To the Graduate Council:

I am submitting herewith a thesis written by James E. Hall, Jr. entitled "A Time Course Study of Transfer Ribonucleic Acids During the Development of Thymic Lumphomas in C57BL/6 Mice." I recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Biomedical Sciences.

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Thesis
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A TIME COURSE STUDY OF TRANSFER RIBONUCLEIC ACIDS DURING THE DEVELOPMENT OF THYMIC LYMPHOMAS IN C57B1/6 MICE

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

James E. Hall, Jr.

August 1978

1383592

ACKNOWLEDGMENTS

I would like to extend my appreciation to the following individuals who helped to make this work possible: L. McAskill for donating normal mice for these studies; L. C. Waters for his donation of aminoacyl tRNA synthetase; L. H. Smith for the donation of thymic lymphoma material and technical information concerning the nature of this tumor type; G. D. Griffin for the time he donated to teach me necessary techniques to carry out this investigation and the patience required to do so and for the many long periods of discussion necessary to overcome the many technical problems encountered; and to G. D. Novelli for his indispensible guidance, advice and consent for this study.

This research was carried out at the Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee whose research is supported by the Department of Energy under contract with the Union Carbide Corporation.

ABSTRACT

The isoaccepting tRNAs for eight different amino acids in normal and lymphoma thymuses from C57Bl/6 mice were compared using reversed-phase column chromatography. Differences were found in the lymphoma tissues for phenylalanine and histidine isoaccepting species. These differences were followed as they appeared during the course of transformation of cells after x-irradiation. The results showed that the differences were not simply gradual increases in the new species and gradual loss of the old but instead the alterations were a very active process.

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CHAPTER I

INTRODUCTION

Differences in the populations of isoaccepting tRNAs between tumor and normal tissues is a well documented phenomenon. Studies of this sort have been conducted on a variety of tumors including mouse mammary tumors[1,2], Mouse plasms cell tumors,[3,4] Ehrlich ascites cells in mice,[5,6] Morris hepatomas in rats,[7] Novikoff Hepatomas in rats,[6] tumors induced by the injection of L-M cells into mice,[8] polyoma and SV_{40} transformed rat embryo cells in culture,[9] SV_{40} transformed African green monkey kidney cells[10] and vaccinia virus infected HeLa cells.[11] In each study the profiles of isoacceptor tRNAs for a series of amino acids were examined from both tumor and normal tissues. Upon comparison of these profiles these studies showed that isoacceptor tRNAs for a few of the amino acids did differ by the appearance of new species and disappearance of some of the normal species. Most of these studies were conducted using fully developed tumors or tumor lines in order to efficiently detect any changes.

In practice these studies differentiated the isoaccepting tRNAs for a specific amino acid by the use of various column chromatographic procedures: reversed-phase column chromatography,[1-9,11] methylated albumin kieselgurh,[2,10] benzoylated DEAE-cellulose[5,6] and DEAE-Sephadex.[6,10] Although these techniques were all capable of showing differences in isoacceptors, reversed-phase column chromatography (RPC chromatography) isolated the isoacceptors into separate distinct peaks better and more consistently so that most of the differences could be detected. For these

reasons RPC chromatography has been the dominant technique used in this field. Additionally, when RPC chromatography is used to cochromatograph normal and tumor tRNAs, these differences can be easily seen and compared. To accomplish this task the normal tRNAs are usually aminoacylated with ^{3}H labelled amino acids while the tumor tRNAs are aminoacylated with ^{14}C labelled amino acids. Only one species of amino acid is used on each chromatographic run; therefore, to examine the complete spectra of tumor isoacceptor tRNAs for all the amino acids a separate chromatographic run must be made for each one.

It is a generally held theory that the differences seen between normal and tumor isoaccepting tRNAs are due to the lack of full post-transcriptional modification of bases in some of the tumor tRNAs.[6,7] This would be due to the lack of specific tRNA modifying enzymes in tumor cells.[12,13] Therefore, as cells are slowly transformed in a tissue there might be a gradual loss of some of the normal modifying enzymes and possibly even the gradual increase in some new forms characteristic of the tumor line. This change could be followed since it would be reflected in the gradual disappearance and appearance of specific isoaccepting tRNAs. The documentation of this gradual change was the directive of this body of experiments.

The method of accomplishing this consisted of first examining the profiles of normal and tumor isoaccepting tRNAs for a series of eight amino acids to detect any differences which might be present. The amino acids used were those which most often showed changes in tRNAs in studies in the literature. This was done using the reversed-phase column chromatographic procedure since it has been the most widely used

and documented, its basic parameters have been better established,[4,14,15,16] the results are very reproducible and it yields the best separations of isoaccepting tRNAs. When the differences had been noted, further experiments were then done using tRNAs extracted from tumors at different stages of development.

A tumor line had to be selected which would lend itself easily to this type of study. Fortunately, L. H. Smith had, previous to my arrival, conducted biochemical experiments with C57Bl/6 mice which had been x-irradiated to induce lymphomas of the thymus. Samples had been taken at several periods during the tumor development and at the terminal stage. These samples were in quantities large enough for use in these experiments and had been kept frozen in liquid nitrogen. Dr. Smith kindly agreed to donate some of this material for use in these experiments.

This tumor type was desirable for these studies since histological and histochemical studies carried out on it[17-20] gave evidence that cells which were transforming into this tumor form moved predictably through different stages of development at usually the same rate. Thus the tumors extracted at the same chronological time would correlate well in tumor developmental stages. These stages as defined by Lagerlöf and Kaplan[18] were: atrophic stage, lymphoma "in situ," lymphoma confined to the thymus, invasive lymphoma and disseminated lymphoma. The periods at which lymphomas were collected in this study: 30, 62, 97 and 157 days after the final radiation treatment coincide with the last four stages of tumor development listed consecutively.

Additionally, the theory that the detectable changes in iso-acceptor tRNA species between normal and tumor cells occurs as a gradual process is reinforced by the findings of Smith,[17] Lagerlöf and Kaplan,[18] and Wilson et al.[19] These studies followed the appearance of alkaline phosphatase activity in this type of thymic lymphoma. The alkaline phosphatase is not present in the thymuses of normal C57B1/6 mice but if they are irradiated with x-rays as in the same procedure used here, alkaline phosphatase appears in the tumor cells. Moreover the rate of appearance of the enzyme closely matches that of the rate of tumor development and has been strongly implicated as being directly caused by the transformation process.

CHAPTER II

MATERIALS AND METHODS

Normal Thymuses

Live normal C57Bl/6 mice were donated by L. McAskill. These were males and females ranging from 7 to 16 weeks of age. They were immediately killed, the thymuses removed, frozen in liquid nitrogen and stored at -80°C until used.

Thymic Lymphomas

Thymic lymphomas from C57Bl/6 mice, males and females, were obtained from L. H. Smith. These had been developed by exposing normal C57Bl/6 mice of 30-35 days of age to four doses of 170 Rads of x-rays at seven day intervals.[20] The total dosage was 680 Rads of 250 kVp x-rays. At average intervals of 30, 62, and 97 days after the last exposure to x-rays, mice were killed, the thymuses removed and frozen in liquid nitrogen. Thymuses were also removed from mice in which the lymphomas had reached the terminal level of development, an average of 157 days after the last exposure to x-rays but ranging from 99 to 270 days. The hymuses were stored under liquid nitrogen for approximately 3.5 years.

Aminoacyl Transfer RNA Synthetases

C57Bl/6 mouse liver aminoacyl tRNA synthetases were donated by L. C. Waters and G. D. Griffin. The general procedure of Mildave[21] as

adapted by Yang and Novelli[4] which included the alteration recommended by Muench and Berg[22] was used to prepare the synthetases.

To 7 grams of C57Bl/6 mouse liver tissue placed in a Potter homogenizer, 50 ml of 0.3 M sucrose, 0.1 M KCl, 0.01 M Triso-HCl pH 7.5, 0.01 M B-mercaptoethanol, 10% glycerol and 0.01 M $MgCl_2$ was added. The material was then homogenized with a teflon pestle. To the solution an additional 20 ml of the 0.3 M sucrose solution was added, the solution was transferred to a centrifuge tube and centrifuged at 16,000 rpm for 20 minutes in a Beckman model L5-65 ultracentrifuge. From this the supernatant was collected, placed in Spinco centrifuge tubes and centrifuged at 45,000 rpm for 90 minutes with a 50 ti rotor. The supernatant was collected and applied to a Sephadex G-100 column which had been pre-equilibrated with 10 mM KH_2PO_4 pH 7.5, 5 mM KCl, 1 mM $MgCl_2$, 2 mM DTT and 15% glycerol. The column volume was 20 times the sample volume. The sample was eluted with the same KH_2PO_4 solution while being held at 4°C. The first peak and the first half of the second peak which were eluted from the column, as detected by monitoring at OD_{260} and OD_{280}, were pooled and applied to a 40 ml DEAE-cellulose column which had been pre-equilibrated with the same KH_2PO_4 solution. The column was washed with two column volumes of the same solution after which the sample was eluted with 1.5 column volumes of 10 mM KH_2PO_4 pH 6.5, 0.25 M KCl, 5 mM $MgCl_2$, 2 mM DTT and 15% glycerol. Fractions were collected, the OD_{280} measured and those fractions containing significant quantities of the enzyme peak were frozen and stored at -80°C.

Isolation of Transfer RNAs

The tRNAs of thymus material were extracted by the Yang and Novelli procedure.[4] The following is written on the basis of using one gram of thymuc material, in practice usually from 1 to 5 grams were processed at any one time. Unless otherwise stated all steps were carried out at 4°C.

One gram of thymus material was crushed to a fine powder under liquid nitrogen. This was transferred to a polypropylene centrifuge tube and the liquid nitrogen was allowed to evaporate off. Then 15 ml of 0.15 M NaCl, 10 mM Triso-HCl pH 7.5, 1 mM EDTA and 1% bentonite was added as well as 3% of total volume (0.45 ml) of DEPC. This was vigorously shaken and allowed to stand for 5 minutes. An equivalent volume of 80% phenol equilibrated with 10 mM Tris-HCl pH 7.5 was added and shaken for 30 minutes. The solution was centrifuged at 12,000 rpm in a Sorvall RC-2 centrifuge with a SS-34 rotor, or a GSA rotor for larger samples, for 30 minutes after which the aqueous phase (upper layer) was carefully removed and placed in a polypropylene tube. The phenol layer was discarded. To the aqueous layer a 10% of volume of 20% K acetate was added and mixed followed by transference to a polycarbonate centrifuge tube. Two and a half volumes of cold 100% ethanol were added, mixed and the solution was allowed to stand at -20°C overnight.

The following day the precipitate was collected by centrifugation at 8000 rpm in a Sorvall centrifuge for 30 minutes. The ethanol was carefully drawn off and discarded, the pellet was air dried and resuspended in approximately 5 ml of 0.25 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 10 mM Na acetate pH 4.5. This was applied to a small DEAE-

cellulose columm which had been pre-equilibrated with the same NaCl-buffer solution. Generally, each column contained 1 ml of DEAE-cellulose for every 20 OD_{260} of material in the resuspended pellet. The column was then washed with 3 column volumes of this beginning solution. The material was eluted with 0.70 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 10 mM Na acetate pH 4.5. One ml fractions were collected, the OD_{260} of each fraction was measured and those containing the tRNAs were pooled. This pooled material was placed in a polycarbonate centrifuge tube, 2.5 volumes of cold 100% ethanol were added, mixed and allowed to stand overnight at -20°C.

The next day the precipitate was collected either by millipore filtration or by centrifugation at 8000 rpm in a Sorvall centrifuge for 60 minutes. The precipitate was resuspended in 10 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA and 1 mM DTT and stored at -20°C.

DNA Content Analysis

The reaction of DNA with diphenylamine in a mixture of acetic and sulfuric acids to produce a blue color was first proposed by Dische[23] as a colorametric method to analyze DNA content. The technique was later revised by Burton[24] and this revised form was used here.

The Dische reagent was prepared by dissolving 2.0 grams of diphenylamine in 80 ml of glacial acetic acid. To this was added 2 ml of concentrated H_2SO_4 at room temperature. This solution was diluted to 100 ml with glacial acetic acid after which 0.5 ml of 2% (v/v) aqueous acetaldehyde was added and mixed well.

Samples were prepared by diluting approximately 1.0 OD_{260} samples to 2 ml with 5% TCA. Standards were prepared by dissolving 20 mg of purified cal thymus DNA in 100 ml of 5% TCA and heating to 60°C gently. Volumes of this solution containing 20, 40, 80 and 160 ug of DNA were diluted to 210 ml with 5% TCA. These standards and the samples were then heated to 90°C for 15 minutes. They were removed and cooled to room temperature. To each was added 0.1 ml of 70% perchloric acid and 2.0 ml of the Dische diphenylamine reagent. The tubes were mixed and heated for 18-24 hours at 30°C after which they were cooled to room temperature. The optical density of each sample was measured at 600 nm. The ug of DNA in each was calculated by comparison with the OD_{260} of the standards. Then using the constant: 20 OD_{260}/mg DNA, the OD_{260} of DNA in each sample was determined. And finally for each tRNA extract, the percentage of the OD_{260} contributed by DNA contaminants was calculated.

Electrophoresis

To run polyacrylamide gel electrophoresis of tRNA extracts the procedure of Loening[25] was followed to prepare the gels and run the electrophoresis with slight alterations by Griffin.[26]

Solutions for preparing gels were made by dissolving 15 g recrystallized acrylamide and 0.375 g bisacrylamide in 100 ml of water. A stock buffer consisted of 0.036 M Tris, 0.030 M NaH_2PO_4 and 0.001 M Na_2 EDTA. To make 7.5% gels, 2 ml of the stock buffer was added to 2.7 ml water and 5 ml of the stock acrylamide solution. This mixture was

degased and 25 ul of TEMED (N,N,N',N' Tetramethylethylene diamine) was added in addition to 0.25 ml of 10% ammonium persulfate (w/v). This was immediately transferred to glass tubes and allowed to set for two hours. After the gels had polymerized, they were placed in an electrophoresis apparatus and pre-electrophoresed at 70 mAmps with 190 pulses/second and 220 volts for 30 minutes. The buffer solution for this and the main run was made by diluting the stock buffer five-fold and adding SDS to a final concentration of 0.2% (w/v).

The samples were prepared by taking 100 ul of each sample containing approximately 1.0 OD_{260} of nucleic acid material and adding 6 ul of the tracking dye, bromphenol blue. To these were added 20 ul of the stock buffer containing 0.2% SDS (w/v) to even out the ionic strengths and 16 ul of 40% sucrose (w/v). These were mixed well and layered onto the top of the pre-electrophoresed gels. Standard gels were also run which contained 1.0 OD_{260} of 4 S and 5 S RNAs. The samples were then electrophoresed into the gels by running at 35 mAmps and 150 pulses/second for 20 minutes at 8-10°C. The current was raised to 70 mAmps and 220 pulses/second and allowed to run for 2.75 hours for the main electrophoresis. At the end of this period the gels were removed and scanned in a spectrophotometer to determine the position and concentration of the nucleic acdis and tracking dye. The nucleic acids were monitored at 260 nm and the tracking dye at 600 nm.

Analytical Aminoacylation

Before aminoacylating a tRNA preparation for application to an RPC-5 column, a small sample was first tested by the following analytical

aminoacylation procedure. This yielded information on the extent of aminoacylation and time needed to aminoacylate a tRNA preparation with a specific labelled amino acid. The general principle and procedure of Moldave[21] was used to aminoacylate the tRNAs with the use of the paper disc method of Bollum[27] and Mans and Novelli.[28]

For each tRNA preparation to be tested, two small test tubes were set up each containing 12.5 umoles Tris-HCl pH 7.5, 12.5 umoles KCl, 0.5 umoles DTT, 1.0 umoles ATP, 1.0-5.0 x 10^{-9} moles of either ^{3}H or ^{14}C labelled amino acid and 50 ul of aminoacyl tRNA synthetase. $MgCl_2$ was also added to each tube but to varying extents based upon which amino acid was to be aminoacylated. The amount of $MgCl_2$ was calculated from a constant for each amino acid which represents the optimal ratio between moles of $MgCl_2$ and moles of ATP. The ratio constants are 0.8 for leucine, 1.5 for glutamate and glycine, 2.0 for methionine, proline, threonine, tyrosine and glutamine, 3.0 for arginine, histidine, tryptophan, serine, valine and asparagine and 5.0 for alanine, aspartate, isoleucine, lysine and phenylalanine. To one of the tubes was added 20 ul (0.20-0.60 OD_{260}) of the tRNA preparation to be tested. The other tube served as a blank. Finally, the total volume in each tube was brought up to 0.250 ml using distilled water. Each tube was mixed and placed in a 30°C water bath.

At 5, 15, 30 and 60 minutes after the start of incubation, a 50 ul sample was withdrawn from each tube and spotted onto a separate Whatman 3 MM filter paper disc. This was allowed to dry for 15 seconds and was dropped into a beaker containing 10% TCA at 4°C. Fifteen minutes after the final pair of discs had been added to the 10% TCA, the wash was

replaced by fresh 5% TCA at 4°C and allowed to stand for 15 minutes. This was followed by another 15 minute wash in fresh 15% TCA. This was then replaced by a 20 minute wash in a solution of 50% ethanol (95%) and 50% anhydrous diethyl ether followed by a 5 minute wash in 100% anhydrous diethyl ether. All of the above washes were constantly stirred by a magnetic stirring device.

After the final wash the discs were removed and dried under a heat lamp. Each was placed in a scintillation vial containing 5 ml of scintillation fluid which consisted of toluene plus 0.4% BBOT. The samples were placed in a scintillation counter, counted and the extent of aminoacylation calculated. The resultant values are in units of picomoles/OD_{260} of tRNA.

Preparative Aminoacylation

To generate a satisfactory chromatographic profile of labelled aminoacyl tRNAs on RPC generally 5000-7000 cpm of ^{3}H and 10,000-20,000 cpm of ^{14}C in discriminatory counting were necessary. The amount of tRNA necessary to achieve this was calculated from the results of the analytical aninoacylation. Again the general reaction conditions of Moldave[21] were followed with special alterations by Yang and Novelli[4] in the preparative aminoacylation procedure.

The correct amount of tRNA was placed in a small plastic tube. For each ml of final reaction volume, the following reagents were also added: 50 umoles Tris-HCl pH 7.5, 50 umoles KCl, 2 umoles DTT, 4 umoles ATP, 1.0×10^{-8} moles of either ^{3}H or ^{14}C labelled amino acid, 100 ul of synthetase and the appropriate amount of $MgCl_2$ in keeping with the ratio

between $MgCl_2$ and ATP determined by the constant for each amino acid. Distilled water was added when necessary to achieve the correct total reaction volume. The tube was mixed and incubated at 30°C in a water bath for the optimal aminoacylating time.

During this period a 3.5 ml DEAE-cellulose column was set up in a 5 ml plastic disposable syringe with a plastic valve and a miracloth disc to retain the packing.[29] The column was washed and equilibrated with 5 column volumes of 0.25 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 10 mM Na acetate pH 4.5. When the appropriate length of time for aminoacylation had elapsed, the tube was withdrawn from the water bath and placed on ice. Two equivalent volumes of the 0.25 M NaCl solution at 4°C were added to the tube, mixed and applied to the column. After the sample had moved onto the column, the column was washed with three column volumes of cold 0.25 M NaCl solution. The aminoacylated tRNAs were eluted from the column with 3 column volumes of cold 0.70 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA and 10 mM Na acetate pH 4.5. One ml fractions were collected, the OD_{260} of each measured and those fractions containing the tRNA peak were pooled.

From these pooled fractions, a 50 ul sample was withdrawn and placed in a tube with 1 ml of the 0.25 M NaCl solution, 4 OD_{260} of calf thymus DNA and 8 ml of 10% cold TCA. This was mixed and held on ice for 15 minutes at the end of which time the precipitate formed was collected by filtration through a glass fiber filter disc. The disc was rinsed with 5 ml of 70% ethanol, dried under a heat lamp and placed in a scintillation vial containing 5 ml of scintillation fluid. The vial was counted in a liquid scintillation counter at discriminatory counting

settings and from the results the extent of charging of the tRNA was determined.

Meanwhile, the pooled fractions of the aminoacylated tRNAs were placed in a polycarbonate centrifuge tube, 10 OD_{260} *E. coli* tRNA and 2.5 equivalent volumes of cold 100% ethanol were added, the tube was mixed and the resultant solution was allowed to stand overnight at -20°C.

On the day the aminoacylated tRNAs were to be chromatographed on RPC-5, the tube was removed from storage and centrifuged for 60 minutes at 8000 rpm in a Sorvall centrifuge. The ethanol was carefully withdrawn and discarded, the pellet was partially air dried and was resuspended in 2-4 ml of 0.35 *M* NaCl, 10 *mM* $MgCl_2$, 1 *mM* EDTA, 3 *mM* B-mercaptoethanol and 10 *mM* Na acetate pH 4.5. This was kept on ice until application to the RPC-5 column.

Reversed-Phase Column Chromatography

Preparation of Packing

The procedure for preparation of the RPC-5 packing was that of G. D.Novelli[30] as adapted from that of Pearson *et al.*[10]

Four ml (2.5 grams) of Adogen 464 was dissolved with 250 ml of chloroform and placed in a large plastic jar. To this 100 grams of plaskon powder was added, the jar was tightly stoppered and vigorously shaken for 2 hours. At the end of this period the slurry was poured into a large glass evaporating dish and placed under a heat lamp in a ventillated hood. During this drying period the slurry was constantly stirred with a large ceramic spatula. When the packing was thoroughly dry, it was transferred to a glass container for storage.

Column Packing

Novelli[30] and Pearson et al.[10] were generally followed in their procedure for the packing of a column.

When a column was to be packed the desired amount of RPC-5 packing was weighed out and ground to a fine powder with a mortar and pestle. This was transferred to a beaker containing approximately 500 ml of 1 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 3 mM B-mercaptoethanol and 10 mM Na acetate pH 4.5. The slurry was stirred overnight at 4°C. The following day a Chromatronix LC-6M-13 column was partially filled with 0.35 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 3 mM B-mercaptoethanol and 10 mM Na acetate pH 4.5. To it the slurry of RPC-5 packing was added, the top plunger put back in place and the RPC-5 was packed under pressure (250-500 psi) by increasing the flow rate of the 0.35 M NaCl solution through the column. The plunger was removed, more packing was added, the plunger replaced and fluid pumped through again. This was repeated until the column had been adequately filled.

Many column volumes of this 0.35 M NaCl solution were pumped through the column after which many volumes of the 1 M NaCl solution were run through to further compact the column and wash off any extraneous material adhering to the packing. Finally the column was washed with several column volumes of the 0.35 M NaCl solution after which the column was closed off until needed for a chromatographic run.

Chromatography

The basic chromatographic conditions of Yang and Novelli[4] were followed with recommended alterations by Novelli[30] and Griffin.[26]

A packed 25 cm x 0.63 cm RPC-5 column at 22°C was washed with 60 ml of 1 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 3 mM B-mercaptoethanol and 10 mM Na acetate pH 4.5 at 150-190 psi at a flow rate of 1 ml/minute before each chromatographic run. This was folllowed by equilibration with 60 ml of 0.35 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 3 mM B-mercaptoethanol and 10 mM Na acetate pH 4.5. A sample containing a mixture of tRNAs from normal C57B1/6 mice were aminoacylated with a ^{14}C amino acid and mixed tRNAs from C57B1/6 mice having thymic lymphomas were aminocylated with the same amino acid but carrying a ^{3}H label. The two labelled mixed tRNAs were combined and applied to the column. The sample was followed by a 5-10 ml wash of the 0.35 M NaCl solution. Immediately following this a NaCl gradient was started. This was established by placing 100 ml of the beginning and final solutions in a gradient device and pumping from it into the column. The salt graidents contained the same concentrations of $MgCl_2$, EDTA, B-mercaptoethanol and Na acetate as before. The gradients ranged from 0.35 M to 0.70 M NaCl. They were all increasing linear concentration gradients. As the sample was applied to the column 3 ml fractions were collected, these were generally the first five fractions. One milliliter fractions were collected during the application of the NaCl gradient to the column. All but the last 10-20 ml of the gradient were applied followed by the 1 M NaCl solution wash collected as ten 2-ml fractions.

To each fraction coming off the column was added 2 OD_{260} of calf thymus DNA/ml of fraction volume and 6 ml of cold 10% TCA. These were vigorously mixed and allowed to stand on ice for 15 minutes to allow the aminoacyl tRNAs to precipitate. The precipitates were collected by

filtration through glass fiber filter discs. Each fraction tube was then rinsed with 5 ml of 70% ethanol and this was also passed through the filters. The discs were then dried for 15 minutes under heat lamps, placed in scintillation vials, 5 ml of scintillation fluid (toluene plus 0.4% BBOT) was added to each vial and the vials were counted in a liquid scintillation counter at discriminatory counting settings for five minutes each in order to determine the counts of ^{14}C and ^{3}H in each vial.

From the raw counts, background and spillover counts were subtracted giving the final values which were plotted as cpm of ^{14}C or ^{3}H versus fraction number. From these types of plots the populations of normal and thymic lymphoma isoaccepting tRNAs for any particular amino acid could be seen and compared.

Chemicals and Isotopes

The ^{14}C and ^{3}H labelled amino acids were obtained from Schwarz/Mann Bioresearch Labs (Orangeburg, N. Y.) and Amersham/Searle Corporation (Arlington Heights, Ill.). Whatman GF/C glass fiber filter discs and Whatman 3 MM paper filter discs were used to collect TCA precipitated tRNAs. Crude E. coli tRNA was received from G. D. Novelli. Adogen 464 [methyltrialkyl (C_8-C_{10}) ammonium chloride] was obtained from Ashland Chemical Company (Columbus, Ohio). Plaskon CTFE 2300 powder (polychlorotrifluoroethylene) was obtained from G. D. Novelli, originally obtained from Allied Chemical Company (Morristown, N. J.). Calf thymus was from the Sigma Chemical Company (St. Louis, Mo.). Chromatronix columns were from Lab Data Control (Fla.). The general reagents used were standard reagent grade chemicals.

CHAPTER III

RESULTS

Before samples of thymic lymphoma tRNAs could be chromatographed on the RPC-5 column, it was necessary to break in the column with 20 OD_{260} of E. coli tRNAs to saturate any nonspecific irreversible binding sites. This was followed by three chromatographic runs of aminoacylated rat liver tRNAs to test for reproducibility of elution profiles. This proved successful so that analytical and preparative aminocylations of thymic lymphoma tRNAs were begun.

It was soon observed that there was some difficulty in obtaining the usual extent of aminocylation of tRNAs in analytical aminoacylations. This lack became even more critical in preparative aminoacylations where the extent of aminoacylation was less than one-tenth of what was expected by calculations based on the analytical aminoacylations. The reactions were repeated but the same results occurred. The general procedure of Yang and Novelli[4] called for 0.5-2.0 OD_{260} of tRNA to be used per milliliter of reaction volume in order to achieve aminoacylations of 20,000 to 80,000 cpm, and this was with a 50% dilution of the labelled amino acid by cold amino acid. In this work 0.5 ml reaction volumes were used, thus only 0.2 to 0.4 OD_{260} of tRNAs were added in the first attempts, since 10,000 to 20,000 com of aminoacylated tRNAs were sought. These tests yielded very little aminoacylated tRNAs.

Since the rat liver tRNAs which had previously been aminoacylated by this procedure to a high level were run with 1.79 OD_{260} of tRNA in the aminoacylation reaction, it was felt that perhaps the concentration

of tRNA should be increased to 0.5 to 1.0 OD_{260}/0.5 ml reaction volume in these aminoacylation reactions. This proved to be unsuccessful in that very little increase in aminoacylated tRNAs was found. This was also the case as the amount of tRNA used was increased further to 1.5 to 2.0 OD_{260}/0.5 ml of reaction volume and again when raised to 3.5 to 4.0 OD_{260}/0.5 ml of reaction volume. Each time the overall amount of aminoacylated tRNA increased slightly but nowhere near the minimum levels needed for chromatography. Finally, these minimum levels were approached when 8.0 OD_{260} of tRNA was used. This was an incredible amount of tRNAs as compared to the amounts recommended by Yang and Novelli.[4] It was obvious that the aminoacylation conditions needed to be optimized for the thymus tRNAs since the source of tumor tissue was definitely limited.

Attention was then focused on the aminoacyl synthetases since they may be the restrictive factor in an in vitro aminoacylation reaction. The general procedure[4] for this reaction calls for 0.50 to 0.10 mg of synthetase/0.250 ml of reaction volume. This was contained in 50 ul of synthetase stock solution. A series of analytical aminoacylation reactions were run using 50, 40, 30 and 20 ul quantities of synthetase stock in 0.250 ml reaction volumes. It was found that the 50 ul quantity gave better aminoacylation by 50% above the 20 and 30 ul of synthetase reactions and 20% above the 40 ul of synthetase reaction based on the overall quantity of tRNA aminoacylated. Unfortunately, this preparation of synthetase was too limited to increase the concentration further for additional tests.

At this time a large amount of synthetase was obtained from L. C. Waters. Using 10, 25, 50 and 75 ul of this preparation in a series of analytical aminoacylations, it was found that only 10 ul was needed to achieve the same extent of aminocylation (quantity of amino acid attached/OD_{260} tRNA in the reaction) as was achieved with 50 ul of the previous preparation. Additional amounts of synthetase did increase the overall quantity of tRNA aminoacylated but only slightly so that it was felt that 10 ul/0.250 ml reaction volume would prove to be the most efficient quantity to be used to avoid waste. Since the analytical aminoacylations were still very low, one-third to one-fifth of those usually reported in the literature[31] and in personal communications[26,30,32] it was additionally felt that some other reagent might also be in non-optimal amounts. This experiment also showed that if lack of adequate aminoacylation was due to non-specific absorbance of the labelled amino acid to the synthetase molecules then the reactions with the larger amounts of synthetase would have aminoacylated less. This was not the case.

Since some of the labelled amoni acids used up to this point were old, aminoacylation reactions were run comparing new labelled amino acids with the older stocks. No difference was observed. Even though there was no indicated need, new labelled amino acids were used from this point on to avoid any loss of label, degradation of amino acids or pH changes that might possibly occur in older preparations.

A careful check was run on the amount of tRNA in a reaction relative to the other reagents. The experiments conducted previously using increasing quantities of tRNA were run in increasing overall reaction

volumes in order to accommodate the larger volumes of tRNA solution. In this experiment the reaction volume was held constant. The results showed that with a three-fold increase in the amount of tRNA there was a three-fold increase in the quantity of tRNA aminoacylated; however, the extent of amino acid attached/OD_{260} of tRNA remained the same. These reactions were run using 0.176 and 0.528 OD_{260} of tRNA in an analytical aminoacylation procedure. For later preparation aminoacylation reactions run with greater concentrations of tRNA, 6 to 8 OD_{260} tRNA/ml, this rule did not apply. For them a two-fold increase in tRNA many times led to no overall increase in aminoacylated tRNAs and at times even a decrease.

In some cases it has been reported that low aminoacylation of tRNAs with labelled amino acids has been due to dilution of the labelled amino acids by cold amino acids from the active site of the synthetase molecules.[25] With this in mind, a series of aminoacylation reactions were run in which the total amino acid content remained constant but the percentage which was labelled was varied. The range was 5, 10, 25, 50 and 100% labelled amino acid. It was found that the amount of amino acid attached/OD_{260} tRNA remained fairly constant. There was a slight decrease on the 5 and 10% labelled amino acid reactions but this could be accounted for by experimental error.

An experiment was done to determine if possibly the amino acid acceptor stem of the tRNAs has been damaged such as by the loss of the CCA terminal. This was conducted by running an aminoacylation reaction with the normal amount of ATP but also containing CTP at one-tenth the concentration of ATP so that the tRNAs could be repaired. The outcome showed this had no effect.

Many of the aminoacylation reactions done to this point had contained not only the labelled amino acid to be attached but also small quantities of the nineteen other amino acids in the unlabelled form. This was done to help prevent aminoacylation of tRNAs by the incorrect amino acid. When this assortment of amino acids was removed there was no positive or negative effect on aminoacylation of the desired labelled amino acid.

It was found that in preparative aminoacylations, the parameters established for the use of aminoacyl synthetases were not the same as in the analytical aminoacylations. One of the main problems was the quantity of synthetase used. The 10 ul of synthetase/0.250 ml of reaction volume level for analytical aminoacylations were inadequate. First attempts to optimize this quantity for preparative aminoacylation was done by regulating the amount of synthetase relative to the amount of tRNA in the reaction volumes. This was tried in several experiments by varying the ul of synthetase/ul of tRNA at levels of from three-fold greater to one-tenth as much synthetase as tRNAs. The results were generally equivalent. It was eventually found that this estimation procedure was inadequate. With further tests 25 ul of synthetase/0.250 ml of reaction volume was found to aminoacylate sufficiently. If the tRNAs still aminoacylated poorly with this level then some other reagent was more likely the limiting factor.

In preparative aminoacylations the length of time of amino-acylation was varied in an attempt to optimize this factor. When the time was increased to 0.5 hours there was little extra aminoacylation achieved above the quantity obtained at one hour. When the time was

dropped to 15 minutes there was usually a low extent of aminoacylation even though 15 minutes was plenty of time to reach an end point in the analytical aminoacylations.

An attempt was made to use plastic instead of glass for any hardware which came in contact with the tRNAs to avoid the adsorptive effects of glass. This proved slightly beneficial and was continued.

There is an inherent problem in the aminoacylation reaction in relation to the aminoacylated tRNAs due to the fact that while the synthetases require a pH of 7.5 for optimal activity this pH can promote deacylation of certain aminoacylated tRNAs. To try to avoid this the reaction volume immediately after the reaction time was completed was diluted three-fold with the 0.25 M NaCl solution containing 10 mM Na acetate pH 4.5 which was the initial application solution for the DEAE-cellulose purification to follow. This helped slightly and was continued.

Several articles in the literature report that aminoacylated tRNAs, after the aminoacylation reaction, are recovered and purified by processing them through the phenol extraction procedure.[2,7,33] This method was tested several times but was discontinued since there was firstly, no added protection of aminoacylated tRNAs and secondly, because free labelled amino acids were carried through the purification along with the aminoacylated tRNAs thus contaminating the final product.

Aminoacylated tRNAs were found to remain intact better if they were immediately applied to an RPC-5 column after resolubilizing them after the final ethanol precipitation step. This did not allow time to test the quantity of ^{3}H and ^{14}C cpm applied to the column immediately before chromatography. Instead, these data had to come from the previous

day's evaluation of the extent of aminoacylation. Thus, the amount of ^{3}H and ^{14}C counts applied to the column listed in the figure legends are probably 5 to 20% too high which leads to some inaccuracy in the estimates of recovery.

It was also found at this time that if the 10 OD_{260} of E. coli tRNAs used as a "carrier" was added to the aminoacylated tRNA solution immediately before the thanol precipitation step instead of just before TPC-5 chromatography, recovery of aminoacylated tRNAs from the ethanol was enhanced. This was probably due to more efficient precipitation.

To check if many of the problems seen in the aminoacylation reactions were due to personal error, G. D. Griffin was asked to run an analytical aminoacylation reaction of the same tRNA preparations with his own reagents. He obtained slightly higher levels of aminoacylation but not appreciable amounts. Following this a preparation of mouse liver tRNAs was obtained from Dr. Griffin and also a precipitation from L. C. Waters. These were aminoacylated under my reaction conditions and were found to be aminoacylated to an extent many times greater than those levels achieved in analytical aminoacylations of my tRNA preparations. In addition this was achieved with only one-fourth the amount of synthetase used normally.

These large numbers of aminoacylation experiments were a drain on the limited amount of thymic lymphoma material available. Because unusually large amounts of tRNAs were needed in each reaction, a number of extractions had to be done from thymuses requiring a lot of time. Since it was difficult to believe that the reaction conditions couldn't be better optimized and the wasting of material was definitely to be

avoided, only small quantities of thymuses were extracted at any one time.

In addition to the eight amino acids which were successfully aminoacylated and chromatographed, attempts were made to aminoacylate lysine and tryptophan. Unfortunately, it was not possible to adequate aminoacylate either of these on a preparative scale and, further, the small quantities of aminoacylated lysine that were obtained underwent very quick spontaneous deacylation.

The quality of the tRNA preparations was then checked by an analysis for DNA contaminants by the Dische diphenylamine reaction and by gel electrophoresis. Table I shows the results of the Dische diphenylamine reaction. As can be seen several of the tRNA preparations had large percentages of DNA contaminants which might have been responsible for some of the difficulties encountered in the aminoacylation reaction. There appears to be a great deal of variation in DNA content between extractions with no apparent pattern.

As can be seen in Figure 1, showing the scans of two gels containing samples of two representative tRNA preparations, DNA is not the only major contaminant in the preparations. There appears to be a large amount of material absorbing at 260 nm which is less than 4 S in size. This is possibly degraded tRNAs of various sizes which occur as the result of the thymuses having been frozen, especially in the case of the thymic lymphomas. Additionally, nuclease activity could have contributed to the degradation. These tRNA fragments were probably

TABLE 1

DNA CONTENT IN TRANSFER RNA EXTRACTIONS

Transfer RNA Source	% DNA[1]
Normal thymus	23[2]
	41
	20
	12
	27
Thymuc lymphoma	18
30-day[3]	-
62-day	14
97-day	86
Terminal	40
	4
E. coli[4]	23

[1]DNA analyzed by the Dische diphenylamine colorimetric assay. %DNA = (OD_{260} DNA/OD_{260} tRNA preparation) x 10^2 were OD_{260} is calculated based on the micrograms of DNA determined from the reaction being converted to OD_{260} units according to the assumption that one milligram of DNA yields 20 OD_{260} units.

[2]Results for several different tRNA preparations.

[3]Transfer RNAs extracted from thymic lymphomas removed from mice at the indicated elapsed time after radiation treatment.

[4]*E. coli* tRNAs which had been purified over DEAE-cellulose.

Figure 1. Electrophoresis of normal and thymic lymphoma tRNAs on 7.5% gels. Electrophoresis of 1.0 OD_{260} of normal thymus tRNAs (part A) and 1.0 OD_{260} of thymic gymphoma tRNAs (part B) on 7.5% crosslinked polyacrylamide gels. The samples were electrophoresed for 2.75 hours at 70 mAmps and 220 pulses/second at 8 to 10°C. The voltage was approximately 180 V. The nucleic acids were then located on the gels by scanning at 260 nm. Reference gels were run using rat liver 4 S and 5 S RNAs. Their movement relative to the tracking dye was measured and plotted on the sample electrophoresis scans.

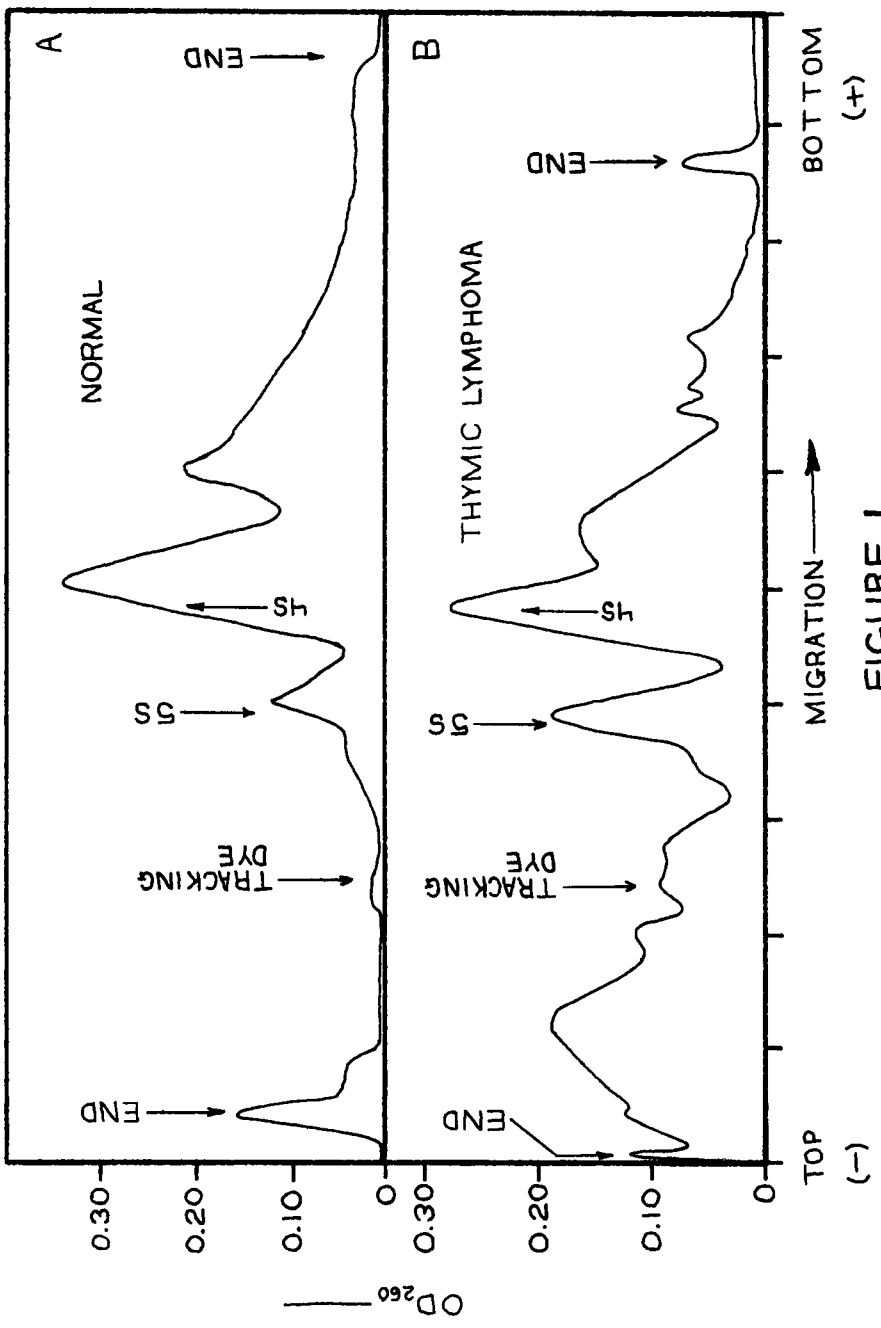

A
NORMAL
END
4S
5S
TRACKING DYE
END
B
THYMIC LYMPHOMA
END
4S
5S
TRACKING DYE
END
0.30
0.20
0.10
0
0.30
0.20
0.10
0
OD260
TOP (-)
MIGRATION
BOTTOM (+)

FIGURE I.

responsible for the interference in the aminoacylation reactions since they would have had a much greater affinity than DNA fragments for the aminoacyl synthetases thus acting as competitive inhibitors. Efforts were not made to try to purify the tRNA preparations further since it would have been extremely difficult to remove the tRNA fragments without loss of some of the intact tRNAs. This arises from the fact that many of the tRNA fragments appear to be only a few bases short of whole tNRAs.

These electrophoretic gels being 7.5% polyacrylamide, the DNA fragments appeared at the top of the gels and in the case of the thymic lymphoma tRNA preparation even short distances into the gel. Normally in good extractions of tRNAs from cells of other types, the ratio of 4 S RNAs/5 S RNAs equals approximately 5.0. In the tRNA preparations shown here, the ratios are 2.6 and 1.5 for the normal and thymic lymphoma tRNA extractions respectively.

Using these tRNA preparations the eight different amino acids were eventually aminoacylated in analytical aminoacylation reactions the results of which are shown in Table II. The extent of aminoacylation of the tRNAs was low considering what can normally be expected, in fact most were less than 50% of the levels which should have been attained.[31] Although between different tRNA preparations there appeared to be some variation in the amount of aminoacylatable tRNAs for specific amino acid, e.g. leucine and lysine, there also appeared to be considerable agreement, i.e tyrosine. As shall be shown later, there were no detectable differences between isoaccepting species of tyrosine tRNAs

TABLE II

EXTENTS OF AMINOACYLATION AS DETERMINED BY ANALYTICAL AMINOACYLATION REACTIONS

Amino Acid	Thymic Lymphoma Preparations[1]	Normal Preparations[2]
Leu	38*, 38*, 20[3]	43*, 48*, 47*
Lys	44, 11	20, 13*, 6* 8*, 7*, 8*
Ser	27	38
Gly	56	18
Asp	22	11
Pro	17	14
Phe	(30-day)[4] 21 (62-day) 16 (97-day) 21 Terminal 18	6, 7
Tyr	(30-day) 10 (62-day) 6 (97-day) 8 Terminal 9,6,8	9,6,4,5
His	(30-day) 4 (62-day) 1 (97-day) 2 Terminal 0.4	14,10

[1]Aminoacylated with ^{3}H amino acids, units are picomoles amino acid/OD_{260} of tRNA preparation.

[2]Aminoacylated with ^{14}C amino acids, units same as above.

[3]A series of values represents several determinations of the extent of aminoacylation using different tRNA preparations.

TABLE II (Continued)

[4]Thumus tRNAs extracted from lymphomas removed from mice at the indicated elapsed time after radiation treatment was terminated.

*Starred values within a series of values for a specific amino acid indicates that these values were determined using the same tRNA preparation.

in normal and cancerous thymuses; therefore, the aminoacylation values between the two can be compared revealing considerable agreement. When a series of analytical aminoacylations were performed on the same tRNA preparation using the same amino acid, the aminoacylation values in the series were in good agreement with each other, i.e. starred values in leucine and lysine values. Since the variations which did arise were small, large differences in aminoacylation with different tRNA preparations were most likely due to the differing amounts of contaminating DNA and degraded tRNAs present.

In practice, after the extent of aminoacylation had been determined for a particular amino acid by an analytical aminoacylation, the preparative aminoacylations were immediately carried out. When enough tRNAs of both types, normal and tumor, had been aminoacylated with labelled amino acids, they were chromatographed on RPC-5 before proceeding on to an analytical aminoacylation with the next amino acid. The following series of figures show the results of RPC-5 chromatograph of all eight amino acids.

Figure 2 shows the results of the chromatography of tRNAs amino-acylated with serine, part A, and glycine, part B. The chromatography of serine tRNAs appear as three major isoaccepting species with small quantities of several lesser forms. These species were very well duplicated in the normal and thymic lymphoma tRNAs and the positions coincided well. There was also a small body of material which did not elute from the column until the 1 M NaCl wash was applied.

Figure 2. RPC-5 cochromatography of normal and thymic lymphoma tRNAs using serine and glycine. Part A is the cochromatography of ^{3}H-thymic lymphoma tRNAs (-----) and ^{14}C-serine normal tRNAs (- - - -). Approximately 32,000 cpm of ^{3}H-serine and 13,000 cpm of ^{14}C-serine were used. The sample was applied in a volume of 10 ml of 0.35 M NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 10 mM Na acetate pH 4.5 and 3 mM B-mercaptoethanol followed by a 10 ml chase of the same salt solution. The 0.4 to -.6 M NaCl gradient was then started using 100 ml of each concentration containing the same additional reagents as above. The flow rate was 1 ml/min at 150 psi with the 25 x 0.63 cm column at 22°C. After all but 20 ml of the gradient had been applied, a 20 ml 1 M NaCl wash containing the same additional reagents was applied. Fractions collected as material eluted were as follows: 3 ml fractions during sample and low salt chase application, 1 ml fractions during the gradient and 2 ml fraction during the 1 M NaCl wash. Recovery was 79% for ^{3}H-serine cpm and 110% for ^{14}C-serine cpm. Part B shows the cochromatography of ^{3}H-blycine thymic lymphoma tRNAs (-----) and ^{14}C-glycine normal tRNAs (- - - -). There were 25,000 cpm of ^{3}H-glycine and 8080 cpm of ^{14}C-glycine. Chromatographic conditions same as in part A except the pump pressure was 170 psi. Recovery was 81% for ^{3}H-glycine cpm and 110% for ^{14}C-glycine cpm.

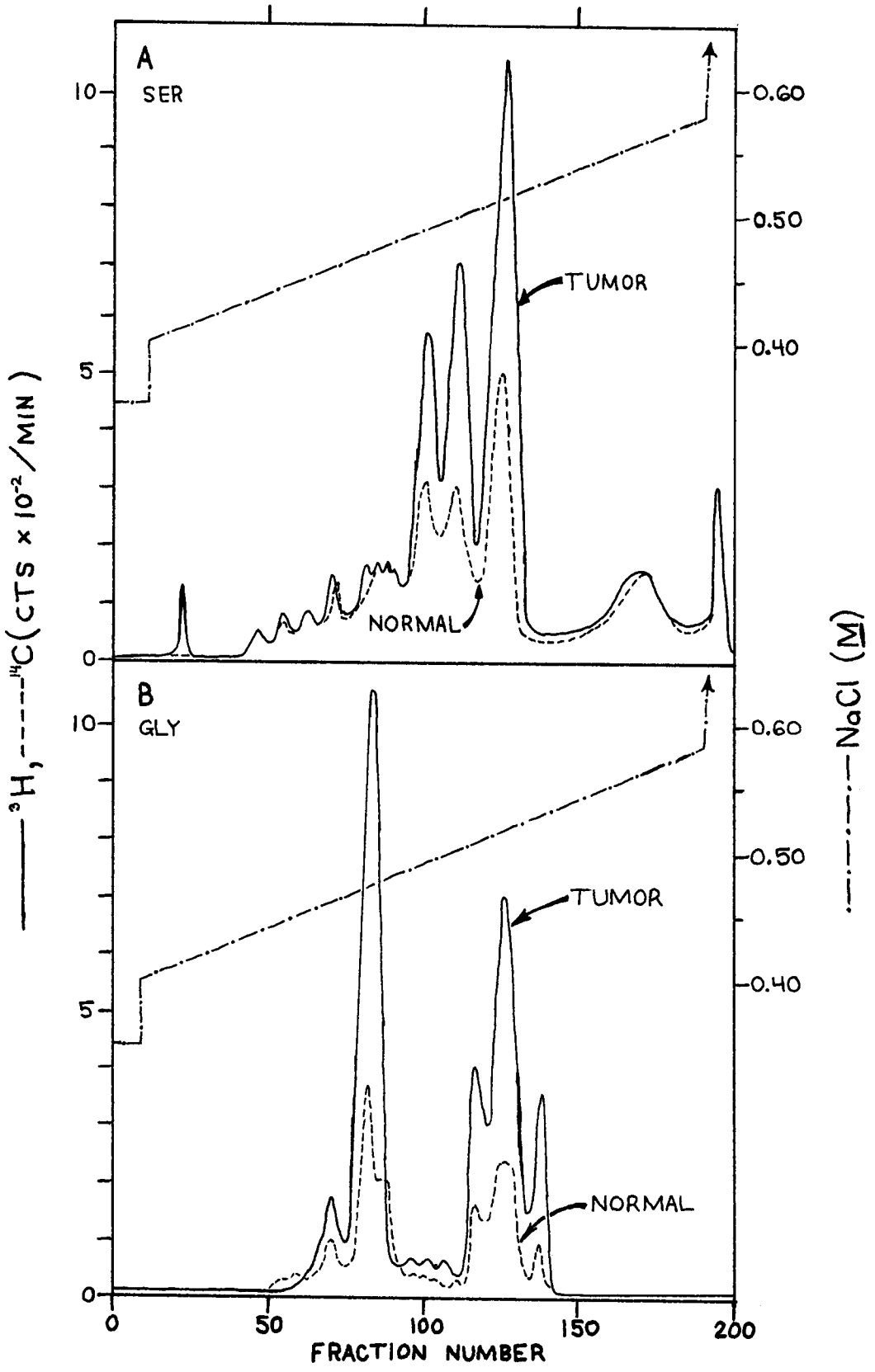

A
SER
TUMOR
NORMAL
B
GLY
TUMOR
NORMAL
10
5
0
0.60
0.50
0.40
0
50
100
150
200
FRACTION NUMBER
——³H, ----¹⁴C (CTS x 10⁻²/MIN)
-·-·-NaCl (M)

FIGURE 2.

In the glycine chromatogram, again there was good duplication in major and minor isoaccepting species from normal and thymic lymphoma tissue. Since only 8080 cpm of ^{14}C-glycine labelled normal tRNAs were used, some of the minor isoaccepting tRNA peaks did not show up as well as their counterparts in the ^{3}H-glycine labelled thymic lymphoma tRNAs. There appear to be two major peaks and three minor peaks. All the recoverable tRNAs eluted from the column came off within the NaCl gradient.

In the next figure, Figure 3, appear the chromatograms of leucine and tyrosine tRNAs. Part A shows that leucine isoaccepting tRNAs consist of three distinctive major forms. There are two very minor forms but they only occur as very small side shoulders on the leading edge of two of the major peaks. The tyrosine chromatogram consists of one major peak with a small side shoulder. This was present in both the normal and thymic lymphoma tyrosine tRNAs.

The profiles of aspartic acid and proline tRNAs appear in Figure 4. For the aspartic acid isoaccepting tRNAs there were definite similarities between the normal and thymic lymphoma forms especially in the one major peak but the several minor peaks only mirrored each other in general. This was due to the low amount of cpm of ^{3}H- and ^{14}C-amino acids chromatographed, since each minor peak is represented by less than 100 cpm. The proline chromatogram in part B shows that there is a wide spread of proline isoaccepting species which are difficult to cleanly separate. There is good agreement on the shape and position of the broad peak as well as the side shoulders. A similar profile was attained

Figure 3. RPC-5 cochromatography of normal and thymic lymphoma tRNAs using leucine and tyrosine. Part A is the cochromatography of ^{3}H-leucine thymic lymphoma tRNAs (-----) and ^{14}C-leucine normal tRNAs (- - - -). There were approximately 40,000 cpm ^{3}H-leucine and 13,000 cpm ^{14}C-leucine. Chromatographic conditions were the same as in Figure 2 (p 33) except pump pressure was 170 psi. Recovery was 87% for ^{3}H-leucine cpm and 153% ^{14}C-leucine cpj. Part B is the cochromatography of ^{3}H-tyrosine thymic lymphoma tRNAs (-----) and ^{14}C-tyrosine normal tRNAs (- - - -). Approximately 24,000 cpm of ^{3}H-tyrosine and 18,000 cpm of ^{14}C-tyrosine were used. Chromatographic conditions same as in Figure 2 except pump pressure was 170 psi. Recovery 109% of ^{3}H-tyrosine cpm and 62% of ^{14}C-tyrosine cpm.

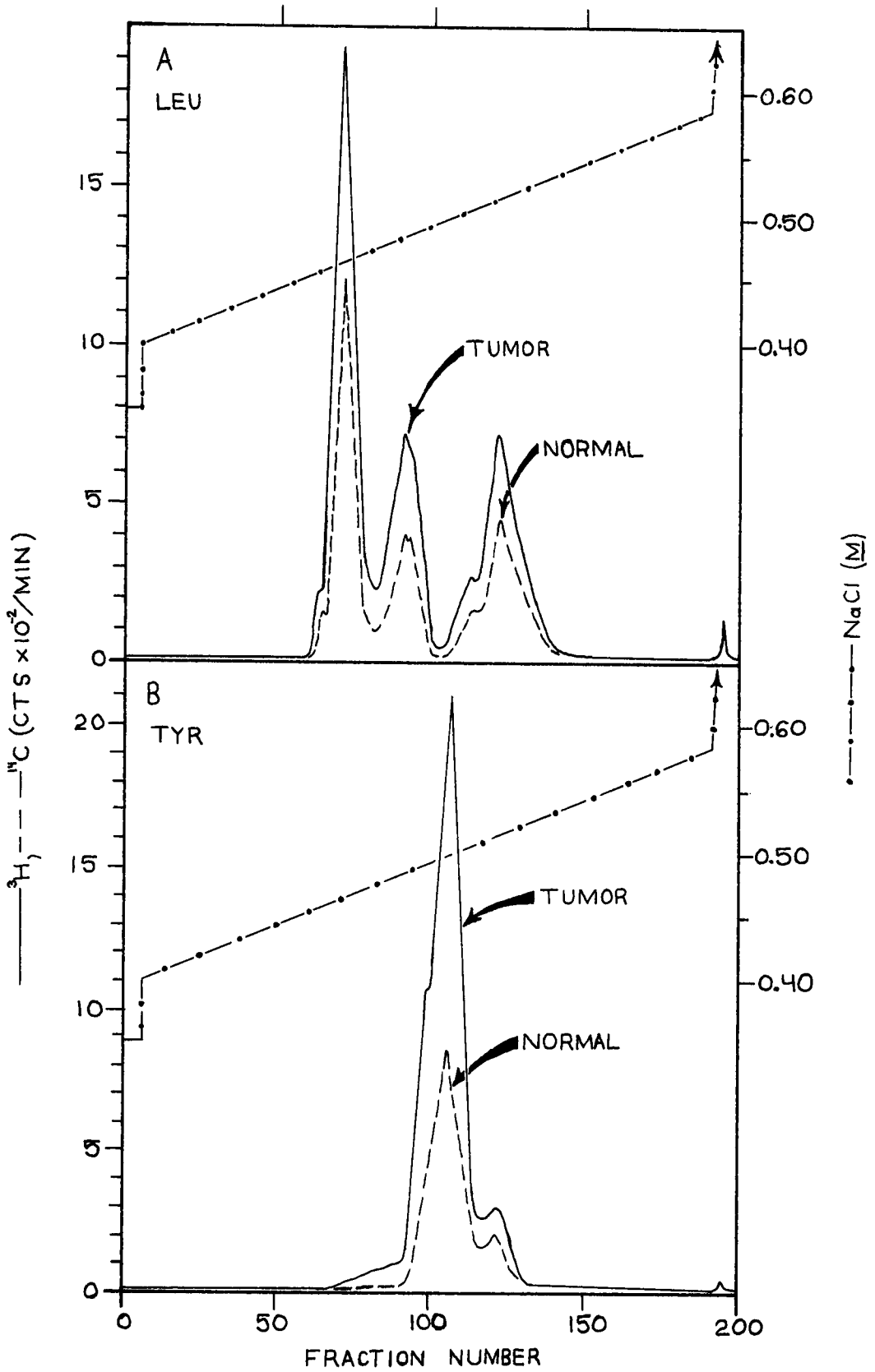

A
LEU
B
TYR
TUMOR
NORMAL
TUMOR
NORMAL
0.60
0.50
0.40
0.60
0.50
0.40
0
5
10
15
0
5
10
15
20
0
50
100
150
200
FRACTION NUMBER
^3H — — ^{14}C (CTS x 10^{-2}/MIN)
NaCl (M)

FIGURE 3.

Figure 4. RPC-5 cochromatography of normal and thymic lymphoma tRNAs using aspartic acid and proline. Part A is the cochromatography of ^{3}H-aspartic acid thymic lymphoma tRNAs (-----) and ^{14}C-aspartic acid and 4400 cpm of ^{14}C-aspartic acid were used. Chromatographic conditions were as listed in Figure 2 (p 33) except a 0.35 to 0.65 M NaCl gradient was used at a pump pressure of 183 psi. Recovery was 67% of ^{3}H-aspartic acid cpm and 72% of ^{14}C-aspartic acid cpm. Part B is the cochromatography of ^{3}H-proline thymic lymphoma tRNAs (-----) and ^{14}C-proline normal tRNAs (- - - -). Approximately 16,000 cpm of ^{3}H-proline and 7500 cpm of ^{14}C-proline were used. Chromatographic conditions were as in Figure 2 except that a 0.35 to 0.70 M NaCl gradient at 155 psi pump pressure was used. Recovery was 93% for ^{3}H-proline cpm and 91% for ^{14}C-proline cpm.

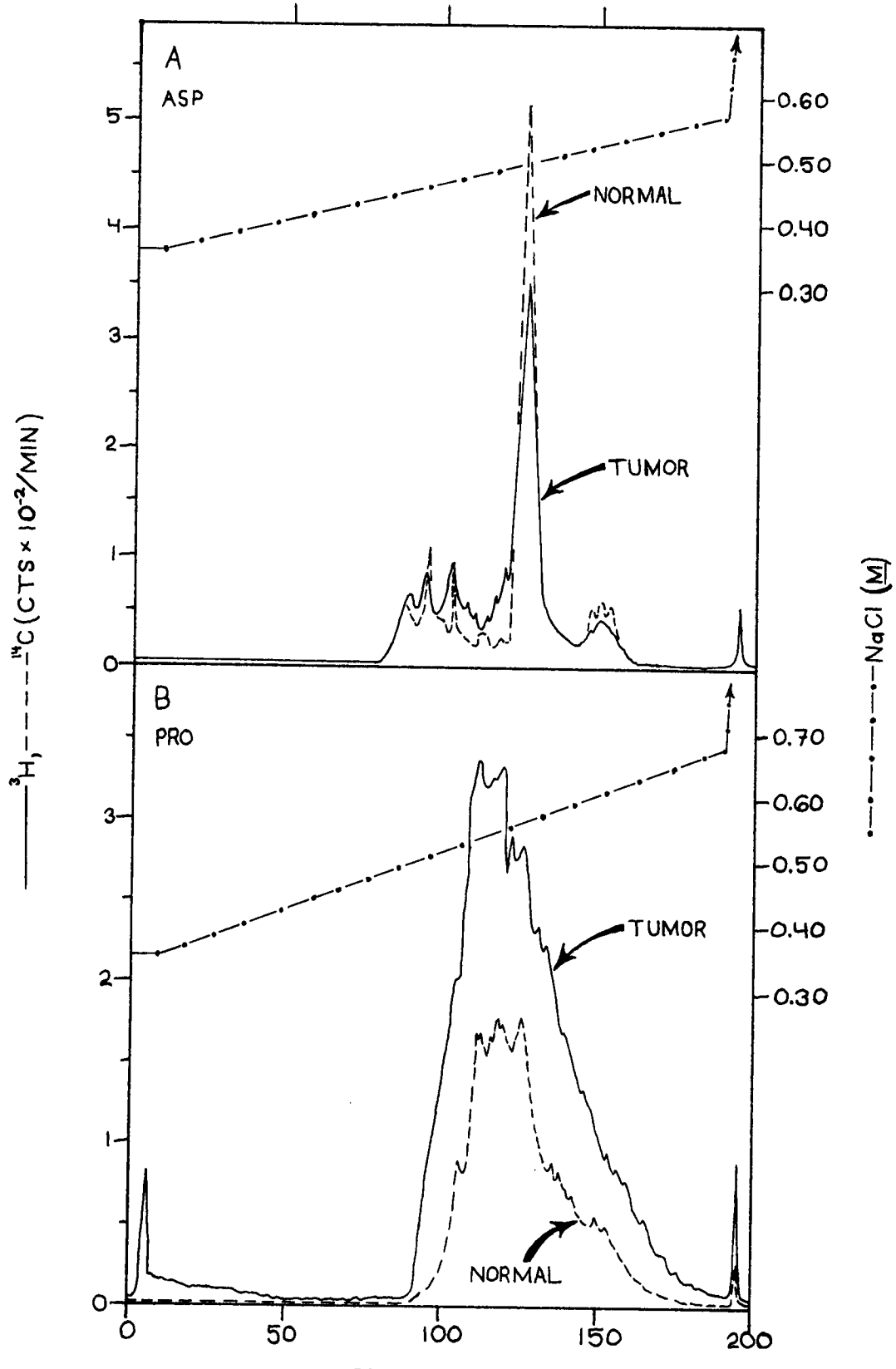

A
ASP
NORMAL
TUMOR
B
PRO
TUMOR
NORMAL
0.60
0.50
0.40
0.30
0.70
0.60
0.50
0.40
0.30
0
50
100
150
200
FRACTION NUMBER
^{3}H, ---- ^{14}C (CTS × 10^{-2}/MIN)
—•—•— NaCl (M)

FIGURE 4.

upon repetition of the experiment. Even though 16,000 cpm of ^{3}H-proline and 7500 cpm of ^{14}C-proline were used in the chromatography, the peak never exceeded more than 350 cpm due to its broadness.

Figure 5 shows the results of chromatographing histine and phenylalanine on RPC-5. The histidine profile shows the first real difference between normal and thymic lymphoma isoaccepting tRNAs. This difference shows up as a shift in position of the first and the last minor peaks and as a slight shift in the large central peak. In addition the relative proportions of the peaks have altered such that the large central peak is the greatest contributing isoaccepting species present. No exact quantitative measurements of each species could be made since the tRNAs were not reverse labelled. Thus only relative proportions can be discussed here.

The phenylalanine chromatogram shown in part B illustrates much more dramatic differences between normal and thymic lymphoma tRNAs. The normal tRNAs consist of three minor species and one very dominant major peak. The tumor tRNAs consist of only one clear minor species but two major peaks. Both major species of the tumor coincide with species of the normal tRNAs, one a major and one a minor peak; however, the minor species of tumor tRNAs appears at a new location. Thus the tumor species show some changes from the normal both in position and relative proportion of the peaks.

Abnormal alterations in tRNA profiles which were found differed from normal in two classes of tRNAs, those which carried histidine and those which carried phenylalanine. These alterations were examined further to determine at what period during tumor development they appeared.

Figure 5. RPC-5 cochromatography of normal and thymic lymphoma tRNAs using histidine and phenylalanine. Part A shows the cochromatography of ^{3}H-histidine thymic lymphoma tRNAs (-----) and ^{14}C-histidine normal tRNAs (- - - -). Approximately 10,900 cpm of ^{3}H-histidine and 6000 cpm of ^{14}C-histidine were applied to the column. Chromatographic conditions were the same as in Figure 2 (p 33) except a 0.35 to 0.60 M NaCl gradient at 190 psi was used. Recovery was 105% for ^{3}H-histidine cpm and 148% for ^{14}C-histidine cpm. Part B shows the cochromatography of ^{3}H-phenylalanine thymic lymphoma tRNAs (-----) with ^{14}C-phenylalanine normal tRNAs (- - - -). Approximately 15,800 cpm of ^{3}H-phenylalanine and 4800 cpm of ^{14}C-phenylalanine were applied to the column. Chromatographic conditions were as in Figure 2 except that a 0.45 to 0.70 M NaCl gradient at 183 psi was applied. Recovery was 89% for ^{3}H-phenylalanine cpm and 136% for ^{14}C-phenylalanine cpm.

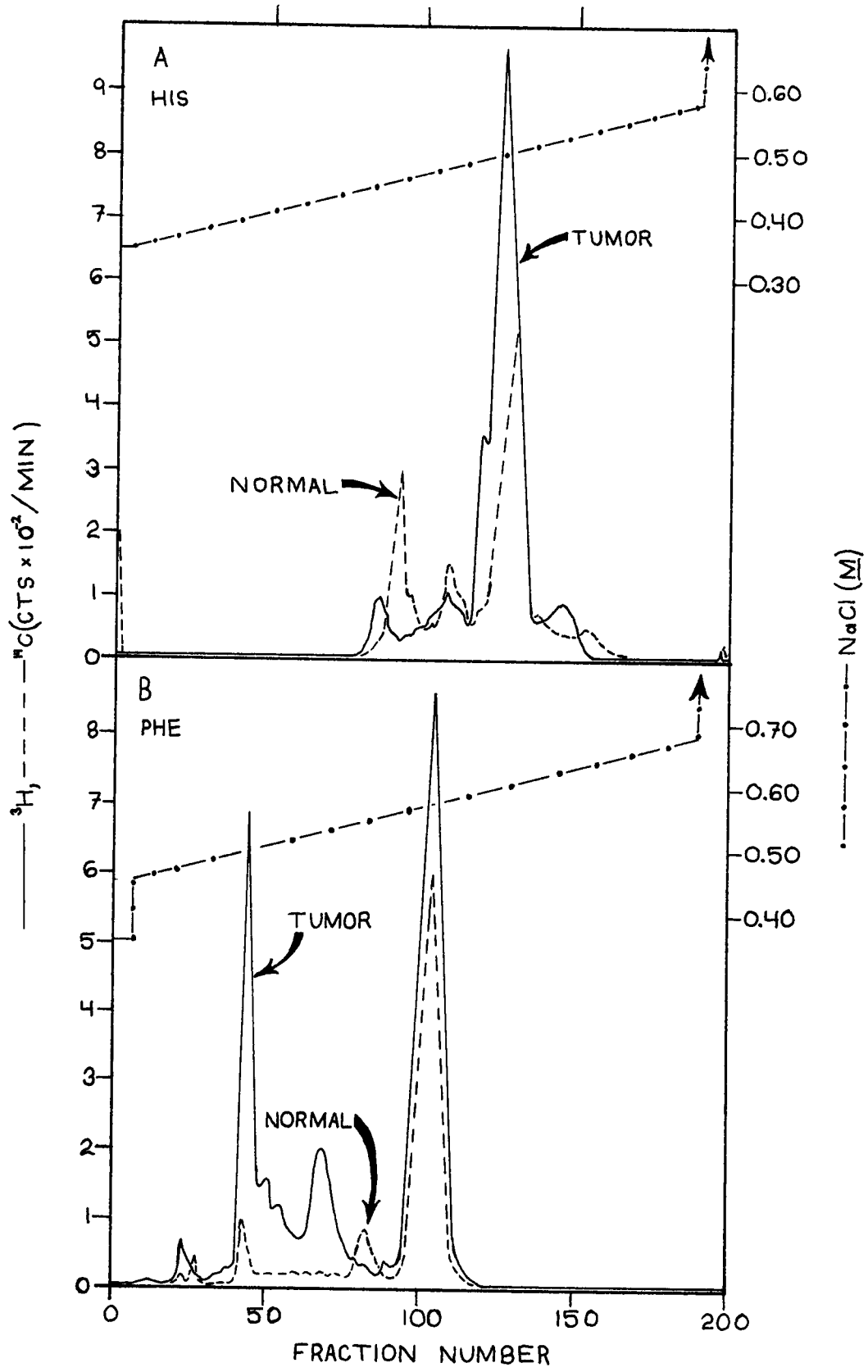

A
HIS
TUMOR
NORMAL
B
PHE
TUMOR
NORMAL
0.60
0.50
0.40
0.30
0.70
0.60
0.50
0.40
0
50
100
150
200
FRACTION NUMBER
——³H; - - - - ¹⁴C(CTS x 10⁻²/MIN)
— · — · — NaCl (M)

FIGURE 5.

Figure 6 shows the results of examining tRNAs from cells taken from thymuses at 30, 62 and 97 days after the final radiation treatment. The terminal stage is also shown for comparison. These tRNAs were aminoacylated with labelled phenylalanine. Unfortunately, in the chromatograph shown in part A there was very poor recovery of the ^{14}C-phenylalanine normal tRNAs; however, enough can be seen to determine the positions of the peaks. The tumor species consist of only two major peaks and an insignificant minor peak. For comparisons of different chromatograms the positions of peaks must be viewed as relative to the positions of peaks of the normal species. Thus, the two major peaks coincide with the major and a minor peak of the normal species. At this stage the tumor species have been altered into the two major species but not the minor species seen in the terminal case, part D.

At the 62 day period, part B, the profile of tumor species has changed drastically. It consists of two minor species, one which coincides with a minor peak of the normal and one which does not, and a large major peak which was present at the 30 day period but no in such quantity. This same profile is maintained at the 97 day period shown in part C. Lastly, in the terminal stage the second major peak has re-emerged but the first minor peak has disappeared. The first major peak has remained although it is not as large.

Next the profiles os histidine isoaccepting tRNAs were examined at the various stages of tumor development, Figure 7. Again the terminal stage profile is shown for comparison, part D.

Figure 6. RPC-5 cochromatography of tRNAs from different stages of thymic lymphoma development compared to normal tRNAs using labelled phenylalanine. Chromatographic conditions for all runs were as listed in Figure 2 (p 33) except a 0.45 to 0.70 M NaCl gradient at 190 psi was used. ^{3}H-phenylalanine thymic lymphoma tRNAs are represented as (-----) and ^{14}C-phenylalanine normal tRNAs as (- - - -). Part A shows the chromatography of thymic lymphoma tRNAs extracted from thymuses 30 days after the final radiation treatment. Approximately 14,000 cpm of ^{3}H-phenylalanine and 6000 cpm of ^{14}C-phenylalanine were used. Recovery was 136% for ^{3}H-phenylalanine cpm and 25% for ^{14}C-phenylalanine cpm. Part B shows the chromatography of thymic lymphoma tRNAs removed 62 days after the final radiation treatment. Approximately 19,000 cpm of ^{3}H-phenylalanine and 8400 cpm of ^{14}C-phenylalanine were applied to the column. Recovery was 66% for ^{3}H-phenylalanine cpm and 94% for ^{14}C-phenylalanine cpm. Part C shows the chromatography of thymic lymphoma tRNAs removed 97 days after the final radiation treatment. Approximately 24,000 cpm of ^{3}H-phenylalanine and 9500 cpm of ^{14}C-phenylalanine were applied. Recovery was 28% for ^{3}H-phenylalanine cpm and 102% for ^{14}C-phenylalanine cpm. Part D shows the chromatography of thymic lymphoma tRNAs removed at the terminal period of development. See Figure 5 (p 41) for details.

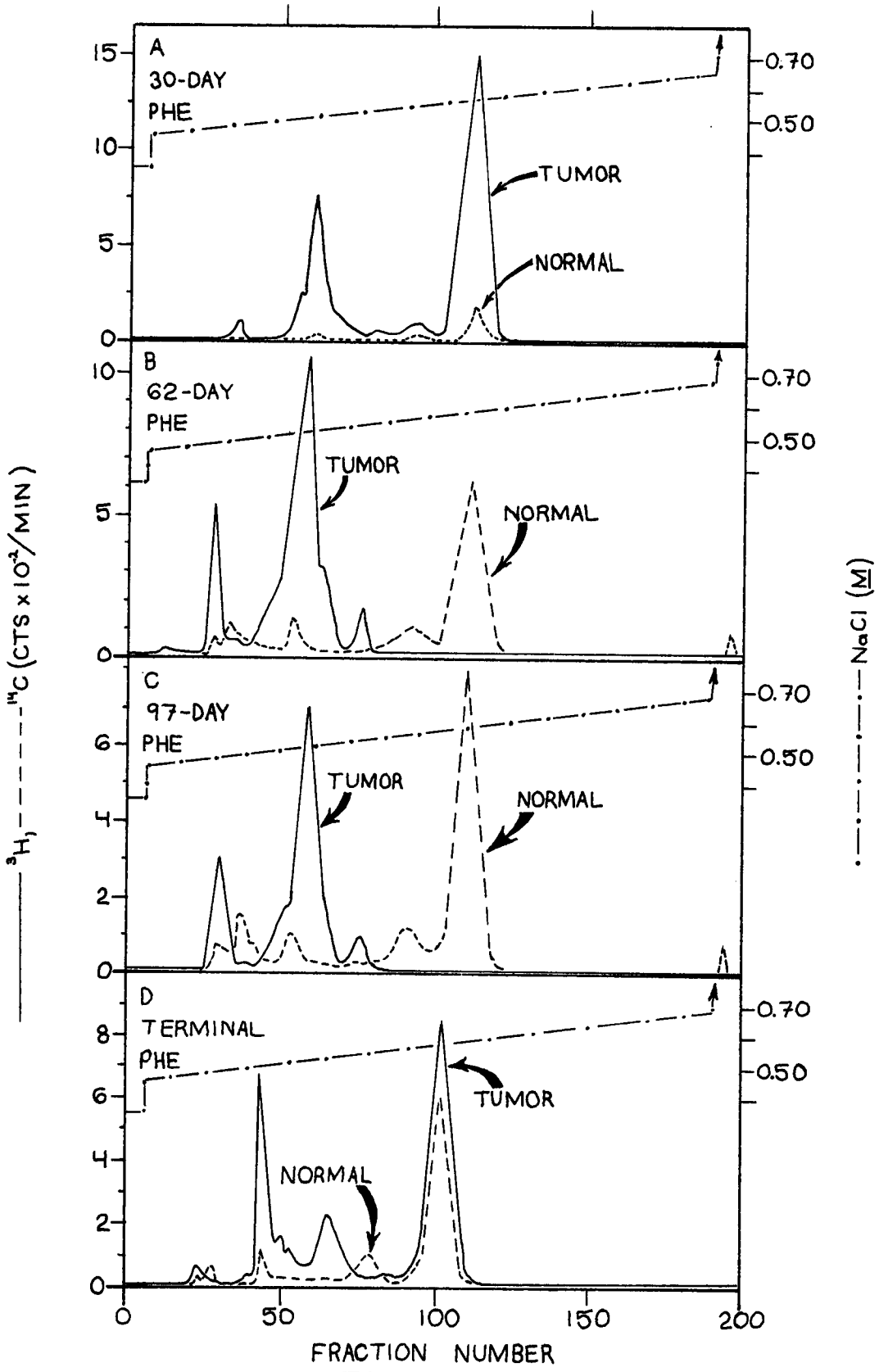

A
30-DAY
PHE
TUMOR
NORMAL
B
62-DAY
PHE
TUMOR
NORMAL
C
97-DAY
PHE
TUMOR
NORMAL
D
TERMINAL
PHE
TUMOR
NORMAL
0.70
0.50
FRACTION NUMBER
^3H, ——— ^{14}C (CTS x 10^{-2}/MIN)
——·——·—— NaCl (M)

FIGURE 6.

Figure 7. RPC-5 cochromatography of tRNAs from different stages of thymic lymphoma development compared to normal tRNAs using labelled histidine. Chromatographic conditions for all runs were as listed in Figure 2 (p 33) except a 0.35 to 0.60 M NaCl gradient at 165 psi was employed. ^{3}H-histidine thymic lymphoma tRNAs are represented as (-----) and ^{14}C-histidine normal tRNAs as (- - - -). Chromatograms of tRNAs removed from thymuses 30, 62 and 97 days after the final radiation treatment are shown in parts A, B, and C respectively. Part D shows tRNAs from thymic lymphomas in the terminal stage, approximately 157 days after the final radiation exposure. Part A shows the cochromatography of 20,000 cpm of ^{3}H-histidine thymic lymphoma tRNAs with 6000 cpm of ^{14}C-histidine normal tRNAs. Recovery was 62% of ^{3}H-histidine cpm and 92% of ^{14}C-histidine cpm. Part B shows the cochromatography of 17,700 cpm of ^{3}H-histidine thymic lymphoma tRNAs with 7600 cpm of ^{14}C-histidine normal tRNAs. Recovery was 92% of ^{3}H-histidine cpm and 154% of ^{14}C-histidine cpm. Part C shows the cochromatography of 17,700 cpm of ^{3}H-histidine thymic lymphoma tRNAs with 9000 cpm of ^{14}C-histidine normal tRNAs. Recovery was 86% of ^{3}H-histidine cpm and 63% of ^{14}C-histidine cpm. Part D shows the cochromatography of tRNAs from the terminal stage of thymic lymphoma development. See Figure 5 (p 41) for details.

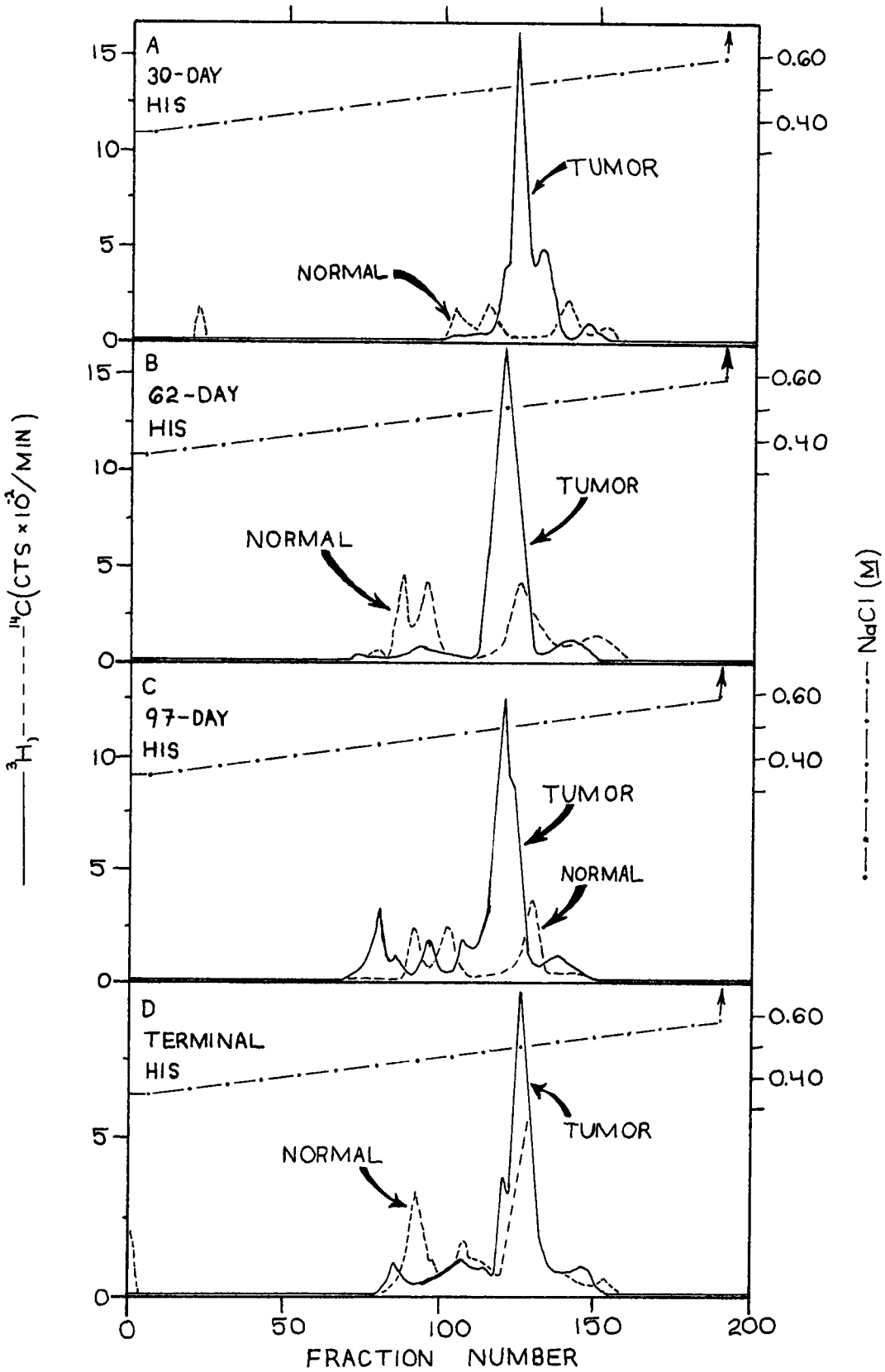

A
30-DAY
HIS
TUMOR
NORMAL
B
62-DAY
HIS
TUMOR
NORMAL
C
97-DAY
HIS
TUMOR
NORMAL
D
TERMINAL
HIS
TUMOR
NORMAL
15
10
5
0
0.60
0.40
0
50
100
150
200
FRACTION NUMBER
^{3}H, ——— ^{14}C (CTS × 10^{3}/MIN)
NaCl (M)

FIGURE 7.

The profile of the tumor tissue at the 30 day time point, part A, is characterized by one major peak with a side shoulder and a small minor peak. None of them coincide with any of the four peaks of normal tRNA species. At 62 days, part B, the main peak has shifted over to the position of its side shoulder in part A. This is slightly askew to the position of one of the normal peaks. The minor peak is also present and occupies its same position. Themajor major and minor peaks are present at the 97 day period in the same positions, part C, however there are additionally two minor peaks present neither of which coincide with peaks of the normal material. These same peaks remain in the same positions and relative quantities up into the terminal period, part D.

The last set of profiles in this series are those of tyrosine tRNAs at the various stages of tumor development, Figure 8. These act as controls for the two previous series, Figures 6 (p 44) and 7 (p 46). The profile of tyrosine isoaccepting tRNAs in thymic lymphoma tissues remained the same throughout the different periods of development. Thus the differences seen in the profiles of histidine and phenylalanine isoaccepting tRNAs were true differences and not artifacts of the procedure for extr-action, storage, aminoacylation or chromatography of tumor tRNAs. This was also supportive evidence, although not conclusive, against the possibility that the differences seen were due to a secondary non-specific effect such as radiation sickness. It can be seen that the group of tRNAs as a whole, both from normal and thymic lymphoma tissues, can move very slightly up or down the chromatogram. It appears

Figure 8. RPC-5 cochromatography of tRNAs from different stages of thymic lymphoma development compared to normal tRNAs using labelled tyrosine. Chromatographic conditions for all runs were as listed in Figure 2 (p 33) except a pump pressure of 180 psi was maintained. ^{3}H-tyrosine thymic lymphoma tRNAs are represented by (-----) and ^{14}C-throsine thymic lymphoma tRNAs are represented by (- - - -). Chromatograms of tRNAs removed from thymuses 30, 62 and 97 days after the final radiation treatment are shown in parts A, B, and C respectively. Part D shows tRNAs from the terminal stage of thymic lymphoma development. Part A shows the cochromatography of 30,500 cpm of ^{3}H-tyrosine thymic lymphoma tRNAs with 9000 cpm and 129% for ^{14}C-tyrosine cpm. Part B shows the cochromatography of 25,500 cpm of ^{3}H-tyrosine thymic lymphoma tRNAs with 9700 cpm of ^{14}C-tyrosine normal tRNAs. Recovery was 145% of ^{3}H-tyrosine cpm and 76% of ^{14}C-tyrosine cpm. Part C shows the cochromatography of 24,000 cpm of ^{3}H-tyrosine thymic lymphoma tRNAs with 15,700 cpm of ^{14}C-tyrosine normal tRNAs. Recovery was 117% of ^{3}H-tyrosine cpm and 66% of ^{14}C-tyrosine cpm. For details on part D see Figure 3 (p 36).

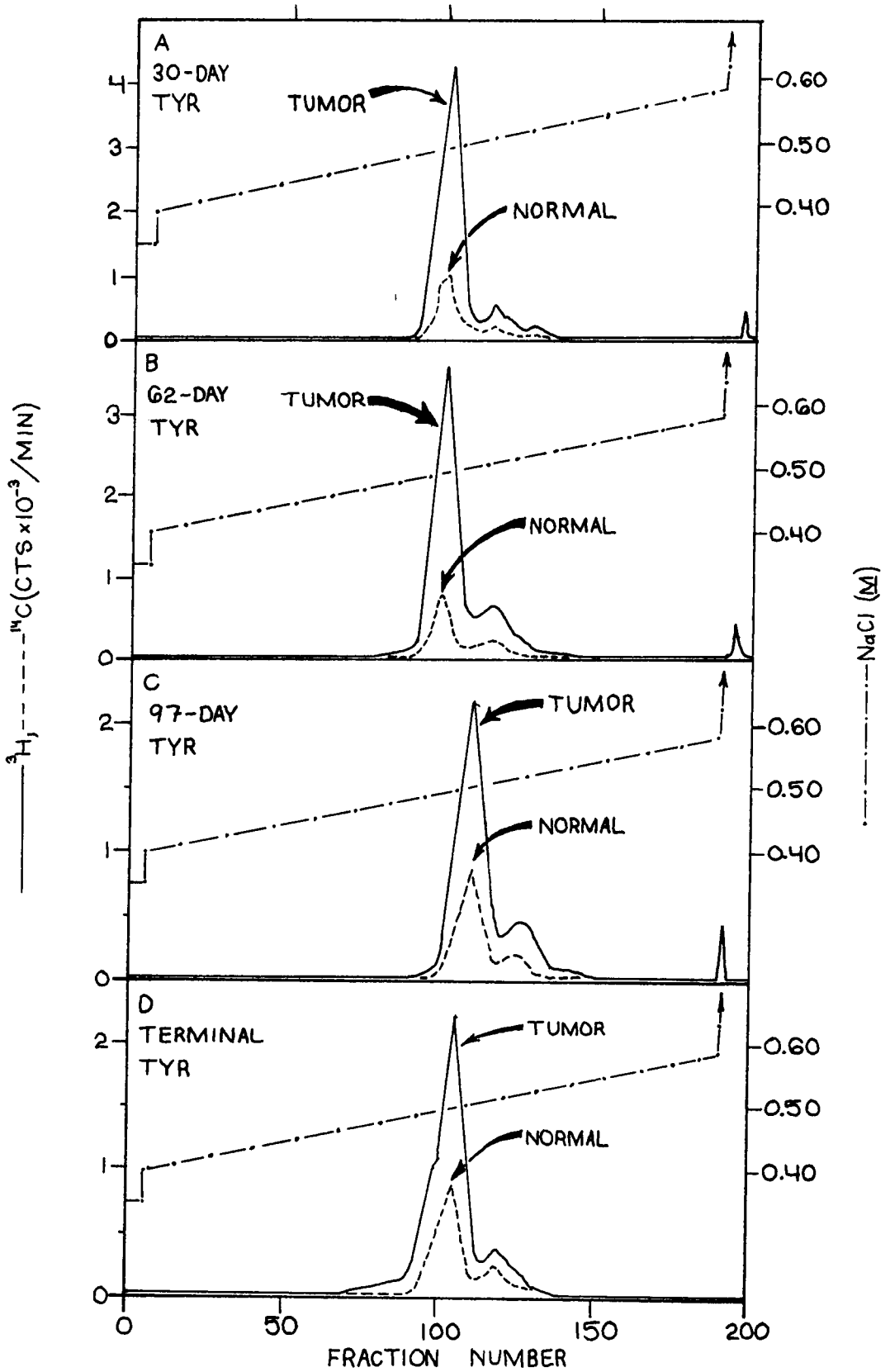

A
30-DAY
TYR
TUMOR
NORMAL
B
62-DAY
TYR
TUMOR
NORMAL
C
97-DAY
TYR
TUMOR
NORMAL
D
TERMINAL
TYR
TUMOR
NORMAL
0.60
0.50
0.40
0
50
100
150
200
FRACTION NUMBER
^{3}H, ----- ^{14}C (CTS x 10^{-3}/MIN)
—·—·— NaCl (M)

FIGURE 8.

this was a normal occurrence which was due to very slight experimental variations in the salt gradient and size of sample load but not reflective of differences in tRNA isoaccepting species.

CHAPTER IV

DISCUSSION

In examining the profiles of RPC-5 chromatographs of normal and terminal thymic lymphoma tRNAs it was found that differences occurred with the isoacceptors for two of the eight amino acids examined, phenylalanine and histidine. The isoacceptors for these two amino acids commonly show changes in different tumors[2,8-11] so that these results were not unexpected. A closer inspection was then made of the isoacceptor isoacceptor tRNAs for phenylalanine and histidine at various stages of tumor development. The changes were found to appear at various times of development and were not constant once they did occur since some would appear and then disappear before the terminal stage. Other species which were present in the normal tissues would disappear in the early stages of tumor development but would then re-emerge at the terminal stage. These odd occurrences never would have been detected if only the terminal tumor isoacceptor tRNAs were examined which is the case in most studies of this nature.

Previous to these experiments, differences in isoaccepting tRNA species were thought to be a process of gradual change in which the tumor isoaccepting species would slowly appear as the normal species gradually disappeared for those isoacceptors which did show change at the terminal stage of tumor development. This would have occurred at a rate equivalent to the overall rate of transformation of the normal cells to tumor cells and in fact would have been reflective of it. This

obviously proved not to be the case since species appeared and disappeared at different stages of tumor development. This disappearance and re-emergence of species is the most interesting and baffling occurrence seen in this study especially when it involved major peaks as in the case of phenylalanine.

By examining the profiles of tyrosine tRNAs at the various periods of tumor development after the final radiation treatment it was demonstrated that changes seen in phenylalanine and histidine tRNAs were not likely to be artifacts of any extraction or aminoacylation procedure since the same pyrosine profile was reproducible in all stages of development. This also tends to negate the possibility that the differences observed with the phenylalanine and histidine tRNAs were due to radiation sickness and not tumor development.

Since differences seen in isoaccepting tRNAs are usually reflective of differences in post-transcriptional modification of bases in the tRNAs, the changes seen here are probably reflective of the changes which occurred in the type of modifying enzymes present in the cells. There must have been continual changes in the spectra of modifying enxymes present in the developing tumors but it was odd that some may have been lost and then regained later. What may actually have been the case was that the production of certain modifying enzymes were stopped in early tumor development which led to the build-up of under-modified isoaccepting tRNAs which would have appeared in the chromatograms as increases in some of the peaks already present or new peaks. Then at a later stage of development the tumor cells began to manufacture their

own form of modifying enzymes. These would then have modified the bases of the under-modified isoacceptors to yield the finished species thus leading to the re-appearance of that species in the chromatography profile and the decrease or disappearance of the peak representing the under-modified form. Or alternatively, the new forms of modifying enzymes in the tumors could have modified the under-modified tRNAs to new forms which may have chromatographed the same as the old species even though they were different. This later explanation seems less likely since RPC-5 has been shown to differentiate very minor differences between isoacceptors most of the time.[16]

Of course these ideas have no firm support in this experiment but they are implied. Certainly the old theory of gradual change does not explain this phenomenon. Further experiments are needed to clarify this discrepancy. They would involve the isolation of the several species of isoacceptors for a specific amino acid, either phenylalanine or histidine, which appear at the various stages of tumor development. Each would need to be characterized to detect the exact differences between them.

It was unfortunate that this study was conducted using tRNAs extracted from thymuses. Because the cells of the thymus consist mostly of a nucleus surrounded by very little cytoplasm they are an excellent source of DNA but not tRNAs. Any tRNA preparation made from them inevitably contains a lot of contaminating DNA of differenr sizes. It is recommended that future experiments performed along the same lines be conducted using a different organ as a tRNA source so that much purer tRNA preparations can be made. This will become especially important in studies involving the isolation of specific isoacceptors.

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APPENDIX

APPENDIX

LIST OF ABBREVATIONS

DEAE-cellulose	Diethylaminoethyl-cellulose
tRNAs	Transfer ribonucleic acids
DTT	Dithiothreitol
RPC-5	Reversed-phase column chromatography type 5
DEPC	Diethyl-pyrocarbonate
TCA	Trichloroacetic acid
Tris	Tris(hydroxymethyl)amino-methane
EDTA	Ethylene-diamine-tetra-acetic acid
OD_{260}	Optical density of a sample measured at 260 nm with a 1.0 cm light path
OD_{280}	Same as OD_{260} but measured at 280 nm
OD_{600}	Same as OD_{260} but measured at 600 nm
Trizma Base	Same as Tris
SDS	Sodium dodecyl-sulfate
ATP	Adenosine triphosphate
BBOT	2,5-bis-2-(-5ert-butylbenzoxazolyl)-thiophene

VITA

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