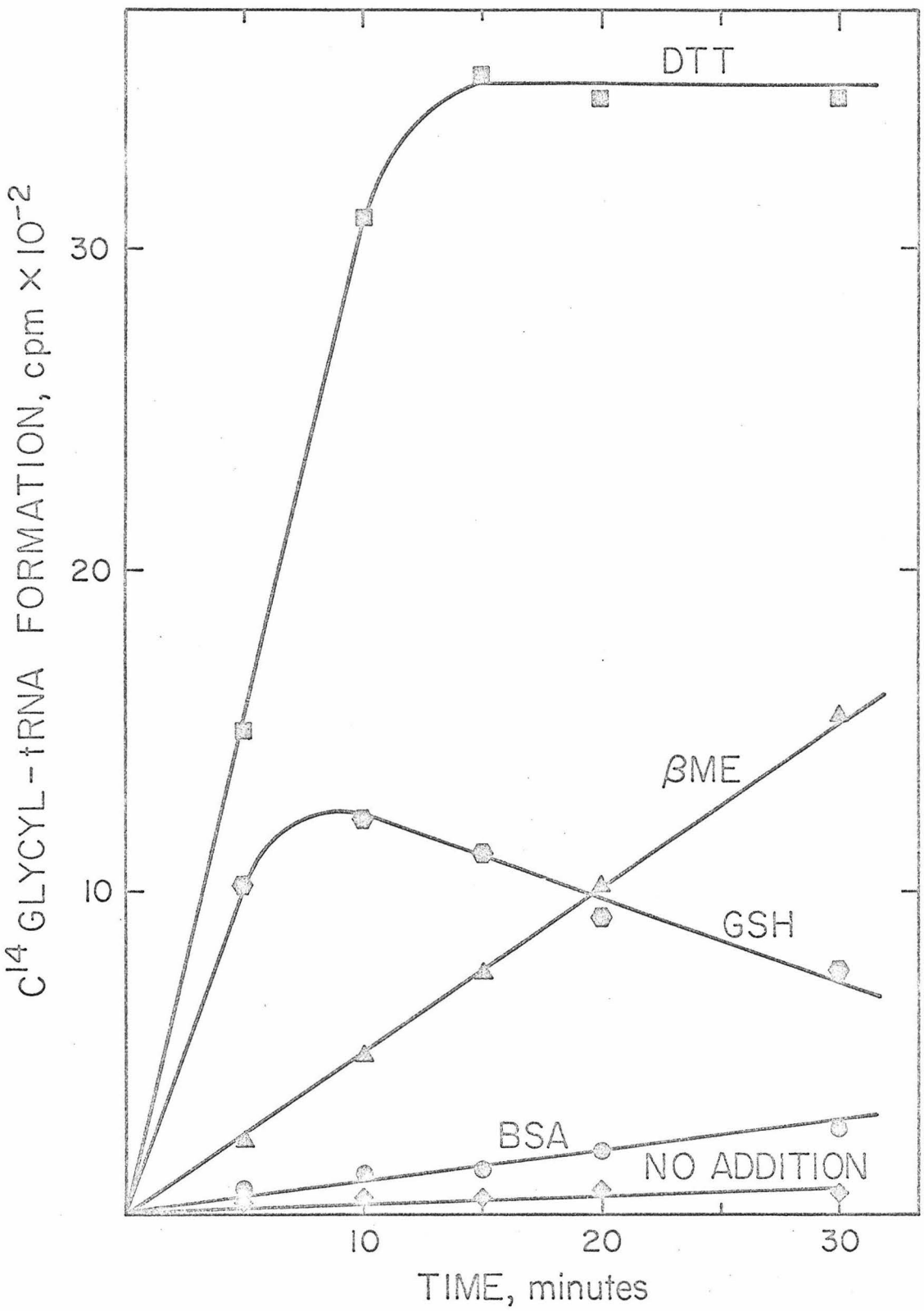
Glycine

Unlike the amino acyl-tRNA formed with the 4 other amino acids which display type IV kinetics in the charging reaction, glycyl-tRNA is quite stable. The apparent explanation of the kinetics in this case is rather bizarre. In preliminary experiments a non-linear relationship was found between the concentration of enzyme and the initial rate of charging glycine to tRNA. This, taken with some evidence from preincubation experiments, suggested that the enzyme was being oxidized, and that a reagent should be added to keep the sulfhydryl groups reduced. However, reduced glutathione proved to be a poor choice. Figure 11 shows the effect of adding different reagents to inhibit the oxidation of sulfhydryl groups. With dithiothreitol (DTT) saturation kinetics are observed; less effective agents (such as mercaptoethanol or BSA) produce reduced initial rates, compared to DTT, as though the enzyme were limiting. Type IV kinetics are observed only with glutathione.

The attachment of an amino acid to tRNA is a reversible reaction (53) and exogenous amino acid will exchange, in the presence of enzyme, with amino acid already acylated to tRNA (55). In yeast an enzyme has been characterized which catalyzes the exchange of free glycine with the glycyl residue in glutathione, in the presence of ATP, ADP and Mg^{++} (56). I assume that in Neurospora a similar enzyme

FIGURE 11. THE EFFECT OF SULFHYDRYL REDUCING AGENTS ON THE ATTACHMENT OF GLYCINE TO tRNA

The standard reaction mixture was used to compare the charging of glycine to tRNA in the presence of different additions. Dithiothreitol (DTT), β-mercaptoethanol (βME) and glutathione (GSH) were each used at a final concentration of 5.0 μmoles/ml; 1.9 mg of BSA per ml of reaction was used.


DTT
βME
GSH
BSA
NO ADDITION
30
20
10
10
20
30
TIME, minutes
C14 GLYCYL-tRNA FORMATION, cpm X 10-2

exists. At late times in the charging reaction of glycine to tRNA (after 10 minutes at 30°C) unlabeled glycine--released from the exchange of labeled glycine with glutathione--dilutes the labeled glycine remaining in the mixture, and by exchange with the labeled glycine attached to tRNA, lowers the specific activity of the glycine isotopic label. This hypothesis accounts for the fact that type IV kinetics is observed for glycine only when reduced glutathione is used as the required sulfhydryl reducing agent.

Table 12 summarizes the sets of optimal conditions determined for assaying the total acceptor activity of Neurospora tRNA for the 20 amino acids of protein.

Chromatography of Amino Acyl-tRNA

If transfer RNA modifications were involved in the regulation of the ethionine-mediated metabolic shift described above, then alterations in the chromatographic profile of the tRNA specific for at least one amino acid should be observed. This prediction was tested by comparing, on MAK columns, the chromatographic profiles of the amino acyl-tRNA from vegetative, control mycelium (-20 hours) with that of ethionine-treated cultures (+5 hours) which produce tyrosinase at a rapid rate.

For 18 of the 20 amino acids at least 2 such comparisons were made: 1) H^3aa_x-$tRNA_C$ vs $C^{14}aa_x$-$tRNA_E$ and 2) $C^{14}aa_x$-$tRNA_C$ vs H^3aa_x-$tRNA_E$ (where "x" is one of 18 amino acids, "C" denotes tRNA extracted from vegetative control cultures and "E" tRNA from ethionine-treated

TABLE 12. SUMMARY OF OPTIMAL CONDITIONS FOR AMINO ACYL-tRNA FORMATION IN NEUROSPORA

Condition	Ala	Glu	Gln	Gly	Met	Ser	14 Others
tRNA mg/ml	0.5 - 1.0					< 0.1	0.5 - 10
pH	7.0	7.3 - 7.5					
$Mg(Ac)_2$ μM/ml	25	10	5	50			
ATP "	2.5	5.0	5.0	2.5	5.0	2.5	
DTT "	0	5.0					
PEP "	0	20	20	0	20	0	0

All reaction mixtures are in 0.10 M Tris-HCl, have 1 mg/ml enzyme preparation (in 50% glycerol) and 0.02 μM/ml radioactive amino acid.

cultures). H^3-labeled cysteine and glutamine are not available; the chromatography of the C^{14}-labeled amino acyl-tRNA was performed for these using H^3 glycyl-tRNA as a marker. Figure 12 contains representative profiles of each amino acyl-tRNA comparison.

These profiles provide evidence on several parameters of Neurospora tRNA: 1) the amount of tRNA specific for each amino acid; 2) the number of separable components that compose this amount; 3) the relative proportion of each component. Finally, they indicate whether any of these parameters are changed for the tRNA extracted from ethionine-treated cultures. Figure 13 summarizes diagrammatically the MAK chromatographic mobilities of the 40 Neurospora amino acyl-tRNA's I have found. The rest of the data derived from the chromatographic profiles are summarized in Table 13.

No qualitative changes in profile are observed; for each amino acid the same number of components are found in the 2 types of culture and they have identical chromatographic mobilities. However, significant quantitative differences for 10 amino acyl-tRNA's do exist between the different cultures.

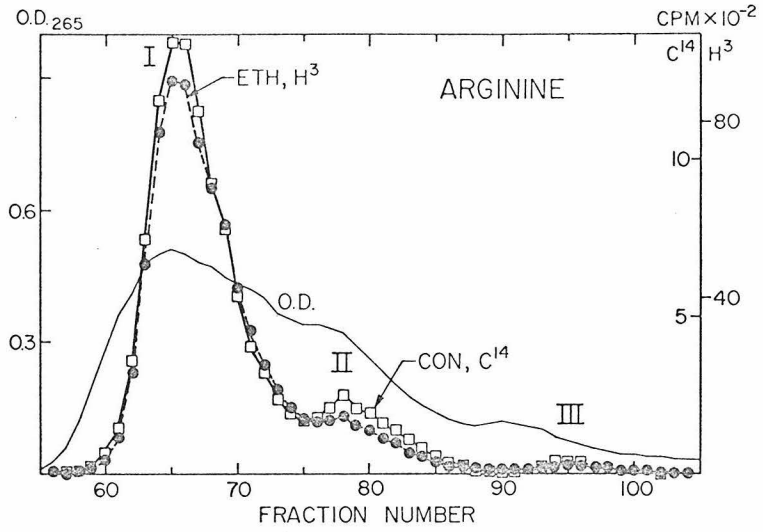
After ethionine treatment, the amount of serine, valine, and methionine-specific tRNA appears to increase, and that for glutamic acid, glutamine, histidine, lysine, proline, tryptophan and tyrosine decreases. The amount of tRNA for each of the 5 amino acids with one amino acyl-tRNA component showed some change.

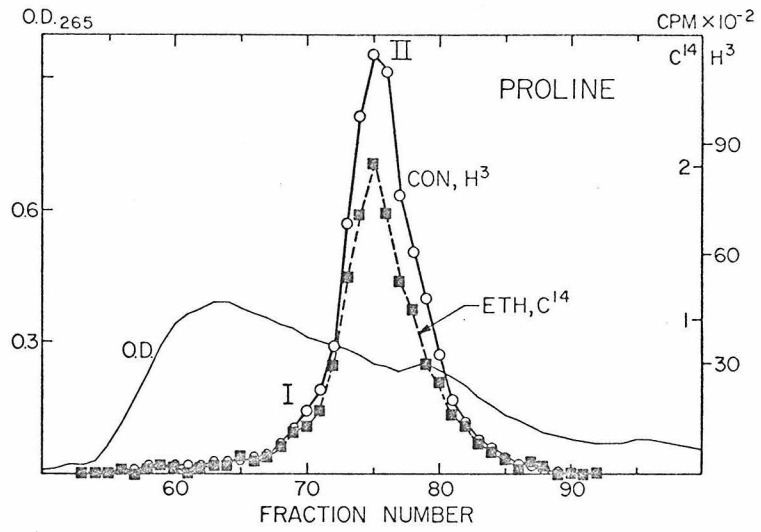
The relative proportion of the components for 14 of the 15 amino acids with multiple amino acyl-tRNA components remains the same.

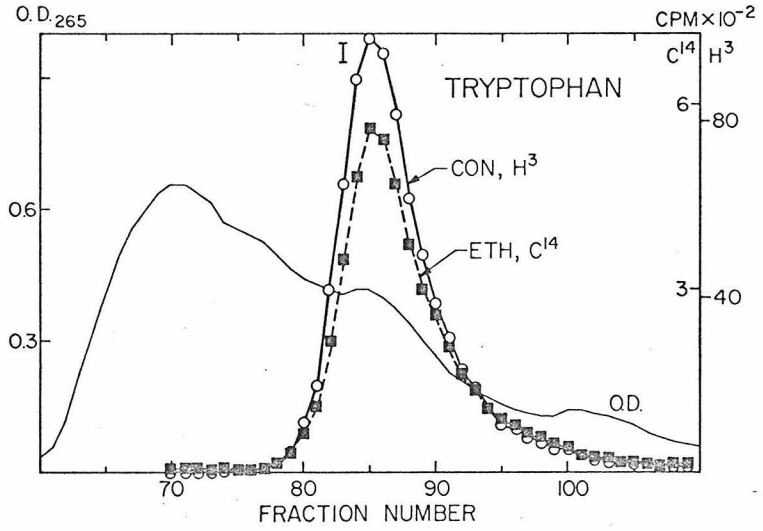
FIGURE 12. CHROMATOGRAPHIC PROFILES OF AMINO ACYL-tRNA's FROM VEGETATIVE, CONTROL AND ETHIONINE-TREATED CULTURES

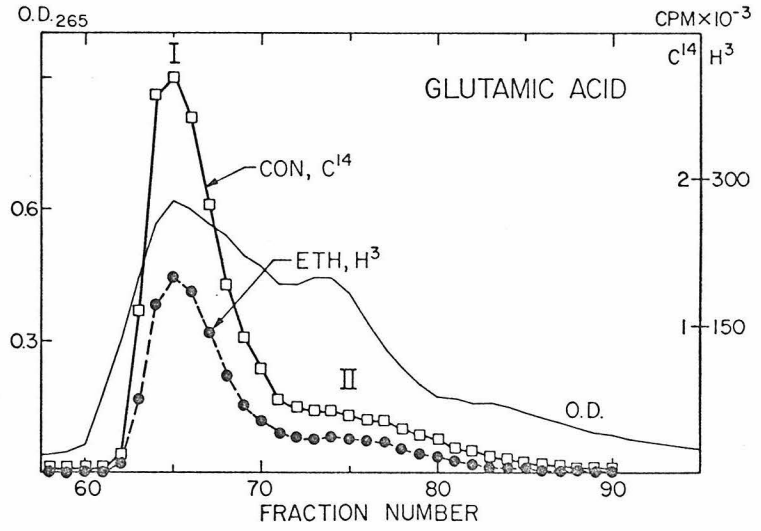
For 18 of the 20 amino acids, tRNA extracted from one culture was charged with C^{14} amino acid and co-chromatographed with tRNA extracted from the other kind of culture, charged with the same amino acid labeled with tritium. Tritium labeling is not available for the other 2 amino acids, cysteine and glutamine. For these, 2 columns were run for each comparison, using H^3 glycyl-tRNA as a reference marker.

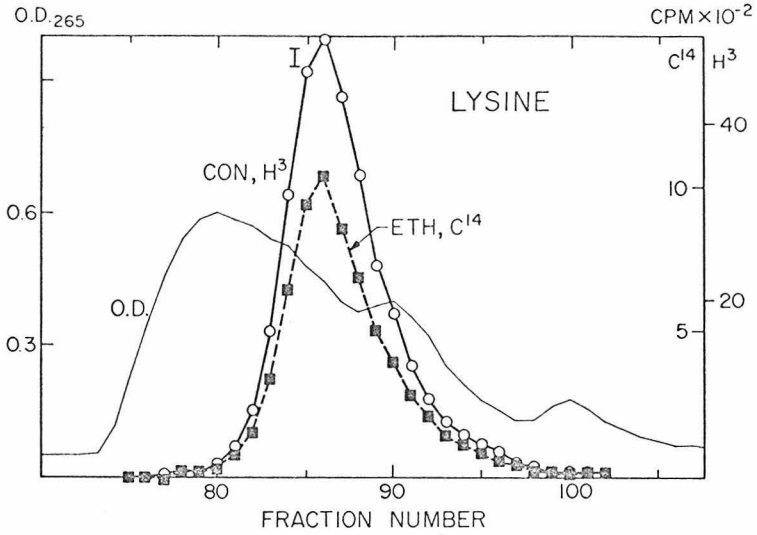
Thin line, $O.D._{265}$; heavy solid line and open symbol, tRNA from vegetative control culture (CON); dashed line and filled symbol, tRNA from ethionine-treated culture (ETH); squares, C^{14} amino acid; circles, H^3 amino acid.

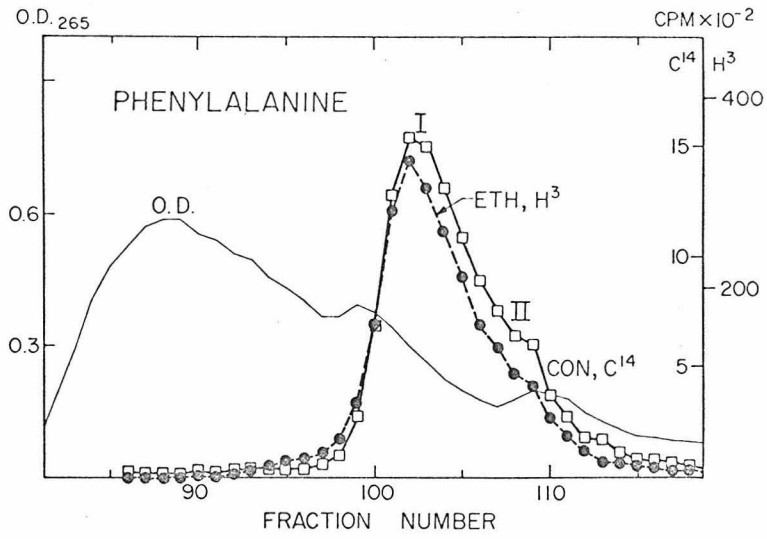

O.D.265
CPM×10-2
C14
H3
ARGININE
I
ETH, H3
O.D.
II
CON, C14
III
0.6
0.3
10
80
5
40
60
70
80
90
100
FRACTION NUMBER

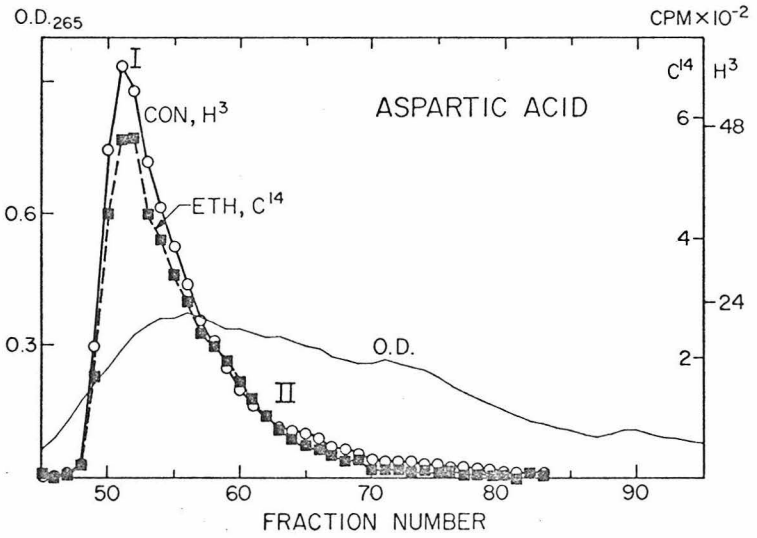

O.D.265
CPM×10-2
C14
H3
PROLINE
II
CON, H3
ETH, C14
O.D.
I
0.6
0.3
2
90
60
1
30
60
70
80
90
FRACTION NUMBER

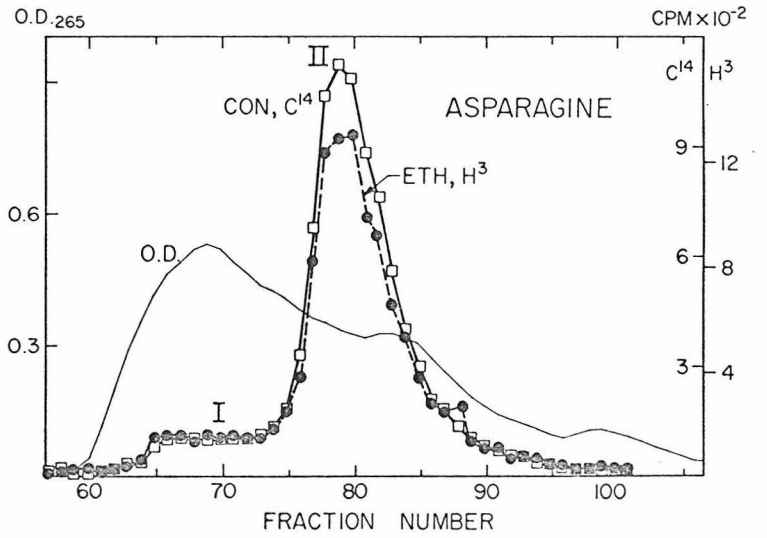

O.D.265
CPM×10-2
C14
H3
TRYPTOPHAN
I
CON, H3
ETH, C14
O.D.
0.6
0.3
6
80
3
40
70
80
90
100
FRACTION NUMBER

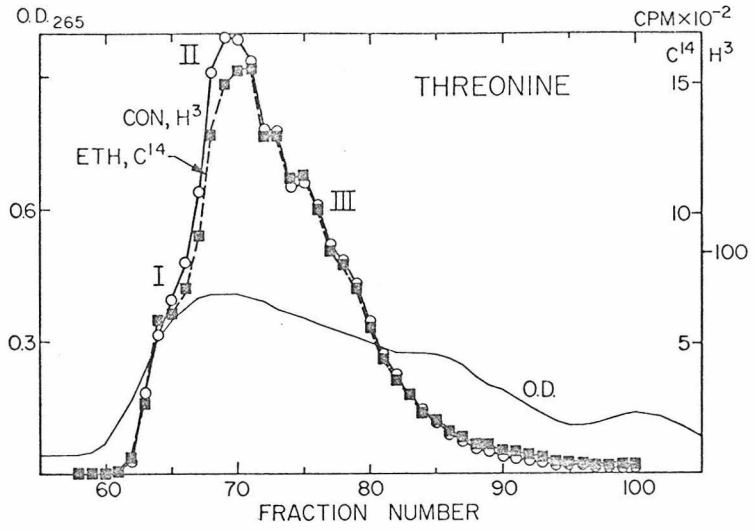

O.D.265
CPM×10-3
C14
H3
GLUTAMIC ACID
I
CON, C14
ETH, H3
II
O.D.
0.6
0.3
2
300
1
150
60
70
80
90
FRACTION NUMBER

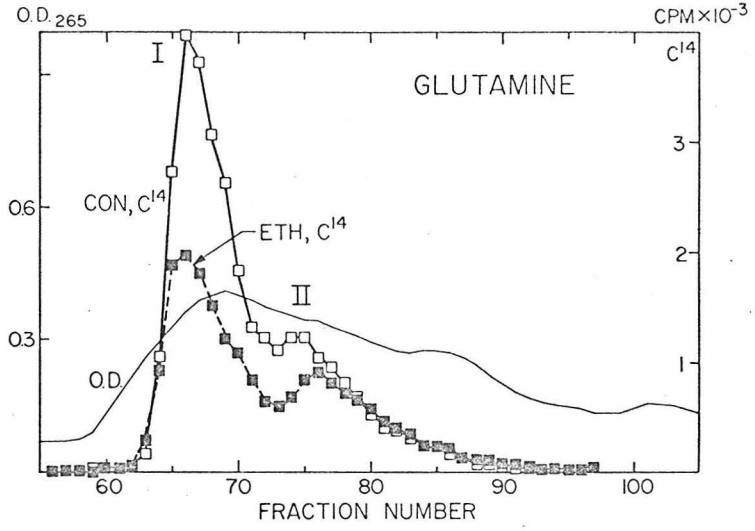

O.D.265
CPM×10-2
C14
H3
LYSINE
I
CON, H3
ETH, C14
O.D.
0.6
0.3
40
10
20
5
80
90
100
FRACTION NUMBER

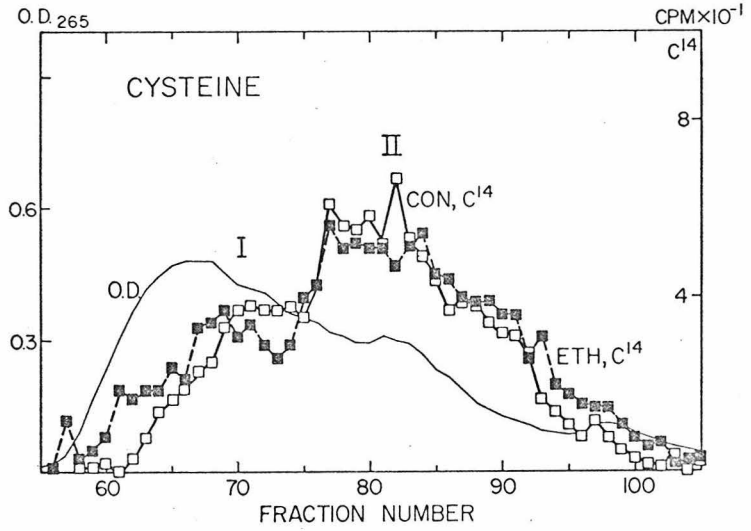

O.D.265
CPM×10-2
C14
H3
PHENYLALANINE
I
ETH, H3
O.D.
II
CON, C14
0.6
0.3
400
15
10
200
5
90
100
110
FRACTION NUMBER

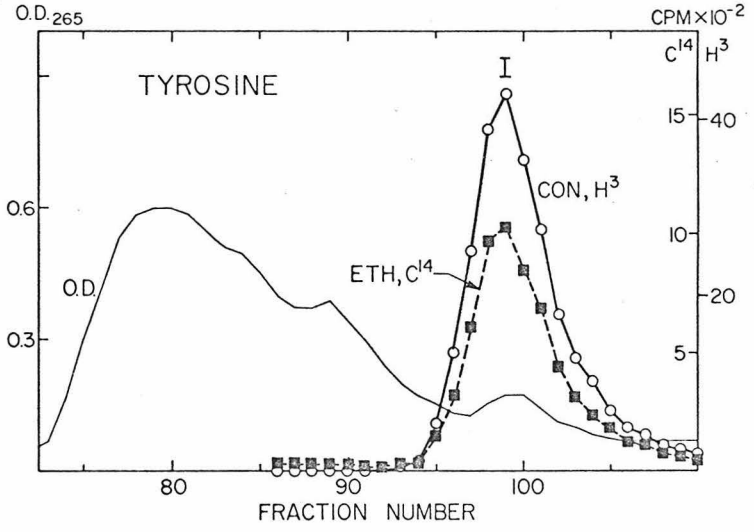

O.D.265
CPM×10-2
C14
H3
ASPARTIC ACID
I
CON, H3
ETH, C14
O.D.
II
0.6
0.3
6
48
4
24
2
50
60
70
80
90
FRACTION NUMBER

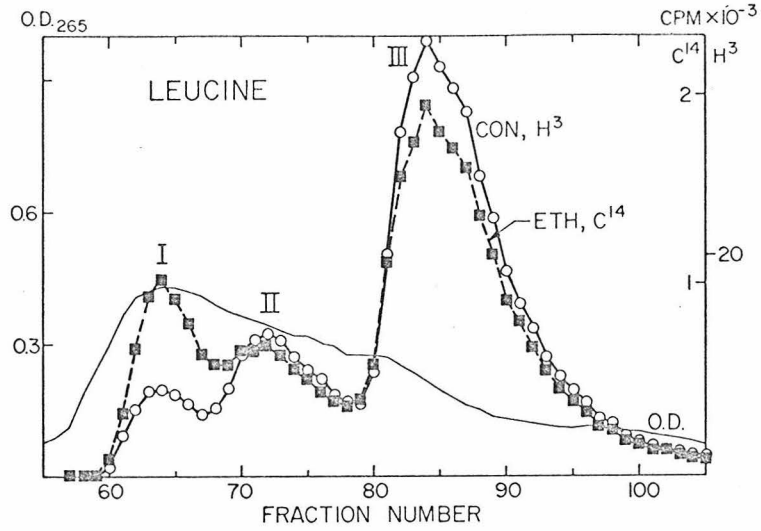

O.D.265
CPM×10-2
C14
H3
II
CON, C14
ASPARAGINE
ETH, H3
O.D.
I
0.6
0.3
9
12
6
8
3
4
60
70
80
90
100
FRACTION NUMBER

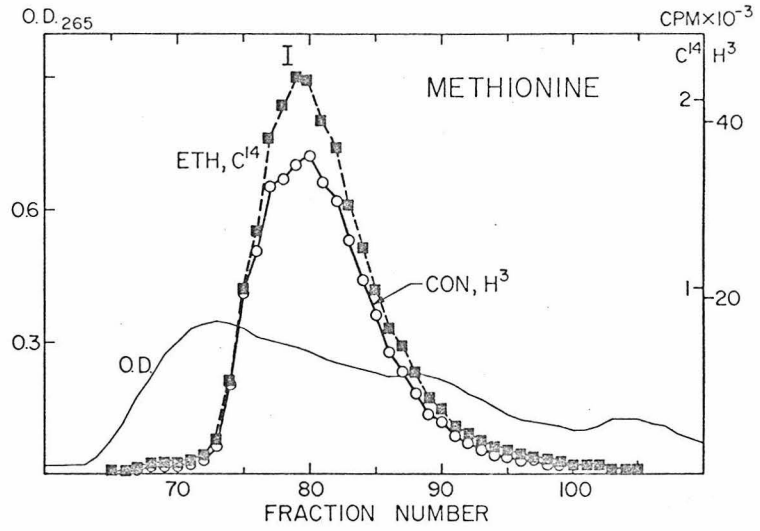

O.D.265
CPM×10-2
C14
H3
THREONINE
II
CON, H3
ETH, C14
III
I
O.D.
0.6
0.3
15
10
100
5
60
70
80
90
100
FRACTION NUMBER

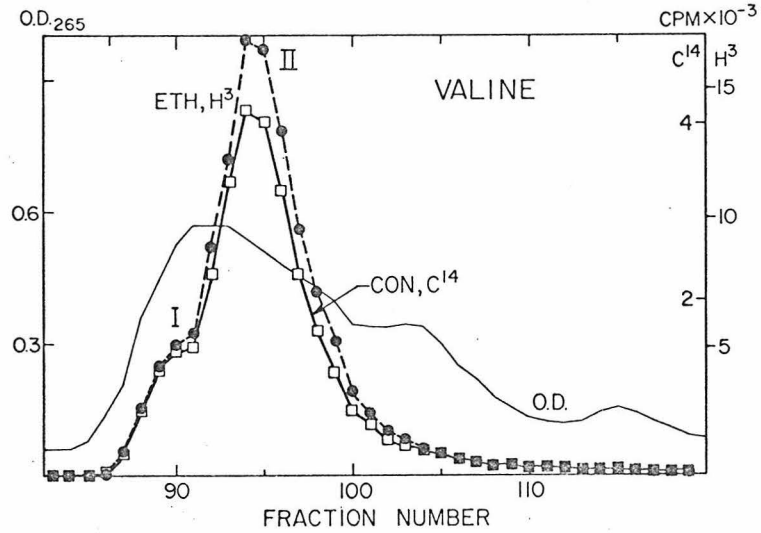

O.D.265
CPM×10-3
C14
GLUTAMINE
I
CON, C14
ETH, C14
II
O.D.
0.6
0.3
3
2
1
60
70
80
90
100
FRACTION NUMBER

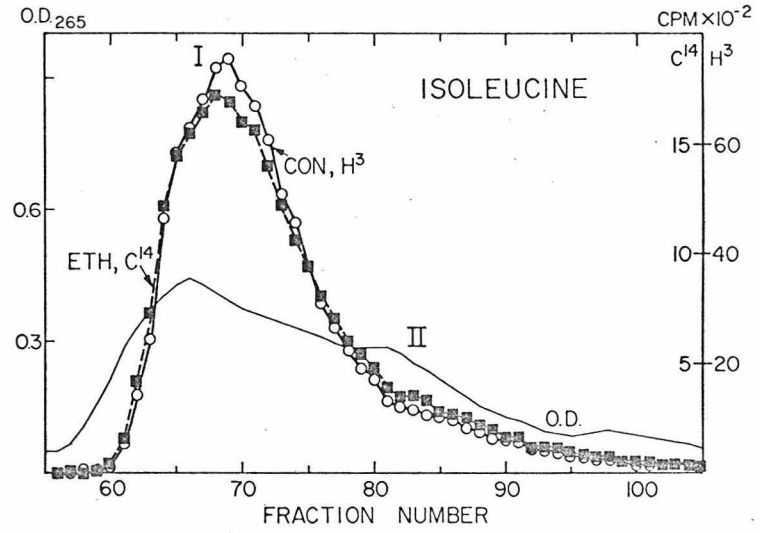

O.D.265
CPM×10-1
C14
CYSTEINE
II
CON, C14
I
O.D.
ETH, C14
0.6
0.3
8
4
60
70
80
90
100
FRACTION NUMBER

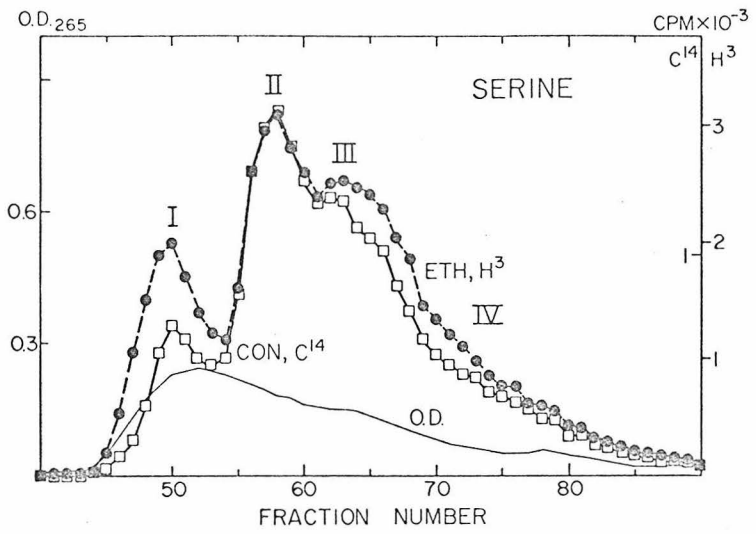

O.D.265
CPM×10-2
C14
H3
TYROSINE
I
CON, H3
O.D.
ETH, C14
0.6
0.3
15
10
5
40
20
80
90
100
FRACTION NUMBER

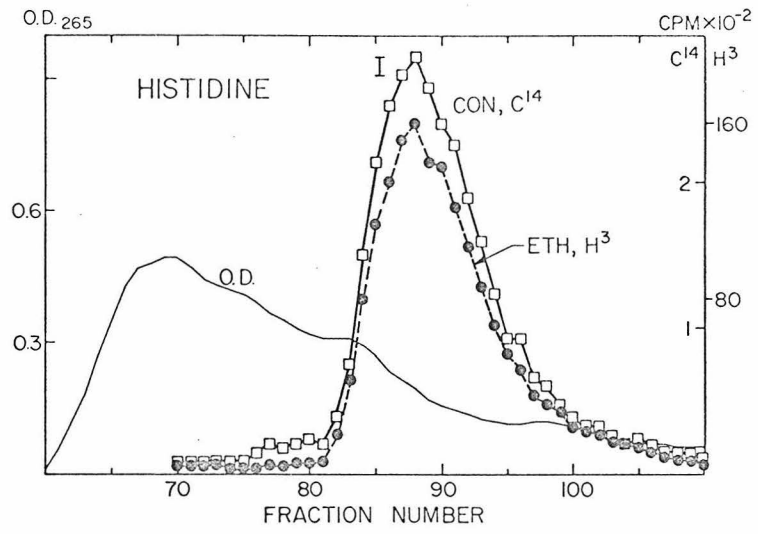

O.D.265
CPM×10-3
C14 H3
LEUCINE
III
CON, H3
ETH, C14
I
II
O.D.
0.6
0.3
2
1
20
60
70
80
90
100
FRACTION NUMBER

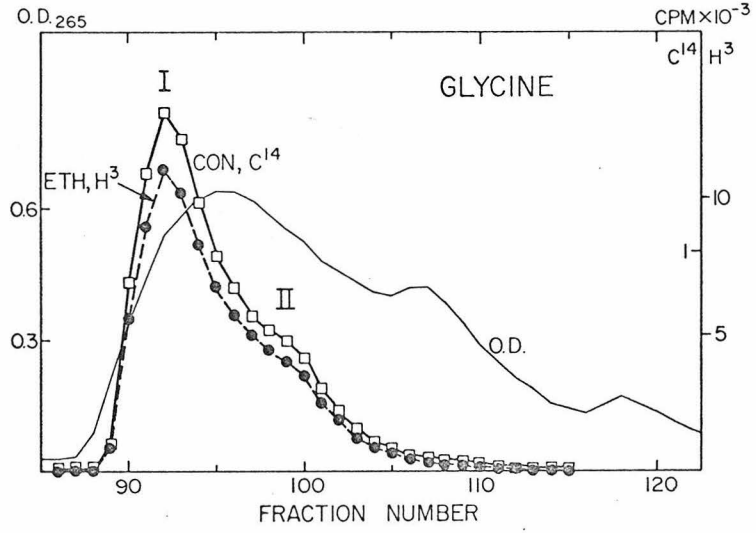

O.D.265
CPM×10-3
C14 H3
METHIONINE
I
ETH, C14
CON, H3
O.D.
0.6
0.3
2
1
40
20
70
80
90
100
FRACTION NUMBER


O.D.265
CPM×10-3
C14 H3
VALINE
II
ETH, H3
I
CON, C14
O.D.
0.6
0.3
4
2
15
10
5
90
100
110
FRACTION NUMBER


O.D.265
CPM×10-2
C14 H3
ISOLEUCINE
I
CON, H3
ETH, C14
II
O.D.
0.6
0.3
15
10
5
60
40
20
60
70
80
90
100
FRACTION NUMBER


O.D.265
CPM×10⁻³
C¹⁴ H³
SERINE
I
II
III
IV
ETH, H³
CON, C¹⁴
O.D.
0.6
0.3
3
2
1
50
60
70
80
FRACTION NUMBER


O.D.265
CPM×10⁻²
C¹⁴ H³
HISTIDINE
I
CON, C¹⁴
ETH, H³
O.D.
0.6
0.3
160
80
2
1
70
80
90
100
FRACTION NUMBER


O.D.265
CPM×10⁻³
C¹⁴ H³
GLYCINE
I
II
ETH, H³
CON, C¹⁴
O.D.
0.6
0.3
10
5
1
90
100
110
120
FRACTION NUMBER

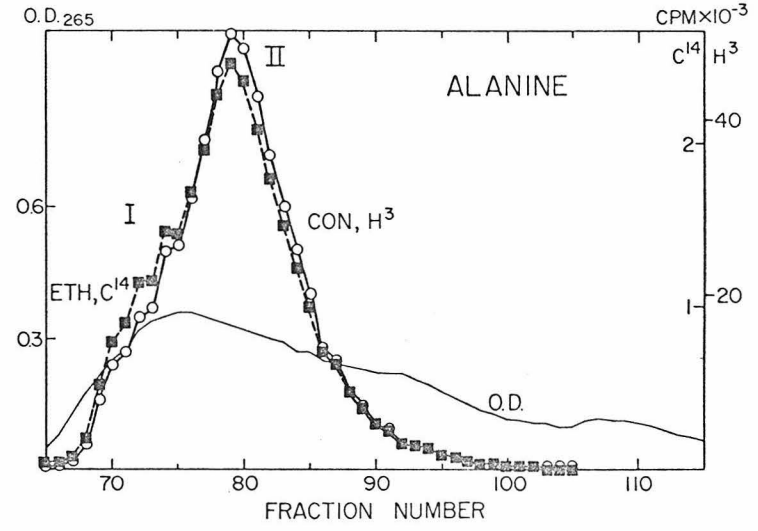

O.D.265
CPM×10⁻³
C¹⁴ H³
ALANINE
I
II
CON, H³
ETH, C¹⁴
O.D.
0.6
0.3
40
20
2
1
70
80
90
100
110
FRACTION NUMBER

FIGURE 13. SUMMARY OF THE ELUTION PROFILES OF THE 40 AMINO ACYL-tRNA's IN NEUROSPORA

The position of each tRNA component on MAK for the 20 amino acids of protein is diagrammatically indicated relative to the optical density profile.

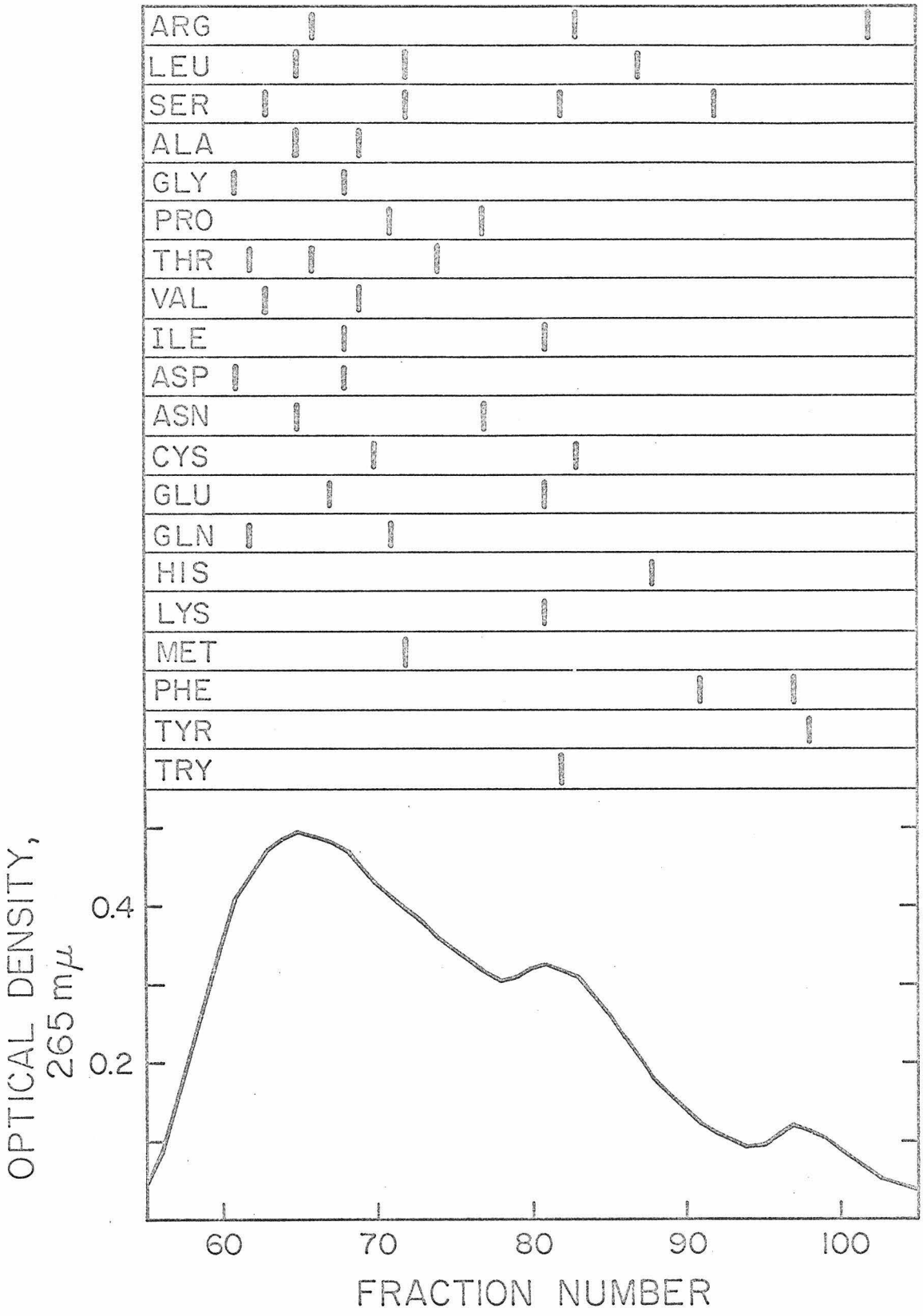

ARG
LEU
SER
ALA
GLY
PRO
THR
VAL
ILE
ASP
ASN
CYS
GLU
GLN
HIS
LYS
MET
PHE
TYR
TRY
OPTICAL DENSITY, 265 mμ
0.4
0.2
60
70
80
90
100
FRACTION NUMBER

TABLE 13. SUMMARY OF DATA FROM CHROMATOGRAPHIC PROFILES

Amino acid	Relative acceptor activity (E/C)100		Relative proportion of components			
			I	II	III	IV
			%			
Ala	98.8	C	11	89		
		E	11	89		
Arg	92.1	C	84	14	2	
		E	85	13	2	
Asn	90.2	C	11	89		
		E	11	89		
Asp	84.4	C	15	85		
		E	17	83		
Cys	106	C	20	80		
		E	26	74		
Glu	51.6	C	79	21		
		E	78	22		
Gln	66.1	C	72	28		
		E	60	40		
Gly	94.1	C	77	23		
		E	77	23		
His	79.0	C	100			
		E	100			
Ile	99.6	C	91	9		
		E	90	10		
Leu	99.4	C	12	21	67	
		E	13	20	67	
Lys	70.8	C	100			
		E	100			
Met	121	C	100			
		E	100			
Phe	85.0	C	81	19		
		E	84	16		
Pro	74.0	C	10	90		
		E	9	91		

TABLE 13 (continued)

Amino acid	Relative acceptor activity (E/C)100		Relative proportion of components			
			I	II	III	IV
			%			
Ser	120	C	18	43	25	14
		E	16	40	29	15
Thr	96.2	C	6	62	32	
		E	7	61	32	
Tryp	85.0	C	100			
		E	100			
Tyr	69.6	C	100			
		E	100			
Val	117	C	9	91		
		E	8	92		

Data for this table comes from profiles in Figure 12 as well as from relevant profiles not presented. Relative acceptor activity is the amount of amino acyl-tRNA from ethionine-treated cultures (E) relative to vegetative controls (C). Relative proportion is determined by the number of counts under each peak or shoulder and expressed as percentage of the total.

The sole exception, glutamine-tRNA seems to lose some of component I but none of component II (Table 14). Thus the relative proportion in this case changes considerably.

The observation of a relative increase in the proportion of component I for leucyl-tRNA (Figure 12) is an artifact. It is characteristic, not of the source of tRNA, but of the C^{14} label. Presumably, the C^{14} leucine, but not the H^3 leucine is slightly contaminated with some other amino acid.

The chromatographic differences in the proportions of the 4 seryl-tRNA components (Figure 12) are not reproducible. Each of 7 different chromatographic profiles of seryl-tRNA show unique proportions of the 4 components. This result can be explained by postulating either that in each case the tRNA chromatographed was not completely charged or that interconversion of the 4 components occurs. Sueoka and Gartland have observed interconversions of tryptophanyl-tRNA components in E. coli (57).

Time Course of Quantitative Changes in Acceptor Activity

In order to explore further the quantitative differences indicated by the chromatographic data, tRNA was extracted from 5 cultures grown for various lengths of time with no ethionine and from 7 cultures after varying lengths of ethionine treatment; the tRNA was assayed, by the filter paper disc method, for specific acceptor activity (total μμmoles of amino acid attached per $O.D._{260}$ unit of unfractionated tRNA). Each of the 12 tRNA samples was assayed individually for each of the 20

TABLE 14. NATURE OF CHANGE IN PROPORTION OF GLUTAMINE tRNA COMPONENTS

Sample	Distribution of components					
	Percentage			μμmoles/O.D.$_{260}$		
	I	II	Total	I	II	Total
Vegetative control	72	28	100	47.8	18.6	66.4
Ethionine treated	60	40	100	26.3	17.6	43.9
Net change				-21.5	-1.0	-22.5

The specific acceptor activity of tRNA from control cultures for glutamine, determined by direct assay, is divided between the two components according to chromatographic evidence. The ethionine-treated culture yielded tRNA with 66.1% as much activity for glutamine and this is also apportioned in the table according to chromatographic evidence.

amino acids. The data are presented in Table 15. Table 15 includes in addition the same data expressed as the molar per cent activity, i.e., each specific activity is expressed as the percentage it represents of the total acceptor activity of the particular sample. The total acceptor activity is the sum of the 20 individually determined specific acceptor activities for any one sample.

If at "0" time (defined previously as 72 hours of growth) no ethionine is added, the total acceptor activity decreases slightly after 20 hours of additional shaking (Figure 14). The loss occurs mainly by reduction of the specific acceptor activity for asparagine, glutamine, and cysteine. If, however, ethionine is added at "0" time, then after 8 hours, 30.3% of the total acceptor activity is lost. The reduced level remains unchanged for at least an additional 10 hours (Figure 14). By extrapolation, this total activity value after 10 hours in ethionine would be reached by the untreated sample in two days (44-48 hours).

For 4 of the amino acids (valine, tryptophan, aspartic acid, and phenylalanine), no pattern of significant change in specific acceptor activity occurs whether or not ethionine is added. Fifteen or 20 hours after ethionine treatment, the molar per cent of these four accordingly increases. An additional three, previously mentioned (cysteine, asparagine, glutamine) lose acceptor activity whether or not ethionine is added. Ethionine does specifically stimulate a loss of specific acceptor activity for the remaining 13 amino acids. The time course for one representative of each class is shown graphically in Figure 15. In Table 16 an average value is listed for the specific acceptor

TABLE 15. SPECIFIC ACCEPTOR ACTIVITY AS A FUNCTION OF TIME FOR CONTROL AND ETHIONINE-TREATED CULTURES

Culture (hours)	Alanine		Arginine		Asparagine	
	μμM/O.D.$_{260}$	M%	μμM/O.D.$_{260}$	M%	μμM/O.D.$_{260}$	M%
-20,C	89.4	6.71	122	9.16	33.6	2.52
-10,C	83.7	6.54	111	8.68	39.0	3.05
-5,C	85.8	6.60	108	8.31	29.8	2.29
0,C	86.2	7.06	111	9.09	29.5	2.42
20,C	90.4	7.83	98.6	8.54	19.6	1.70
2,E	84.8	6.67	107	8.41	31.6	2.48
4,E	84.4	6.57	109	8.48	29.9	2.33
6,E	86.2	6.91	105	8.42	28.5	2.28
8,E	85.3	6.77	115	9.13	33.0	2.62
10,E	67.3	7.54	66.3	7.43	19.3	2.16
15,E	60.1	6.73	79.6	8.92	24.1	2.70
20,E	56.5	6.42	77.0	8.75	22.7	2.58
	Aspartic acid		Cysteine		Glutamic acid	
-20,C	37.4	2.81	22.7	1.70	36.9	2.77
-10,C	42.5	3.32	15.6	1.22	34.1	2.67
-5,C	33.8	2.60	17.0	1.31	33.5	2.58
0,C	33.0	2.73	16.7	1.37	29.3	2.40
20,C	28.6	2.48	10.9	0.94	27.5	2.38
2,E	34.8	2.74	17.1	1.34	36.8	2.89
4,E	42.0	3.27	18.1	1.41	32.9	2.56
6,E	35.2	2.82	15.7	1.26	31.8	2.55
8,E	40.0	3.17	11.0	0.87	32.5	2.58
10,E	16.3	1.83	12.4	1.39	23.9	2.68
15,E	28.2	3.16	9.47	1.06	20.4	2.28
20,E	29.8	3.39	8.06	0.92	20.2	2.30
	Glutamine		Glycine		Histidine	
-20,C	68.7	5.16	55.8	4.19	25.8	1.94
-10,C	69.8	5.46	45.2	3.53	21.2	1.66
-5,C	63.7	4.90	54.0	4.20	19.9	1.53
0,C	60.9	4.99	53.7	4.40	16.9	1.38
20,C	48.2	4.18	46.4	4.02	13.7	1.19

TABLE 15 (continued)

	Glutamine		Glycine		Histidine	
2,E	66.3	5.21	53.9	4.24	20.1	1.58
4,E	69.3	5.39	56.9	4.43	19.0	1.48
6,E	62.9	5.04	48.4	3.88	16.6	1.33
8,E	69.8	5.54	61.8	4.90	24.6	1.95
10,E	37.6	4.21	46.4	5.20	10.0	1.12
15,E	44.3	4.96	22.1	2.48	7.96	0.89
20,E	47.7	5.42	20.1	2.29	7.54	0.86

	Isoleucine		Leucine		Lysine	
-20,C	90.6	6.80	116	8.70	51.7	3.88
-10,C	98.6	7.71	116	9.07	40.8	3.19
-5,C	96.5	7.43	127	9.78	44.6	3.43
0,C	83.7	6.86	110	9.01	36.8	3.02
20,C	91.6	7.94	119	10.31	28.3	2.45
2,E	93.5	7.35	119	9.36	39.2	3.08
4,E	96.3	7.49	112	8.71	42.8	3.33
6,E	103	8.26	111	8.90	38.8	3.11
8,E	92.8	7.36	111	8.81	44.7	3.55
10,E	58.8	6.59	90.2	10.10	21.9	2.45
15,E	67.9	7.60	84.9	9.51	20.6	2.31
20,E	74.7	8.49	78.5	8.93	23.9	2.72

	Methionine		Phenylalanine		Proline	
-20,C	84.1	6.31	18.6	1.40	34.2	2.57
-10,C	81.7	6.39	19.1	1.49	34.0	2.66
-5,C	84.0	6.47	23.2	1.79	35.9	2.76
0,C	75.2	6.16	29.7	2.43	34.2	2.80
20,C	86.2	7.47	27.7	2.40	33.4	2.89
2,E	82.6	6.49	24.5	1.93	33.2	2.61
4,E	87.9	6.84	26.6	2.07	40.5	3.15
6,E	92.0	7.37	32.0	2.57	38.6	3.09
8,E	83.5	6.63	22.9	1.82	39.1	3.10
10,E	54.1	6.06	17.0	1.90	25.0	2.80
15,E	75.6	8.47	34.2	3.83	22.8	2.56
20,E	64.1	7.29	31.5	3.58	21.4	2.43

TABLE 15 (continued)

	Serine		Threonine		Tryptophan	
-20,C	136	10.21	81.4	6.11	57.5	4.31
-10,C	131	10.24	70.3	5.50	56.8	4.44
-5,C	129	9.93	74.4	5.73	60.0	4.62
0,C	123	10.08	74.3	6.09	54.7	4.48
20,C	114	9.88	63.7	5.52	53.8	4.66
2,E	126	9.91	71.4	5.61	57.9	4.55
4,E	121	9.41	71.0	5.52	65.5	5.10
6,E	111	8.90	73.3	5.88	57.3	4.59
8,E	110	8.73	78.0	6.19	59.6	4.73
10,E	103	11.54	53.9	6.04	46.7	5.23
15,E	62.7	7.02	34.4	3.85	51.5	5.77
20,E	57.7	6.56	30.9	3.51	63.7	7.24

	Tyrosine		Valine		Total
-20,C	53.2	3.99	117	8.78	1332.6
-10,C	53.5	4.18	115	8.99	1278.9
-5,C	53.4	4.11	125	9.62	1299.0
0,C	55.5	4.55	106	8.68	1220.6
20,C	36.8	3.19	116	10.05	1154.4
2,E	52.2	4.10	120	9.43	1271.9
4,E	50.2	3.91	110	8.56	1285.3
6,E	42.2	3.38	118	9.46	1247.5
8,E	39.7	3.15	106	8.41	1260.3
10,E	38.0	4.26	84.5	9.46	892.60
15,E	21.1	2.36	121	13.55	892.93
20,E	21.8	2.48	122	13.87	879.80

The underlined values represent significant deviations from the means expressed in Table 16. The probability of obtaining random deviations as large, i.e. (deviation)/(standard deviation) > 3, is < 0.27%. C, control cultures; E, ethionine-treated cultures.

FIGURE 14. TIME COURSE OF TOTAL ACCEPTOR ACTIVITY

Plot of data in column labeled "Total" of Table 15. Solid circles, untreated control cultures; open circles, ethionine-treated cultures.

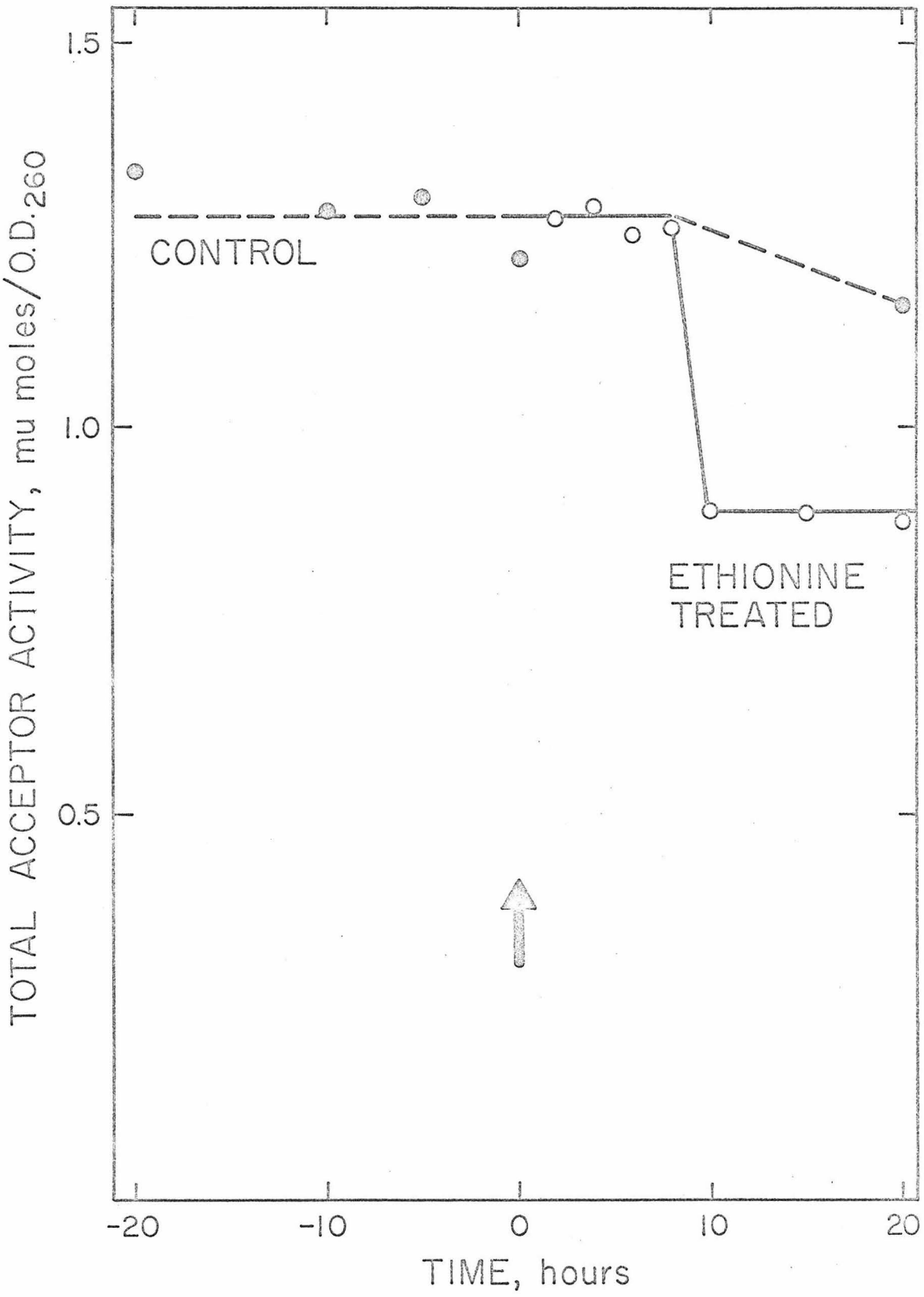

TOTAL ACCEPTOR ACTIVITY, mu moles/O.D.260
1.5
1.0
0.5
CONTROL
ETHIONINE
TREATED
-20
-10
0
10
20
TIME, hours

FIGURE 15. TIME COURSE OF SPECIFIC ACCEPTOR ACTIVITY FOR ALANINE, ASPARAGINE, TRYPTOPHAN

Alanine (triangles) represents 13 amino acids for which specific acceptor activity declines in response to ethionine. Valine (squares) represents 4 amino acids, for which the specific acceptor activity remains unchanged. The specific acceptor activity for asparagine (circles) and 2 other amino acids declines whether or not ethionine is added. Solid symbols, untreated controls; open symbols, ethionine-treated.

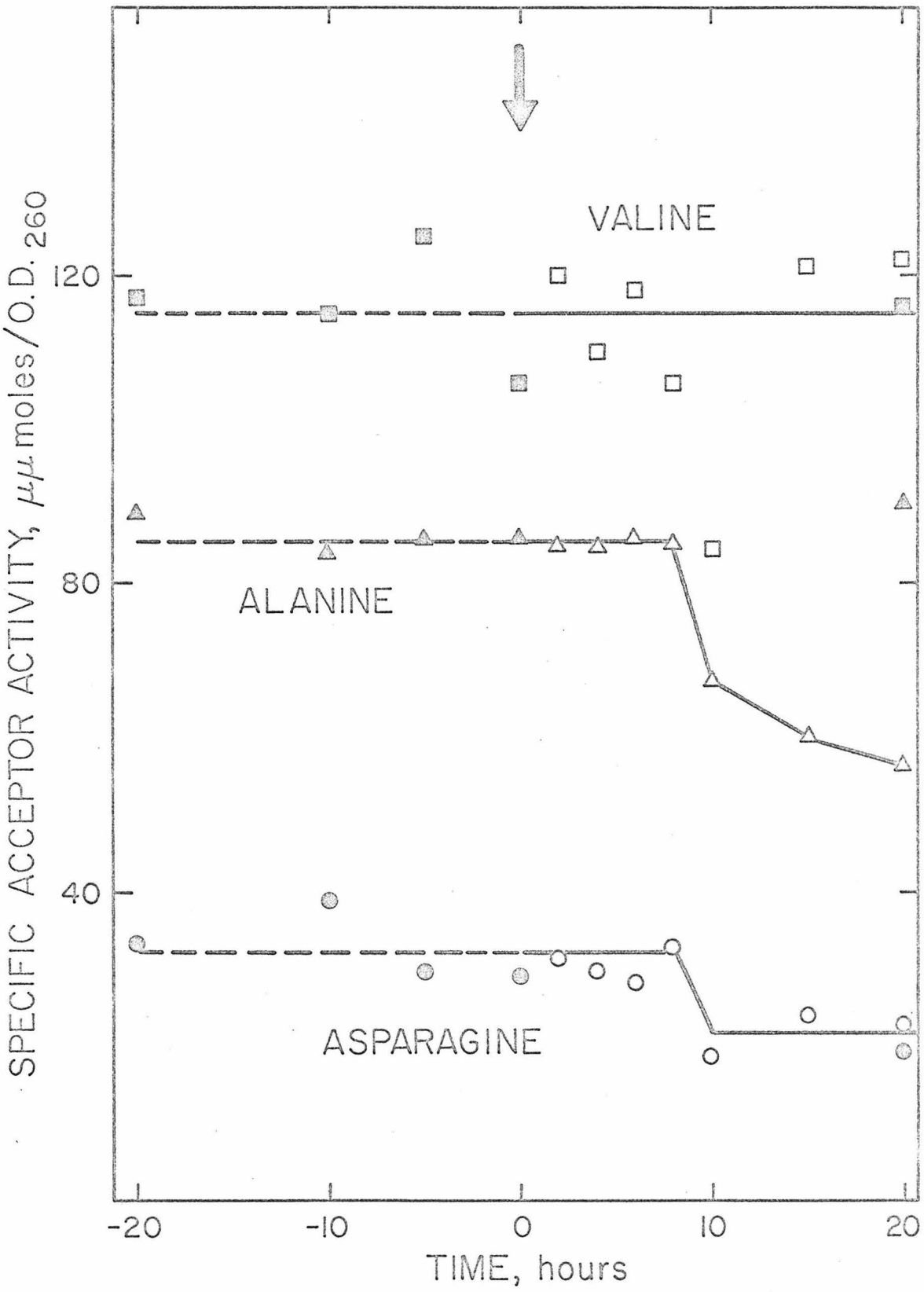

VALINE
ALANINE
ASPARAGINE
SPECIFIC ACCEPTOR ACTIVITY, μμ moles/O.D. 260
120
80
40
-20
-10
0
10
20
TIME, hours

TABLE 16. AVERAGE VALUES OF SPECIFIC ACCEPTOR ACTIVITY FOR 20 AMINO ACIDS IN NEUROSPORA

Amino acid	Specific acceptor activity			
	μμmoles/O.D.$_{260}$	±	standard deviation	% of total
Alanine	85.7	±	1.7	6.7
Arginine	111		5.6	8.7
Asparagine	31.9		3.4	2.9
Aspartic acid	37.4		3.7	2.5
Cysteine	16.7		3.3	1.3
Glutamic acid	33.5		2.6	2.6
Glutamine	66.4		3.5	5.2
Glycine	53.8		5.2	4.2
Histidine	20.5		3.4	1.6
Isoleucine	94.0		5.8	7.4
Leucine	115		5.7	9.0
Lysine	42.8		4.8	3.4
Methionine	83.9		4.9	6.6
Phenylalanine	24.6		4.8	1.9
Proline	36.2		2.8	2.9
Serine	123		9.2	9.7
Threonine	74.3		3.8	5.8
Tryptophan	58.7		3.2	4.6
Tyrosine	50.0		5.8	3.9
Valine	115		6.8	9.0
	1274.4			99.9

Averages were computed from -20, -10, -5, and 0 hour controls and 2,4,6 and 8 hour ethionine-treated values in Table 15.

activity and molar per cent it represents for each of the 20 amino acid-specific tRNA's of Neurospora. The values from the -20, -10, -5, 0, 2, 4, 6, 8 hour time points of Table 15 were used to compute these averages.

The Attachment of Amino Acid Analogs to tRNA

The biochemical response of Neurospora mycelium to the addition of para-fluorophenylalanine (pFphe) is similar to the response to addition of ethionine (eth) (29). The rate of general protein synthesis, for example, changes little after short times (up to 5 hours) of treatment (58). Since these analogs do not act primarily by reducing the rate of protein synthesis, it seemed likely that they were being incorporated into proteins in place of phe and met respectively, and were reducing the functional activity of such proteins. I decided to investigate one step in this proposed sequence: the attachment of these analogs to tRNA.

A considerably higher concentration of analog is required to saturate the synthetase enzyme than is required for the respective amino acid (Figures 16 and 17). The amount of enzyme which is saturated with 0.04 μM/ml of methionine does not even approach saturation with 6.5 times the concentration of ethionine. The differential is even greater in the case of phe and pFphe. At a concentration of 1.5 μM/ml, pFphe is approaching saturation whereas phe saturates the enzyme at 0.005 μM/ml. While the K_M for each of these substrates has not been determined, i.e., initial rates were not measured, it does appear that the

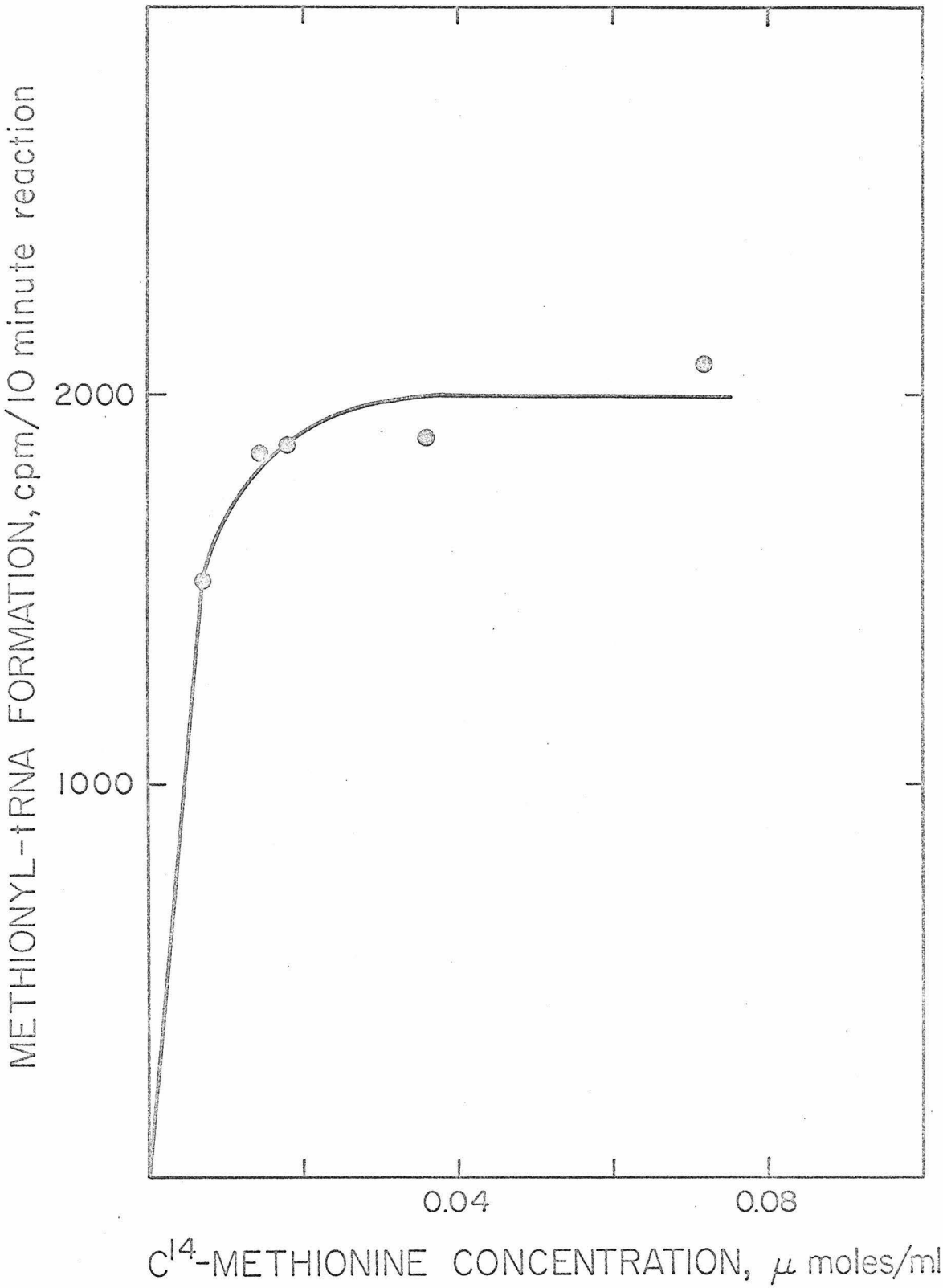
FIGURE 16. CONCENTRATION CURVE FOR CHARGING OF ANALOGS TO tRNA

Rate of reaction as a function of increasing concentration of ethionine (circles) and para-fluorophenylalanine (triangles) respectively.

FIGURE 17. CONCENTRATION CURVE FOR CHARGING OF METHIONINE TO tRNA

Rate of methionyl-tRNA formation as a function of methionine concentration. Methionine appears to saturate the enzyme at about 0.04 μmole/ml.

ANALOG-tRNA FORMATION, cpm/15min reaction
3000
2000
1000
0.5
1.0
C14-ANALOG CONCENTRATION, μ moles/ml
p-FLUOROPHENYLALANINE
ETHIONINE


METHIONYL-tRNA FORMATION, cpm/10 minute reaction
2000
1000
0.04
0.08
C14-METHIONINE CONCENTRATION, μ moles/ml

enzymes have much less affinity for the analogs than for their respective amino acids.

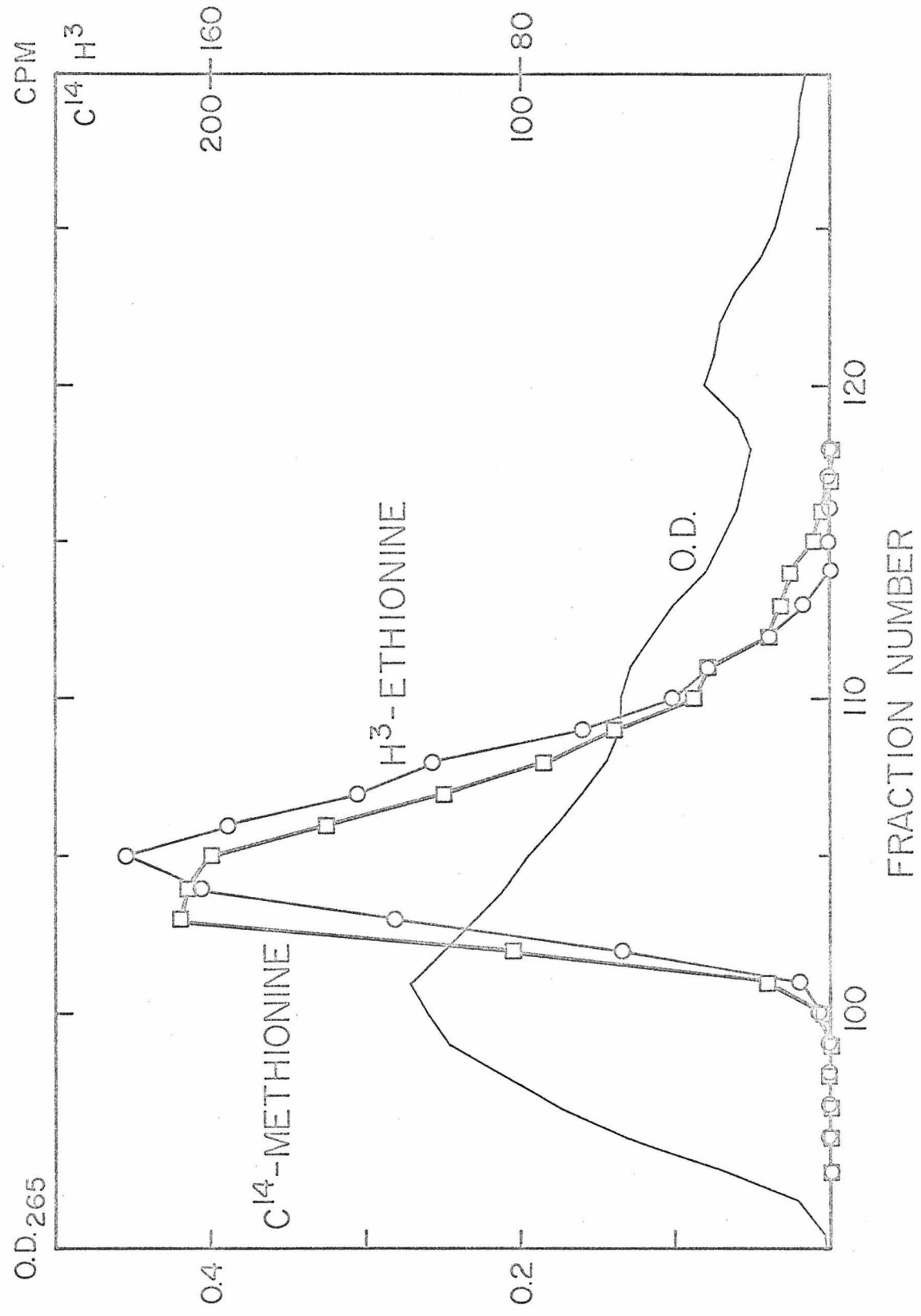
Next I examined whether in both cases the amino acid and its analog were attached to the same tRNA component(s). Figure 18 shows the results of co-chromatographing H^3 phe-tRNA and C^{14} pFphe-tRNA and also C^{14} met-tRNA and H^3 eth-tRNA. In both cases the analog-tRNA complex elutes reproducibly at a slightly higher salt concentration than does the amino acyl-tRNA.

Three possible explanations of this result are: 1) the analogs are attached to tRNA molecules distinct from those specific for their respective amino acids which coincidentally have similar chromatographic mobilities; 2) in the case of phe and pFphe, pFphe is attached only to the minor, mitochondrial, component of the phe-specific tRNA (59); 3) in both cases the amino acid and its analog are attached to the same tRNA molecules, but the difference in the structure of the analog is sufficient to alter the properties of its complex with the acceptor RNA and cause a shift in chromatographic mobility.

Competition experiments using labeled amino acid and increasing concentrations of unlabeled analog support the third alternative (Figure 19). Competition begins when the concentration of analog is five to ten times that of its respective amino acid. In neither case is there any indication of a plateau (incomplete competition). If the first alternative were correct no competition would be expected, and if the second were correct pFphe would be expected to compete with only 15% of the phe acceptors. A serious objection to this interpretation of the

FIGURE 18. CHROMATOGRAPHIC PROFILES OF AMINO ACYL-tRNA VS. ANALOG-tRNA

Co-chromatography on MAK of C^{14} met-tRNA with H^3 eth-tRNA and H^3 phe-tRNA with C^{14} pFphe-tRNA. H^3 eth was used at 0.4 μmole/ml to prepare H^3 eth-tRNA. C^{14} pFphe was used at 1.5 μmole/ml.


CPM
C14 H3
200
160
100
80
O.D.265
0.4
0.2
C14-METHIONINE
H3-ETHIONINE
O.D.
100
110
120
FRACTION NUMBER

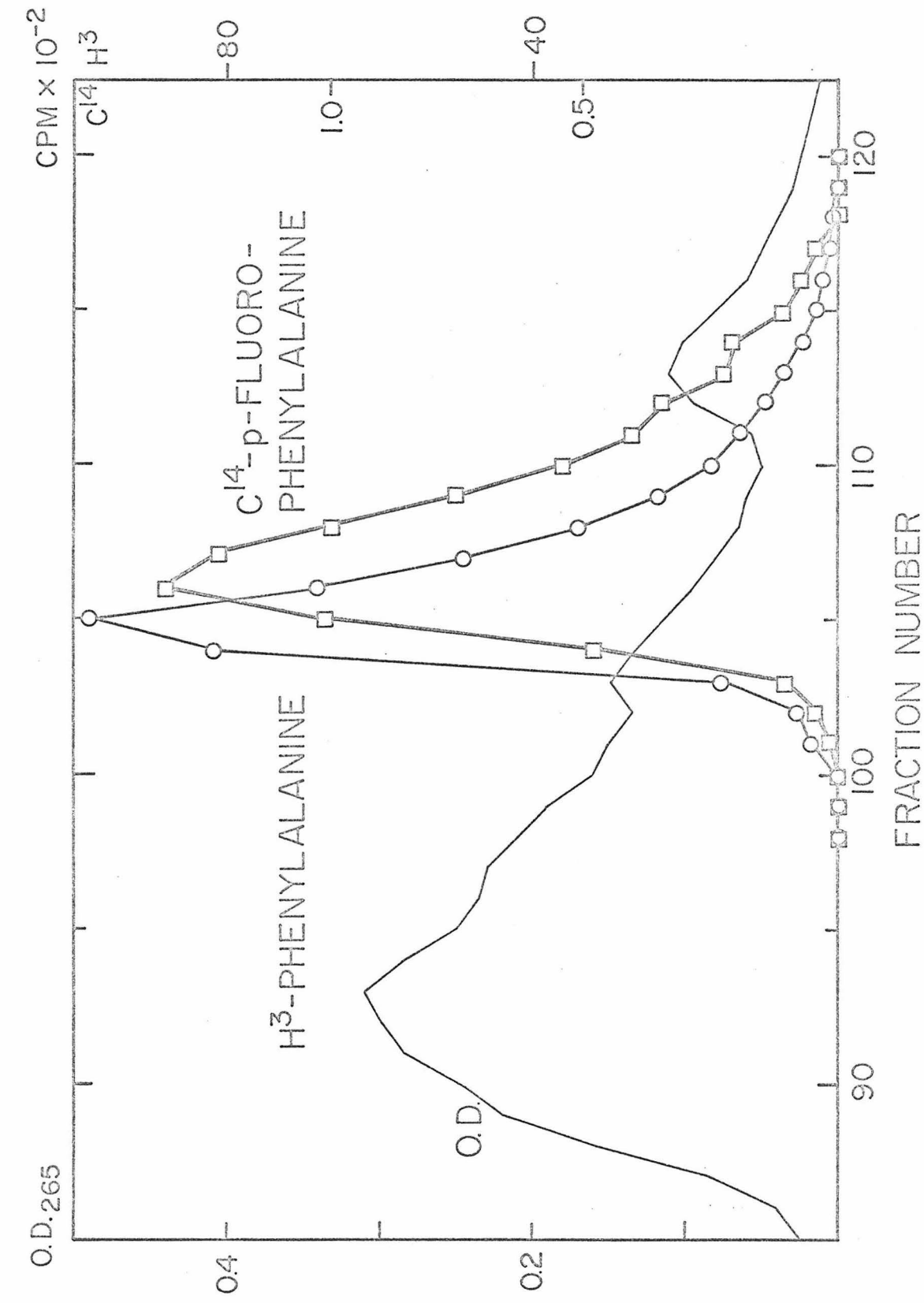
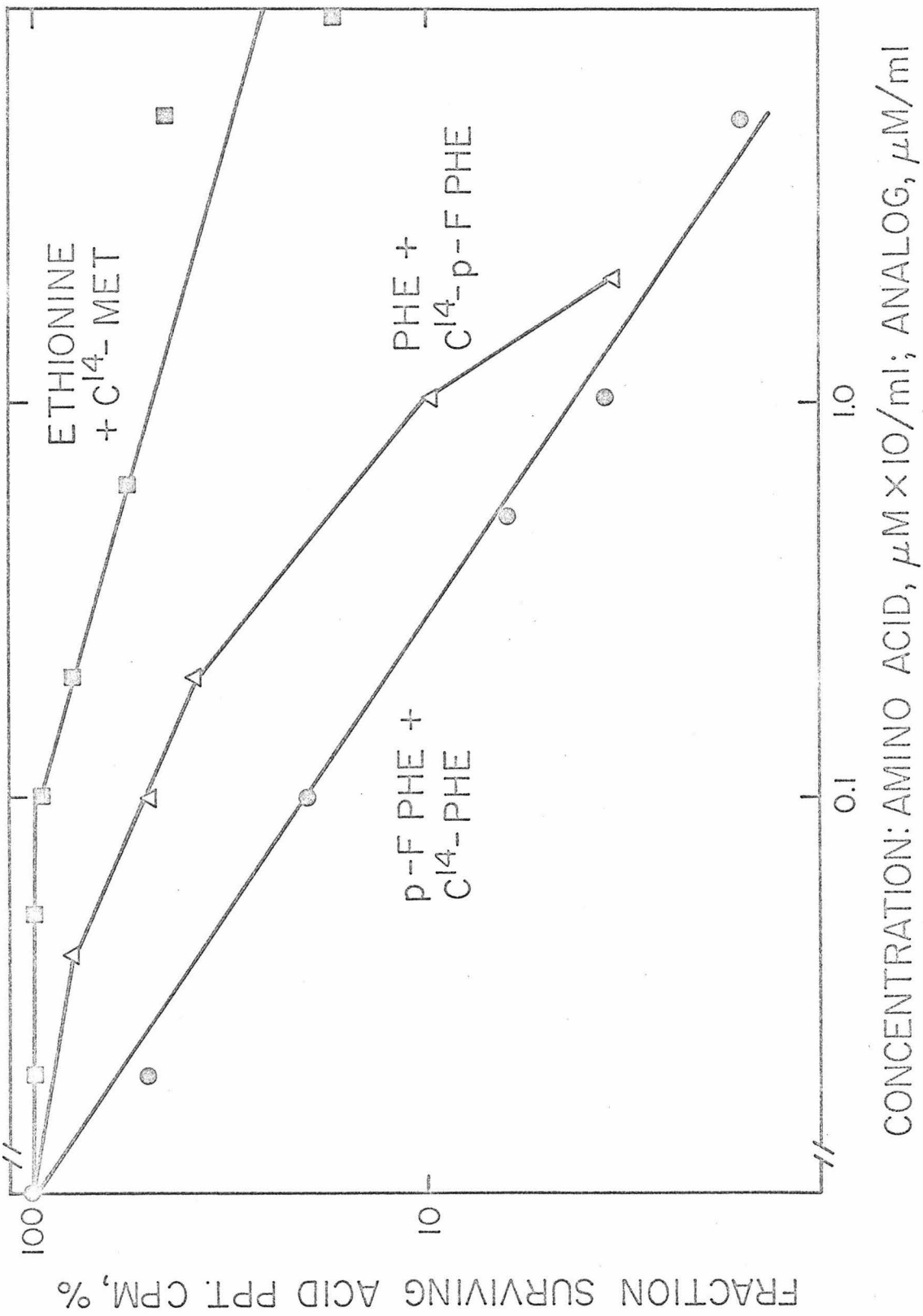

CPM x 10⁻²
C¹⁴
H³
80
40
1.0
0.5
C¹⁴-p-FLUORO-PHENYLALANINE
H³-PHENYLALANINE
O.D.
O.D.265
0.4
0.2
90
100
110
120
FRACTION NUMBER

FIGURE 19. COMPETITION EXPERIMENTS BETWEEN ANALOGS AND THEIR RESPECTIVE AMINO ACIDS

Experiments measuring competition for attachment to tRNA were performed in both directions. A constant amount of labeled amino acid, for example, is charged to tRNA in the presence of an increasing concentration of unlabeled analog. When all of the acceptor sites are occupied, the amount of radioactivity is measured. Squares, increasing ethionine concentration - 0.018 μmoles/ml C^{14} methionine; circles, increasing pFphe concentration - 0.002 μmoles/ml C^{14} phe; triangles, unlabeled phe in the presence of 1.425 μmoles/ml of C^{14} pFphe. Each value is expressed as the percentage it is of the radioactivity attached in the absence of competing unlabeled material.


ETHIONINE
+ C14- MET
PHE +
C14- p-F PHE
p-F PHE +
C14- PHE
100
10
1.0
0.1
FRACTION SURVIVING ACID PPT. CPM, %
CONCENTRATION: AMINO ACID, μM × 10/ml; ANALOG, μM/ml

experiment is that the presence of the analog might noncompetitively inhibit the attachment of the amino acid.

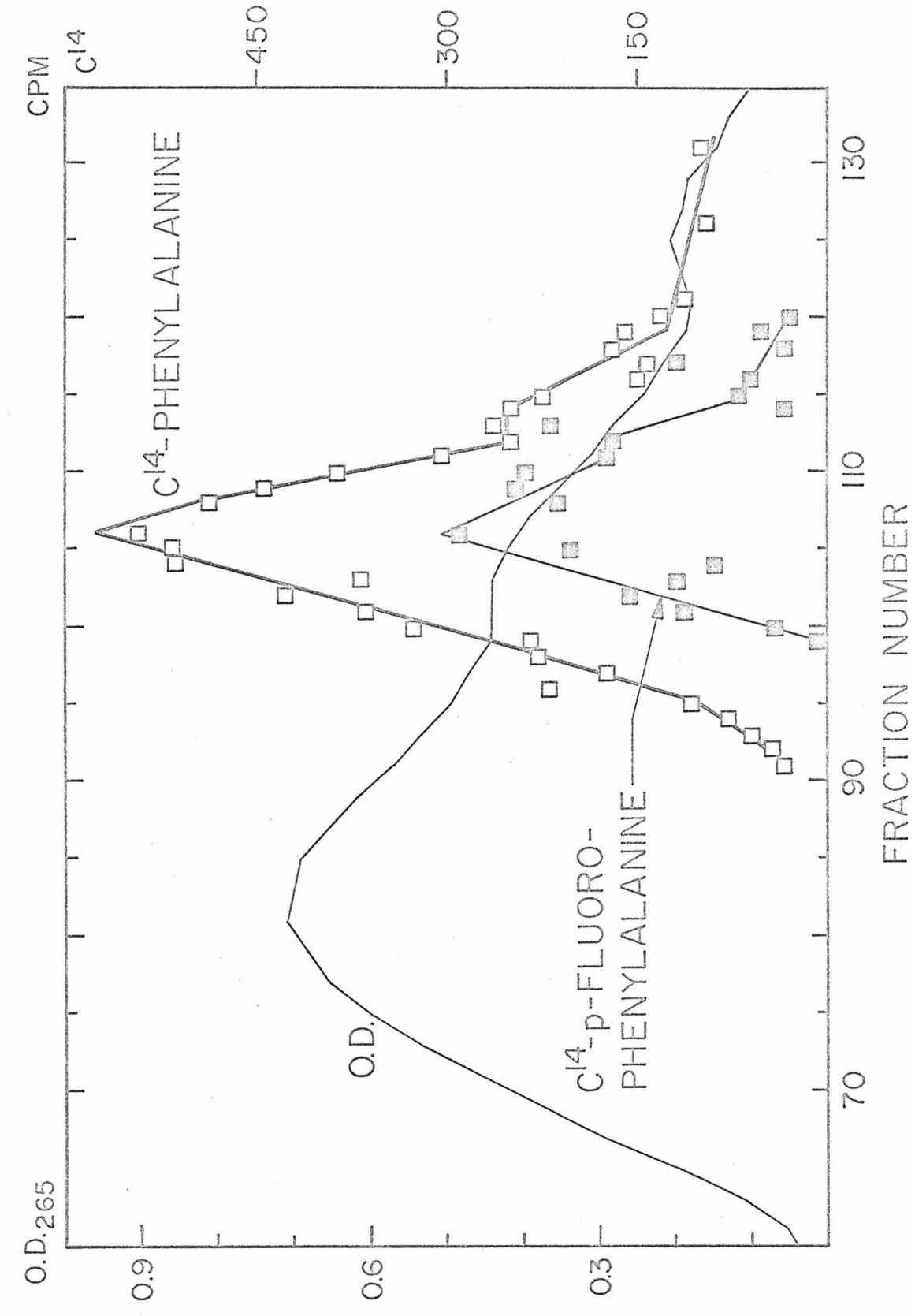
A competition experiment was performed using cold phe to compete with radioactive pFphe (Figure 19). Complete competition is indicated in this direction as well, with the same reservation in interpretation as above.

Additional support for the third explanation is provided by the assay of fractionated uncharged tRNA for both phe and pFphe acceptor activity. If the first or second alternative were correct, then the same shift in position should be observed with acceptor activity as is observed with amino acyl-tRNA. However, the acceptor activity for pFphe is found in the same position as that for phe (Figure 20).

I conclude from this that the chemical differences between these amino acids and their respective analogs are sufficient to cause a structural difference in their complexes with identical tRNA molecules. It is of interest that MAK chromatography has enough resolving power to detect such differences.

FIGURE 20. FRACTIONATION OF phe AND pFphe ACCEPTOR ACTIVITIES

Each fraction from the chromatography on MAK of uncharged tRNA was dialyzed against 0.2 M Tris buffer, pH 7.3 - 0.1 M $Mg(Ac)_2$ overnight and an aliquot of each was assayed for phe acceptor activity (open squares). The tRNA from each fraction of interest (99-120) was concentrated by ethanol precipitation, using DNA as carrier, and assayed for pFphe acceptor activity (solid squares).


CPM
C14
450
300
150
C14-PHENYLALANINE
O.D.
C14-p-FLUORO-
PHENYLALANINE
O.D.265
0.9
0.6
0.3
70
90
110
130
FRACTION NUMBER

DISCUSSION

General and Comparative Aspects of *Neurospora* tRNA

Much data concerning the tRNA complement of *E. coli* (8, 60, 61) yeast (62, 63) and mammalian tissue (9, 62) is already available. This is not true for the tRNA of *Neurospora*. In order to be able to recognize modifications of the tRNA, if they occur during gross metabolic alterations, i.e., biochemical differentiation, I examined several aspects of the biochemistry of tRNA from vegetative *Neurospora* mycelium including: conditions for assaying maximal activity, specific acceptor activities, and the nature of its chromatographic heterogeneity.

The efficiency of extraction of sRNA using the procedure described in METHODS is approximately 25%. I have assumed this material to be a random sample of the total since its characteristics, e.g., leucine specific acceptor activity, are reproducible from one extraction to another and similar to sRNA extracted by other methods which were not used routinely because of their greater complexity or inefficiency.

The total acceptor activity provides an estimate of the extent of transfer RNA in the preparation which operationally is total soluble RNA. If one assumes an average *Neurospora* tRNA molecular weight of 30,000 and an optical density (at 260 mμ) of 24 for a solution containing 1 mg/ml of pure transfer RNA, then there are 1389 μμM of tRNA per $O.D._{260}$ unit. If each RNA molecule is capable of accepting one amino acid molecule, then the maximum acceptor activity of pure tRNA

is 1389 μμM of amino acid per $O.D._{260}$ unit of RNA. The average value for the total acceptor activity of Neurospora soluble RNA which I have measured is 1274 ± 34. Thus about 92% of the RNA soluble in 1.0 M NaCl is tRNA, defined by its amino acid acceptor activity. The remainder of the soluble RNA is probably composed of ribosomal fragments and/or inactive (denatured) tRNA. The acceptor activity reported here averages 8-9 times higher than the only published data (59) on Neurospora tRNA (Table 17, columns 1 vs 2). Perhaps the RNA used by Barnett and Brown contained much ribosomal RNA, i.e., was not primarily tRNA; otherwise it must be concluded that they measured only a fraction of the total acceptor activity.

I have included, in Table 17, published values for the specific acceptor activities of tRNA from yeast, rat liver and E. coli. It is clear from comparing these that tRNA specific acceptor activity is a poor criterion for judging evolutionary relatedness, since the values for Neurospora reported in this thesis are more similar to those for rat-liver and E. coli than they are to those for yeast. The values for 5 of 15 amino acids in rat-liver (gly, his, ile, leu, lys) and 7 of 20 in E. coli (ala, arg, his, thr, tryp, tyr, val) are within ± 25% of the values measured for Neurospora, while in yeast the values for only 2 of 16 amino acids are that close (his, thr). The specific acceptor activity for histidine is the only one which is similar in all four organisms.

Virtually nothing is known about the regulation of tRNA synthesis; no model has been presented which accounts for the different

TABLE 17. SPECIFIC ACCEPTOR ACTIVITY OF tRNA FROM SEVERAL ORGANISMS

Amino acid	Neurospora 69-1113a*	Neurospora 74A†	Yeast‡	Rat-liver‡	E. coli A19§
Ala	85.7	9.0	11	64.1	97.6
Arg	111	9.7	67	-	109.1
Asn	31.9	4.7	41	-	15.7
Asp	37.4	2.3	-	-	69.6
Cys	16.7	-	-	-	11.5
Glu	33.5	6.6	20	-	80.0
Gln	66.4	15.3	-	22.4	159.0
Gly	53.8	13.8	9.0	40.2	119.4
His	20.5	6.1	19	16.8	18.7
Ile	94.0	10.5	41	111.4	52.5
Leu	115	7.2	76	101.3	216.0
Lys	42.8	10.7	55	36.1	81.6
Met	83.9	5.5	35	30.5	46.7
Phe	24.6	3.4	35	32.9	45.8
Pro	36.2	5.3	13	48.2	68.0
Ser	123	7.8	83	80.7	72.6
Thr	74.3	-	65	24.3	92.8
Try	58.7	2.9	-	13.1	51.4
Tyr	50.0	4.6	33	15.0	51.5
Val	115	13.1	65	54.2	128.8

*This thesis

†Barnett and Brown - (59)

‡Cantoni et al. - (62)

§Matthaei et al. - (61)

All values are expressed as μμmoles of amino acid attached per $O.D._{260}$ unit. Dash (-) means no value reported.

distributions of acceptor activity in these organisms. A simple hypothesis is that there exists a 1:1 relationship between the distribution of acceptor activity and the amino acid composition of total protein. This naive proposal is certainly not the case in Neurospora (Table 18). The distribution of acceptor activity (expressed in %) shows no striking relationship to the amino acid composition of total soluble protein, nor to the composition of the free amino acid pools. The amino acid composition of whole mycelium is similar to that of protein because the pool contains only 16% of the total amino acids.

Since the distribution of tRNA is not indicative of the average amino acid composition of protein, these parameters may be related by (1) the efficiency of binding of either the amino acid or the tRNA molecule to the amino acyl-tRNA synthetases or (2) by the concentration of these enzymes. I have not specifically investigated this hypothesis but I do have one shred of evidence consistent with it. The concentration of serine required to maximize the rate of its attachment to tRNA is 20 times that required for phenylalanine (0.100 μM/ml vs 0.005 μM/ml); the ratio of % serine in protein to % serine acceptor activity (7.2/9.6 = 0.75) is 2/5 of the same ratio for phenylalanine (3.6/1.9 = 1.90). In other words, where there appears to be an excess of an amino acid specific tRNA, relative to the extent that amino acid is present in protein (the ratio is less than 1.0), some step in the attachment of that amino acid to tRNA may be deficient. Conversely, the reaction should be more efficient when the ratio is greater than unity. This

TABLE 18. RELATIONSHIP OF AMINO ACID ACCEPTOR ACTIVITY TO AMINO ACID COMPOSITION

Amino acid	Acceptor activity (%)	Protein (%)*	Pool (%)*	Whole mycelium (%)*
Ala	6.7	11.0	25.4	13.3
Arg	8.7	5.6	6.7	5.8
Asn + Asp	5.4	11.5	3.2	10.2
Cys	1.3	1.6	4.0	2.0
Glu + Gln	7.8	10.6	14.3	11.2
Gly	4.2	9.2	5.0	8.6
His	1.6	2.6	2.2	2.5
Ile	7.4	3.2	1.2	2.9
Leu	9.0	7.8	2.2	6.9
Lys	3.4	6.9	5.4	6.6
Met	6.6	1.7	2.2	1.8
Phe	1.9	3.6	1.0	3.2
Pro	2.9	3.8	2.5	3.6
Ser	9.7	7.2	14.8	8.4
Thr	5.8	5.7	4.7	5.6
Try	4.6	0.9	0.1	0.8
Tyr	3.9	2.5	1.0	2.2
Val	9.0	4.6	4.0	4.5
Total	99.9	100.0	99.9	100.1

*Data of DeBusk and DeBusk (49)

suggestion may also be relevant to the fact that there is 15 times as much serine as phenylalanine in the endogenous amino acid pool.

Degeneracy of tRNA Adaptors

There is excellent biochemical evidence for degeneracy in the genetic code (64, 65), i.e., more than one RNA codeword exists for 19 of the 20 amino acids. It has also been recognized that the set of anticodons, the tRNA adaptors, is degenerate (66). The degeneracy of the tRNA is commonly reflected by chromatographic heterogeneity of amino acyl-tRNA (67, 68) or by separation of acceptor activities for a given amino acid using countercurrent distribution (69, 70). With few exceptions, the physically separable tRNA components for a particular amino acid exhibit different coding behavior (63, 66). The significant exceptions to this rule are the mitochondrial tRNA's discussed below (71).

In 1966, Crick proposed the wobble hypothesis which systematically accounts for the nature of the degeneracy in the code (72). This hypothesis is based on the observation that in the third position of an mRNA codon, either pyrimidine codes for the same amino acid and similarly, with two exceptions, the purines are interchangeable. This led Crick to postulate that base pairing at the third position of the codon is not of the standard type only, but also can involve other combinations. Table 19 lists the pairing rules predicted by Crick for the third position of the codon. Given these rules and the genetic code, some limits can be placed on the expected number of tRNA

TABLE 19. BASE PAIRING AT THIRD POSITION ACCORDING TO "WOBBLE" HYPOTHESIS

Anticodon	Codon
U	A,G
C	G
A	U
G	U,C
I	U,C,A

components specific for a single amino acid. Phenylalanine, for example, is coded for by UUU and UUC. One tRNA with the anticodon GAA could decode both; thus the minimum number of adaptors for phe is 1. A tRNA with the anticodon AAA could also transfer phe, but no other anticodons could transfer phe unambiguously. Thus the maximum number of phe adaptors is 2. Similar considerations were used to assign upper and lower limits to the number of tRNA components expected for each amino acid (Table 20, columns 9 and 10).

Also listed in Table 20 is the number of separable tRNA components which have been found in various organisms. In each of these organisms there is a strong tendency to accumulate the minimum number of components necessary to decode all of the codons for each amino acid. Apparent exceptions to this rule are the mitochondrial tRNA's (71). In Neurospora, for example, there are 2 separable phe acceptors, one of which is mitochondrial in origin. However, each of these have identical coding properties; they respond both to UUU and UUC (73). This example is therefore not really an exception to the tendency towards a minimum number of anticodons but only an exception to the rule that physically separable tRNA's have different coding properties. The presence of a distinct methionine acceptor for transferring N-formylmethionine into protein is another "pseudo-exception" (74).

In Neurospora, multiple tRNA's exist for 15 of the 20 amino acids. Eleven of these 15 have 2 components and excluding 2 of these, phe and asp, where the second component is known to be of mitochondrial origin, the relative proportion of the multiple components averages 84%

TABLE 20. THEORETICAL AND OBSERVED "DEGENERACY" OF tRNA

Amino acid	Organism							Theoretical	
	N. crassa*	E. coli†	E. coli‡	E. coli§	B. subtilis‖	Yeast¶	Mammals**	Min	Max
Ala	2	2	2	2	1	1	1	2	5
Arg	3	2	2	2	3	1-3		3	7
Asn	2							1	2
Asp	2		2	2	1			1	2
Cys	1-2							1	2
Glu	2		2	2	2			1	2
Gln	2		2					1	2
Gly	2	2		1-2	1	1-3	2	2	5
His	1			2	1	1-2		1	2
Ile	2	3	2	3	3			1	3
Leu	3	3	5	3-4	1	3-4	2-3	3	7
Lys	1	2-3	2	1-2	1	2-3	2	1	2
Met	1	2	2	1-2	1	2		1	1
Phe	2	2	3	1	1	1	2	1	2
Pro	1-2	2		2		2		2	5
Ser	4	3	4	3-4	2-3	4	2	3	7
Thr	3			3-4	1		1-2	2	5
Try	1	1		1	1	1		1	1

TABLE 20 (continued)

Amino acid	Organism							Theoretical	
	N. crassa[*]	*E. coli*[†]	*E. coli*[‡]	*E. coli*[§]	*B. subtillis*[‖]	Yeast[¶]	Mammals[**]	Min	Max
Tyr	1		1	2	2	1	1-2	1	2
Val	2	2	2	2	2	2		2	5
								31	69

[*] This thesis
[†] Söll *et al.* - (89)
[‡] Muench and Berg - (68)
[§] Sueoka and Kano-Sueoka - (8)
[‖] Kaneko and Doi - (78)
[¶] Cherayil and Bock - (88)
[**] Taylor *et al.* - (9)

Underlined values are not within theoretical limits.

to 16% (Table 13). The major component is 2.5 to 10 times more abundant than the minor component. The ratio of components for the 4 amino acids with still higher multiples of tRNA's is also distinctly non-random. It seems reasonable, therefore, that for each amino acid some codons are used more frequently than others. This idea was the basis of the "modulation" hypothesis proposed by Ames and Hartman (75).

Theories of Regulation at the Translation Level

The degeneracy of the genetic code with its associated tRNA degeneracy considered with the critical role of tRNA in protein synthesis suggests, on theoretical grounds, several ways in which tRNA could play a role in the regulation of protein synthesis at the translation level. The "adaptor-modification" hypothesis has been developed by Sueoka et al. (76).

> If the codon recognition of a particular adaptor out of a set of degenerate adaptors for an amino acid is changed by a structural modification, the mRNA of the genes which accomodate the codon corresponding to the modified adaptor should not be translated properly, while mRNA of the other genes which do not accomodate the codon should be translated normally. This means that by modifying a specific sRNA molecule, the function of some of the genes which are transcribed can be shut off and the rest of the genes kept functional at the translation level. This hypothesis, if true, should constitute one of the major principles in differentiation, and may be called the adaptor modification hypothesis. It is noted that this hypothesis may be applied to cases where a drastic change in the metabolic pattern is observed, rather than to cases involving a change in a small number of enzymes.

A related model might involve the appearance of a new tRNA species preceding differentiation, allowing the translation of a previously untranslatable codeword and thus the synthesis of a new class of proteins. Any such model predicts qualitative and/or quantitative differences in the tRNA taken from an organism during different physiological or developmental states, though, of course, such differences alone do not prove any role in regulation.

Derepression of Tyrosinase Synthesis Indicative of Change in Metabolic Pattern

As noted by Sueoka et al. in the above quotation a mechanism for regulating protein synthesis at the translational level involving tRNA is best suited to controlling major metabolic shifts. The response of vegetative cultures of Neurospora to starvation or to treatment with certain amino acid analogs is such a shift. Starvation on solid media results in sexual differentiation (27). In liquid media, treatment with ethionine for 5 hours results in: 1) a 71% reduction in the incorporation of H^3 guanosine into RNA; 2) a 40% loss of total RNA; 3) and a rapid loss in total soluble protein. Since during this same period there is only a slight decrease in the mass of the mycelium there is presumably an increased accumulation of insoluble protein, polysaccharides, and/or lipids.

In addition to these gross metabolic alterations, the synthesis of at least 5 enzymes is stimulated: tyrosinase (29), L-amino acid oxidase (47), NAD'ase (48), ribonuclease and protease (77). The

appearance of proteolytic activity was not measured directly in the experiments reported here but is inferred from the fact that the protein content of mycelium is relatively constant just prior to ethionine treatment and decreases rapidly after treatment. The change in the rate of protein synthesis is too small to account for this decline. The rapid synthesis of tyrosinase after treatment of mycelium with ethionine is used as an assay for this biochemical differentiation.

Nature of Observed tRNA Pattern During Differentiation

In order to test whether alterations of the tRNA complement are involved in the developmental process described above, amino acyl-tRNA from ethionine-treated cultures was compared chromatographically to amino acyl-tRNA taken from vegetative, control cultures.

No qualitative changes of the tRNA complement occur during ethionine treatment. No new peaks appear after treatment and none of the pre-existing peaks disappear or have altered chromatographic mobility. However, the chromatographic profiles indicate quantitative changes for several amino acyl-tRNA's. This indication is most pronounced for glutamine-specific and glutamic acid-specific tRNA. With a single exception, where these quantitative changes affected amino acyl-tRNA with multiple components, e.g., valyl-tRNA (Figure 12), each component changes proportionately, i.e., the relative proportion of the multiple components remains constant. In the lone exception, glutaminyl-tRNA, the entire change, a 1/3 loss of total activity,

affects only the major component. In this case the relative proportion of the two components changes from 72:28 to 60:40.

Since quantitative interpretation of such data alone is perilous, I investigated the time course of these quantitative changes by assaying the specific acceptor activity for each amino acid of tRNA extracted from cultures treated for various times. From 20 hours prior to treatment to 8 hours after treatment no significant changes in specific acceptor activity occur for any of the 20 amino acids. Since for any one amino acid the reproducibility of this assay is only between 5 and 10 per cent ($\pm$), in order to be judged significant I arbitrarily decided that a deviation must exceed 3 times the standard deviation. Some of the variability arises from variability in the efficiency of charging tRNA mixtures prepared by the same procedure on different days.

The specific acceptor activity for 4 amino acids (asp, val, tryp, phe) remains constant even after 20 hours of ethionine treatment. For an additional 3 amino acids (cys, aspn, glun) the specific acceptor activity declines after 10 hours but this is not related to the addition of ethionine since a similar decline occurs in control cultures. However, for the remaining 13 amino acids, ethionine treatment after 10 hours results in significant lowering of the specific acceptor activity (25-50%).

The nature of the changes reported here differs from all published cases of tRNA alterations accompanying differentiation. Most published cases involve either the appearance of new tRNA components or shifts in the mobility of pre-existing components, i.e., qualitative

alterations (1-12). Differential transcription of tRNA cistrons (2) and enzymatic modification of tRNA (8) respectively have been hypothesized to account for such observations. The remaining cases of reported tRNA alterations (78, 79, 80) involve shifts in the relative proportions of multiple tRNA components, similar to the glun-tRNA shift reported here. However, those cases do not appear to involve any net change in acceptor activity.

Proposed Mechanism of Quantitative Alteration of Neurospora tRNA

The following scheme accounts, I believe, for the observations herein reported. Ethionine, added to near-starving, stationary mycelium, stimulates, as part of a complex response to the poor environment, the synthesis of ribonuclease activity and inhibits directly or indirectly RNA synthesis. As a consequence the total RNA content of such mycelium declines. In vitro transfer RNA does not appear to be as sensitive to this RNA'ase as high molecular weight RNA when assayed under conditions that prevent denaturation, i.e., with Mg^{++} present (54). This apparent resistance may be due to the secondary structure of the molecule.

The sRNA extracted from mycelium at any particular time is, presumably, a mixture of intact tRNA molecules and some distribution of partially digested molecules. Since the extraction procedure involves denaturing steps, the efficiency of recovery should be highest for the least digested molecules. With increasing time of ethionine

treatment the average number of "hits" per molecule in the recovered sRNA population must increase.

Nishimura and Novelli have directly demonstrated that partially digested tRNA can retain its amino acid acceptor activity (23, 81, 82). This was suspected from the fact that the early tRNA work done by the Zamecnik group used RNA with an average sedimentation coefficient of 1.85 s (83). In my own experiments I have shown that treatment of tritiated guanosine-labeled Neurospora tRNA with a crude extract of ethionine-treated mycelium results in no loss of specific acceptor activity for some amino acids, even though as much as 20% of the H^3 counts are rendered acid-soluble.

Up to eight hours after the addition of ethionine and the resulting acceleration of net tRNA breakdown, no change in specific acceptor activity is evident as assayed by the conversion of radioactive amino acids to a TCA insoluble form. The chromatographic evidence, however, shows that changes in specific acceptor activity occur by five hours. The contradiction arises, I think, because while partially digested material may retain its acceptor activity, it does not retain its characteristic chromatographic mobility on MAK. This may be explained by the fact that no Mg^{++} is present during the chromatography and under such conditions partially digested material may lose its secondary structure. It may be remembered that in preliminary experiments I discovered that a ribonuclease-free source of amino acyl-tRNA synthetases was necessary in order to prepare amino acyl-tRNA for chromatography

since otherwise the acid-insoluble radioactive material (thought to be amino acyl-tRNA) would not even bind to the column.

The chromatographic evidence indicates that some tRNA molecules are relatively more resistant to degradation in vivo than others, and that the degree of sensitivity, with the exception of glutamine-specific tRNA, is similar for "degenerate" tRNA acceptors; for example, both valine acceptors have the same sensitivity. This may reflect a similarity in nucleotide sequence of degenerate acceptors. Such a similarity of sequence is expected since a single enzyme seems to recognize multiple components (55).

The changes in specific acceptor activity, assayed by the filter paper disc method, of tRNA taken from cultures treated for 10 hours or longer do not reflect precisely the same parameter that the chromatographic changes reveal. Consequently the amino acyl-tRNA's which indicate alterations by the two techniques are not necessarily the same nor are their magnitudes. In the latter case, chromatography, relative ribonuclease sensitivity is reflected. The former is some indirect measure of how much each tRNA component must be digested before its acceptor activity is lost. Presumably, some tRNA molecules are sensitive to digestion in terms of sustaining "hits" but require many of them before they lose acceptor activity. This may be simply a question of the proximity of the nuclease-sensitive sequences of the molecule to the region important in determining amino acid acceptance. Nishimura and Novelli (23, 81, 82) have observed this kind of amino acid acceptor specificity with the ribonuclease elaborated by

sporulating Bacillus subtillis. Digestion of tRNA resulting in similar losses of amino acid transfer ability did not show identical losses of amino acid acceptor activities (84, 85).

For a variety of reasons, it does not seem probable that ethylation of tRNA plays any role in the observed changes of specific acceptor activity. Ethylation of tRNA does occur in rat liver (86) but not in other rat tissues (86) and not in E. coli (87). The insignificance of tRNA ethylation in Neurospora is suggested by direct incorporation experiments and shown indirectly by the fact that even a large concentration of ethionine in the medium reduces tRNA synthesis much more than it affects tRNA methylation. The lack of altered chromatographic mobility for any of the 40 amino acyl-tRNA components of Neurospora also suggests that ethylation does not occur since the chromatography of amino acid analog tRNA has demonstrated the ability of the MAK column to detect small structural changes.

Regulatory Significance of Quantitative Alterations

The final issue to be discussed is whether the quantitative changes of Neurospora tRNA acceptor activity reported here play a role in regulating the system in which they occur. For at least three reasons, I believe, this is not the case. 1) The relative changes in specific acceptor activity observed are small compared to the large net loss (in mg tRNA/g mycelium) of total tRNA. 2) The alterations observed do not generally affect the relative proportion of synonymous tRNA's which is a kind of change, Sueoka postulates (8), that is able to affect

the translation of synonymous codons. The proportions of acceptor activity for particular amino acids relative to the total acceptor activity are affected. If a regulatory function were involved in this kind of alteration, then one would expect that the average amino acid composition of the mycelial proteins should be changed. However, there is no clear correlation between the tRNA changes and the slight changes in overall amino acid composition which appear after ethionine treatment (50). 3) Any theory in which tRNA plays a regulatory role must postulate that the availability of tRNA is limiting the rate of protein synthesis. This requirement is apparently not met in this system. After ethionine treatment of mycelium for five hours, a time when 40% of the general tRNA content of the mycelium has been lost, there is only a 13% decline in the rate of protein synthesis, as measured by the incorporation of radioactive amino acids into a hot TCA-insoluble form.

It may then be asked, what the significance is of the appearance of ribonuclease and its digestion of accumulated RNA. My suggestion is that the excess of tRNA in the mycelium, over what is required to maintain the rate of protein synthesis after 72 hours of growth, is left over from an earlier time when the rate of protein synthesis was much greater. At the time of ethionine addition the mycelium is virtually a closed system; the exogenous supply of carbon, for example, is nearly exhausted (87). As I have already suggested, since the net loss of total RNA and soluble protein exceeds (by a factor of 2-3) the loss of dry weight, there must be an increased accumulation of insoluble

proteins, polysaccharides, and/or lipids. If that is true, it seems reasonable that the catabolic products of the excess RNA and protein are used as building blocks for those molecules.

REFERENCES

1. Baliga, B., P. R. Srinivasan, and E. Borek, Fed. Proc. (abstract), 27, 794 (1968).

2. Doi, R. H., I. Kaneko, and B. Goehler, Proc. Natl. Acad. Sci., 56, 1548 (1966).

3. Holland, J. J., M. W. Taylor, and C. A. Buck, Proc. Natl. Acad. Sci., 58, 2437 (1967).

4. Kwan, C. N., D. Apirion, and D. Schlessinger, Biochem., 7, 427 (1968).

5. Lazzarini, R. A., Proc. Natl. Acad. Sci., 56, 185 (1966).

6. Lee, J. C., and V. M. Ingram, Science, 158, 1330 (1967).

7. Subak-Sharpe, H., and J. Hay, J. Mol. Biol., 12, 924 (1965).

8. Kano-Sueoka, T., and N. Sueoka, J. Mol. Biol., 20, 183 (1966).

9. Taylor, M. W., G. A. Granger, C. A. Buck, and J. J. Holland, Proc. Natl. Acad. Sci., 57, 1712 (1967).

10. Wevers, W. F., B. C. Baguley, and R. K. Ralph, Biochim. Biophys. Acta, 123, 503 (1966).

11. Waters, L. C., and G. D. Novelli, Proc. Natl. Acad. Sci., 57, 979 (1967).

12. Yang, S. S., and D. G. Comb, J. Mol. Biol., 31, 139 (1968).

13. Sueoka, N., and T. Kano-Sueoka, Proc. Natl. Acad. Sci., 52, 1535 (1964).

14. Lee, J. C., and V. M. Ingram, Fed. Proc. (abstract), 27, 800 (1968).

15. Sueoka, N., T. Kano-Sueoka, and W. J. Gartland, Cold Spring Harbor Symp. Quant. Biol., 31, 571 (1966).

16. Anderson, F. W., Fed. Proc. (abstract), 27, 341 (1968).

17. Foft, J. W., W.-T. Hsu, and S. B. Weiss, Fed. Proc. (abstract), 27, 341 (1968).

18. Subak-Sharpe, H., W. H. Shepherd, and J. Hay, Cold Spring Harbor Symp. Quant. Biol., 31, 583 (1966).

19. Capra, J. D., and A. Peterkofsky, J. Mol. Biol., 21, 455 (1966).

20. Hsu, W.-T., J. W. Foft, and S. B. Weiss, Proc. Natl. Acad. Sci., 58, 2028 (1967).

21. Doi, R. H., and B. Goehler, Cold Spring Harbor Symp. Quant. Biol., 31, 457 (1966).

22. Carbon, J., and J. B. Curry, Fed. Proc. (abstract), 27, 341 (1968).

23. Nishimura, S., and G. D. Novelli, Proc. Natl. Acad. Sci., 53, 178 (1965).

24. Littauer, U. Z., M. Revel, and R. Stern, Cold Spring Harbor Symp. Quant. Biol., 31, 501 (1966).

25. Fleissner, E., Biochem., 6, 621 (1967).

26. Wainfan, E., P. R. Srinivasan, and E. Borek, Cold Spring Harbor Symp. Quant. Biol., 31, 525 (1966).

27. Westergaard, M., and H. K. Mitchell, Am. J. Bot., 34, 573 (1947).

28. Hirsch, H. M., Physiol. Plantarum, 7, 72 (1954).

29. Horowitz, N. H., M. Fling, H. Macleod, and Y. Watanabe, Cold Spring Harbor Symp. Quant. Biol., 26, 233 (1961).

30. Kappy, M. S., and R. L. Metzenberg, Biochim. Biophys. Acta, 107, 425 (1965).

31. Horowitz, N. H., and M. Fling, Genetics, 38, 360 (1953).

32. Horowitz, N. H., J. Biol. Chem., 171, 255 (1947).

33. Pall, M. L., Ph.D. Thesis, California Institute of Technology (1967).

34. Vogel, H. J., Microb. Gen. Bull., 13, 42 (1956).

35. Luck, D. J. L., J. Cell. Biol., 16, 483 (1963).

36. Davis, R. H., and F. M. Harold, Neurospora Newsletter, 2, 18 (1962).

37. Barnett, W. E., Proc. Natl. Acad. Sci., 53, 1462 (1965).

38. von Ehrenstein, G., and F. Lipman, Proc. Natl. Acad. Sci., 47, 941 (1961).

39. Holley, R. W., J. Apgar, B. P. Doctor, J. Farrow, M. A. Marini, and S. H. Merrill, J. Biol. Chem., 236, 200 (1961).

40. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton, and R. J. Britten, In "Carn. Inst. of Wash. Publn. 607" (1955).

41. Horowitz, N. H., M. Fling, H. Macleod, and N. Sueoka, J. Mol. Biol., 2, 96 (1960).

42. Gornall, A. G., C. J. Bardawill, and M. W. David, J. Biol. Chem., 177, 751 (1949).

43. Chamber, R. W., Schwartz Technical Brochure, 66 TR1 (1966).

44. Sueoka, N., and T. Yamane, Proc. Natl. Acad. Sci., 48, 1454 (1962).

45. Mandel, J. D., and A. D. Hershey, Anal. Biochem., 1, 66 (1960).

46. Patterson, M. S., and R. C. Greene, Anal. Chem., 37, 854 (1965).

47. Horowitz, N. H., Biochem. Biophys. Res. Commun., 18, 686 (1965).

48. Urey, J., Ph.D. Thesis, California Institute of Technology (1966).

49. DeBusk, B. G., and A. G. DeBusk, Neurospora Newsletter, 11, 3 (1967).

50. Fling, M., Personal Communication.

51. Minigawa, T., B. Wagner, and B. Strauss, Arch. Biochem. Biophys., 80, 442 (1959).

52. Linn, S., and I. R. Lehman, J. Biol. Chem., 240, 1287 (1965).

53. Berg, P., F. H. Bergmann, E. J. Ofengand, and M. Dieckmann, J. Biol. Chem., 236, 1726 (1961).

54. Adams, A., T. Lindahl, and J. R. Fresco, Proc. Natl. Acad. Sci., 57, 1684 (1967).

55. Yamane, T., and N. Sueoka, Proc. Natl. Acad. Sci., 51, 1178 (1964).

56. Snoke, J. E., and K. Bloch, In "Glutathione," S. Colowick, A. Lazarow, E. Racker, D. R. Schwarz, E. Stadtman, H. Waelsch, eds., Academic Press (1954) p. 129.

57. Gartland, W. J., and N. Sueoka, Proc. Natl. Acad. Sci., 55, 948 (1966).

58. Pall, M. L., Personal Communication.

59. Barnett, W. E., and D. H. Brown, Proc. Natl. Acad. Sci., 57, 452 (1967).

60. Muench, K. H., and P. Berg, Biochem., 5, 982 (1966).

61. Matthaei, J. H., H. P. Voigt, G. Heller, R. Neth, G. Schoch, H. Kubler, F. Amelunxen, G. Sander, and A. Parmeggiani, Cold Spring Harbor Symp. Quant. Biol., 31, 25 (1966).

62. Cantoni, G. L., and H. H. Richards, In "Procedures in Nucleic Acid Research," G. L. Cantoni, and D. R. Davies, eds., Harper and Row (1966) p. 617.

63. Söll, D., J. D. Cherayil, and R. M. Bock, J. Mol. Biol., 29, 97 (1967).

64. Nirenberg, M., T. Caskey, R. Marshall, R. Brimacombe, D. Kellogg, B. Doctor, D. Hatfield, J. Levin, F. Rotman, S. Pestka, M. Wilcox, and F. Anderson, Cold Spring Harbor Symp. Quant. Biol., 31, 11 (1966).

65. Khorana, H. G., H. Buchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kossel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, Cold Spring Harbor Symp. Quant. Biol., 31, 39 (1966).

66. Weisblum, B., S. Benzer, and R. W. Holley, Proc. Natl. Acad. Sci., 48, 1449 (1962).

67. Sueoka, N., and T. Yamane, Proc. Natl. Acad. Sci., 48, 1454 (1962).

68. Muench, K. H., and P. Berg, Biochem., 5, 970 (1966).

69. Doctor, B. P., J. Apgar, and R. W. Holley, J. Biol. Chem., 236, 1117 (1961).

70. Thiebe, R., and H. G. Zachau, Biochim. Biophys. Acta, 103, 568 (1965).

71. Barnett, W. E., and D. H. Brown, Proc. Natl. Acad. Sci., 57, 1775 (1967).

72. Crick, F. H. C., J. Mol. Biol., 19, 548 (1966).

73. Barnett, W. E., and J. L. Epler, Cold Spring Harbor Symp. Quant. Biol., 31, 549 (1966).

74. Clark, B. F. C., and K. A. Marker, J. Mol. Biol., 17, 394 (1966).

75. Ames, B. N., and P. E. Hartman, Cold Spring Harbor Symp. Quant. Biol., 28, 349 (1963).

76. Sueoka, N., and T. Kano-Sueoka, In "Developmental and Metabolic Control Mechanisms and Neoplasia," The Williams and Wilkins Co. (1965) p. 114.

77. Drysdale, R. B., Personal Communication.

78. Kaneko, I., and R. H. Doi, Proc. Natl. Acad. Sci., 55, 564 (1966).

79. Vold, B. S., and P. S. Sypherd, Proc. Natl. Acad. Sci., 59, 453 (1968).

80. Mushinski, J. F., and M. Potter, Fed. Proc. (abstract), 27, 802 (1968).

81. Nishimura, S., and G. D. Novelli, Biochem. Biophys. Res. Commun., 11, 161 (1963).

82. Nishimura, S., and G. D. Novelli, Biochim. Biophys. Acta, 80, 574 (1964).

83. Hoagland, M. B., M. L. Stephenson, J. F. Scott, L. I. Hecht, and P. C. Zamecnik, J. Biol. Chem., 231, 241 (1958).

84. Nishimura, S., and M. Nomura, Biochim. Biophys. Acta, 30, 430 (1958).

85. Nishimura, S., and M. Nomura, J. Biochem., 46, 161 (1959).

86. Farber, E., Advances in Cancer Res., 7, 383 (1963).

87. Smith, R. C., and W. D. Salmon, J. Bact., 89, 687 (1965).

88. Horn, G., Personal Communication.

89. Cherayil, J., and R. M. Bock, Biochem., 4, 1174 (1965).

90. Söll, D., J. Cherayil, D. S. Jones, R. D. Faulkner, A. Hampel, R. M. Bock, and H. G. Khorana, Cold Spring Harbor Symp. Quant. Biol., 31, 51 (1966).