antisera prepared against the isomerase do not cross-react with the decarboxylase, and we have shown that antisera prepared against the decarboxylase do not interact with the isomerase. In addition, the amino acid compositions of the two proteins (Table III) are quite different in some respects: the ratio of proline to glycine, for example, is 0.36 in the decarboxylase and 2.0 in the isomerase. Despite these dissimilarities, the two enzymes share more properties than can be readily ascribed to coincidence. Their amino termini are methionine, their apparent molecular sizes are about 93,000 daltons, and their unusually small subunit sizes are in the range from 12,000 to 13,000 daltons. The similarity of the crystalline structures of the enzymes (Figures 4 and 5) suggests that the multimeric proteins may possess similar tertiary and quaternary structures. It therefore appears likely that the two enzymes are the products of homologous structural genes. The validity of this proposal can be tested only by direct comparison of the primary structures of the proteins.

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Elevated Levels of Acceptor Activity of Hepatoma Transfer Ribonucleic Acid†

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ABSTRACT: It was originally observed that phenylalanyl-tRNA of hepatoma 5123 accepted 3- to 4-fold the amount of phenylalanine than isogenic liver tRNA. A comparison of the extents of aminoacylation of liver and hepatoma tRNAs for 15 other amino acids were compared. In 7 cases, the extent of hepatoma aminoacyl-tRNA formation was significantly greater than that for liver. This difference was less striking for another four amino acids, and the remainder gave essentially identical extents of acylation. No instances were found in which greater amounts of amino acid were incorporated into liver tRNA. Control experiments effectively eliminated several sources of artifact which might have accounted for these differences. Periodate oxidation experiments revealed that the ratio of periodate protected:potentially acylatable tRNA was the same in both tissues. It appears, therefore, that hepatoma 5123D contains, per microgram of tRNA, a greater number of tRNA molecules capable of amino acid acceptance than liver, and it is suggested that this may represent differential rates of tRNA processing and maturation in these tissues.

The observation of tumor-specific phenylalanyl-tRNAs in Morris hepatomas 5123C (Gonano *et al.*, 1971) and 5123D (Volkers and Taylor, 1971), and the suggestion from several laboratories (Strehler *et al.*, 1967; Ilan *et al.*, 1970; Kanabus and Cherry, 1971) that processes of differentiation might be accompanied by changes in aminoacyl-tRNA synthetases, led us to investigate whether the phenylalanyl-tRNA synthetases of hepatoma 5123D and rat liver had different properties. No differences were found between the liver and hepatoma enzymes (Ouellette, 1972); however, it was noted that equivalent amounts of tRNA from these tissues differed in their extents of phenylalanine acceptance. Regardless of whether the liver or hepatoma enzyme was used, the aminoacylation of hepatoma tRNA consistently yielded twice the amount of phenylalanyl-tRNA as did liver tRNA. This paper describes a study determining the general nature of this phenomenon, and reveals significant differences between the tRNA populations of these two tissues.

Materials and Methods

The source and maintenance of tissues and the preparation of total tRNA have previously been described (Ouellette, 1972).

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All aminoacyl-tRNA synthetase activities required in this study were found in the 50–75% ammonium sulfate saturated fraction from either tissue. The 0–85% ammonium sulfate saturated fraction employed in the CTP addition experiments were prepared in the same fashion, except proteins were precipitated by the addition of 55.9 g of solid ammonium sulfate/100 ml of solution.

Aminoacylation Assay. Acid-insoluble, radioactive aminoacyl-tRNA was measured by a modification of the tRNA esterification assay (Mans and Novelli, 1961). Sample aliquots of 50 μl were removed from reactions and pipetted onto 2.3-cm diameter Whatman No. 3 filter paper disks, after which the disks were immersed in at least 20 ml/disk of ice-cold 10% (w/v) trichloroacetic acid. Disks were then washed twice with similar volumes of both 5% trichloroacetic acid and ethanol, dried, and counted using a scintillation cocktail containing 6 g of 2,5-diphenyloxazole (New England Nuclear, Boston, Mass.) per l. of toluene.

Radiochemicals. The following radioactive amino acids were purchased from New England Nuclear: L-[^{14}C]lysine (254 Ci/mol), L-[^{14}C]phenylalanine (384 Ci/mol), L-[^{14}C]serine (125 Ci/mol), L-[^{14}C]threonine (170 Ci/mol), L-[^{14}C]tyrosine (446 Ci/mol), L-[^{14}C]arginine (255 Ci/mol), L-[^{14}C]glutamic acid (197 Ci/mol), L-[^{14}C]glutamine (218 Ci/mol), L-[^{14}C]glycine (87.4 Ci/mol), L-[^{14}C]histidine (255 Ci/mol), L-[^{14}C]proline (208 Ci/mol), L-[^{3}H]leucine (55 Ci/mol), L-[^{3}H]isoleucine (1.65 Ci/mol), L-[^{3}H]methionine (4.02 Ci/mol), L-[^{3}H]tryptophan (5.17 Ci/mol, and L-[^{3}H]valine (2.53 Ci/mol).

A uniformly labeled [^{14}C]protein hydrolysate (54 Ci/atom of carbon) was purchased from Amersham/Searle, Arlington Heights, Ill. 60005.

Determination of Extents of Aminoacylation. Reaction mixtures (105-μl) containing 20 μmol of sodium cacodylate (pH 7.4), 2 μmol of magnesium acetate, 1 μmol of KCl, 0.68 mmol of either hepatoma 5123D tRNA or liver tRNA, 2.0 μmol of ATP, and different amounts of specific amino acids were incubated, in the presence of enzyme, for 20 min at 37°. Desalted 50–75% ammonium sulfate precipitates of either liver or hepatoma high-speed supernatants served as the enzyme source. In every case, the extent of amino acid incorporation was found to be independent of the enzyme source. Aliquots of 50 μl were removed from reaction mixtures, and acid-insoluble, radioactive aminoacyl-tRNA was measured as described above.

Analytical RNA determinations were done according to the procedure of Fleck and Begg (1954) as modified by Bernstein (1970).

The association of ribonuclease (RNase) activity with tRNA preparations was monitored by the procedure of Littauer (1971).

Sephadex Chromatography of Rat Liver tRNA. A 1.0 × 35 cm Sephadex G-25 (Pharmacia Fine Chemicals, Piscataway, N. J.) column was equilibrated with distilled water. Fractions (0.75 ml) were collected and assayed for absorbance at 260 nm.

A 2.5 × 75 cm Sephadex G-200 column was equilibrated with TM buffer containing 0.01 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$. A 1.0-ml volume of liver tRNA (89 A_{260} units/ml) was applied to the column, followed by elution with TM buffer. Fractions of 3.0 ml were collected, monitored for absorbance at 260 nm, and assayed for phenylalanine acceptor activity.

Periodate Oxidation of Liver and Hepatoma 5123D tRNAs. The *in vivo* ratio of aminoacylated:unacylated tRNA was estimated in these tissues by a modification of the method of Yegian *et al.* (1966). Tissue (15–20 g) was minced thoroughly and taken up in 150 ml of 0.10 M sodium acetate buffer (pH 5.0), containing 50 μg/ml of bentonite. The tissue was homogenized for 3 min in an equal volume of acetate-buffered saturated phenol. This preparation was then exhaustively phenol extracted at pH 5.0, chloroform extracted, and alcohol precipitated at −20° with 2.5 volumes of 95% ethanol. The total nucleic acids were dissolved in 0.10 M acetate buffer (pH 5.0) and diluted with buffer to a concentration of 75.0 A_{260} units/ml. Then 75.0 A_{260} units each of liver and hepatoma 5123D total nucleic acids were exposed, in darkness, to 0.20 ml of 0.10 M sodium metaperiodate at 25° for 40 min. Following this oxidation period, the nucleic acid solution was made 10% with respect to glycerol and incubated an additional 10 min in order to exhaust the periodate. NaCl was then added to a final concentration of 0.25 M and the nucleic acids were precipitated at −20° overnight with 2.5 volumes of 95% ethanol. Nucleic acids were collected by centrifugation, redissolved in acetate buffer at pH 5.0, and reprecipitated. This washing procedure was repeated three times, and tRNA was then isolated from the total nucleic acids as previously described.

The extent of phenylalanyl-tRNA formation was determined for oxidized tRNA and unoxidized controls.

Results

Preliminary experiments had determined the level of radioactive amino acids to be the limiting factor in measuring the extent of aminoacyl-tRNA formation. Therefore, aminoacyl-tRNA formation was measured by increasing the concentration of amino acid until further addition of the amino acid failed to appreciably increase the extent of incorporation. While absolute maximal extents were not determined in each case, the method used and values obtained suit the comparative nature of these experiments.

The tRNAs of liver and hepatoma were compared, in this fashion, with regard to their extents of specific amino acid incorporation. Table I contains these values for the 16 amino acids employed and for a [^{14}C]protein hydrolysate. In the case of the first eight aminoacyl-tRNAs in Table I, the hepatoma tRNAs are acylated to a significantly greater extent than the homologous tRNAs of liver. For the remaining amino acids, the differences in the levels of aminoacylation are not as great, but it is most important to note that *no* instances were found in which greater amounts of an amino acid were incorporated into liver tRNA. The values obtained using the [^{14}C]protein hydrolysate reinforce this point, and this value is very close to the mean value obtained for the 16 amino acids.

Before the above data could be interpreted in a meaningful manner, certain control experiments were necessary to establish that these observations were not the result of some artifact. The following sets of experiments were designed to test this possibility, and none have revealed a trivial explanation for the acylation data.

Relative to the hepatoma, rat liver synthesizes large amounts of glycogen. Since the tRNA in the above experiments was quantitated by absorption of light at 260 nm, turbidity due to the presence of glycogen could cause the RNA concentration to appear higher than actual. Therefore, it was important to determine the RNA content of the tRNA preparations by another method. The technique selected was a modification of the alkaline hydrolysis method of Fleck and Begg (1954). The liver and hepatoma tRNA preparations contained equivalent amounts of RNA per A_{260} unit.

The possibility of ribonuclease (RNase) contamination of the liver and hepatoma tRNAs was examined by the method

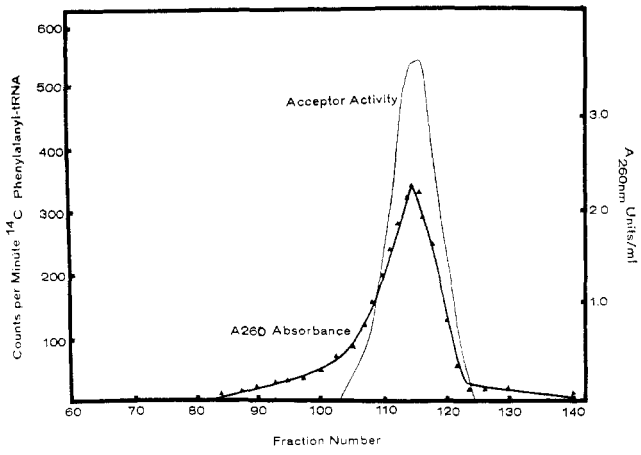

FIGURE 1: Sephadex G-200 column chromatography of buffalo rat liver total tRNA. A 2.5 × 75 cm column was equilibrated with 0.01 M Tris-HCl (pH 7.5)–0.01 M $MgCl_2$. Liver tRNA (89.0 A_{260} units) was added, and the column was eluted with the same buffer. Fractions of 3.0 ml were collected, measured for absorbance at 260 nm, and assayed for incorporation of phenylalanine into acid-insoluble material.

of Littauer (1971). tRNA samples were incubated under alkaline conditions over chloroform for 18 hr at 37°. Phenylalanine acceptance was then determined for the incubated samples and unincubated controls. Neither liver nor hepatoma tRNAs contained appreciable or significant levels of ribonuclease.

All those tRNAs sequenced to date have been shown to contain a common pCpCpA-OH 3′ terminus (Lengyel and Söll, 1969), and the integrity of this 3′ terminus is required for amino acid acceptance. The enzyme CTP (ATP) tRNA nucleotidyltransferase exists in a wide number of cell types and is responsible for turnover and/or repair of the CpCpA-OH terminus in deficient tRNA molecules. This enzyme has been extensively purified and studied in *Escherichia coli* (Carre *et al.*, 1970), and it has been found in 55–85% ammonium sulfate saturated fractions from rat liver (Herbert and Canellakis, 1963). The enzyme is active under the conditions of the aminoacylation assay (Herrington and Hawtrey, 1970) and the addition of CTP to reaction mixtures provides conditions suitable to the repair of tRNAs deficient at the CpCpA-OH terminus. The addition of CTP to reaction mixtures, and therefore the repair of deficient pCpCpA-OH termini, does not significantly increase the extent of liver phenylalanyl-tRNA formation, much less increase it to the level obtainable with hepatoma tRNA.

Bosch *et al.* (1960) have shown that tRNA prepared from rat liver may contain short oligoribonucleotides, and that these molecules, due to their insolubility in alcohol, remain with the tRNA fraction throughout the purification procedure.

Since the presence of these oligonucleotides might have caused the liver tRNA concentration to appear higher than actual, it was important for proper interpretation of the acylation data to exclude the possibility of such contamination. In the same vein, it was equally necessary to determine whether or not the liver tRNAs were contaminated significantly with either 28S or 18S rRNAs. These two possibilities were examined by chromatographing liver tRNA on Sephadex G-25 and Sephadex G-200. The liver tRNAs under study consisted almost entirely of a 4S population corresponding directly with the peak of amino acid acceptor activity. There was a slight amount of contamination in the 4–5S range, and this is taken to be precursor tRNA or 5S rRNA (Figure 1). It seems highly unlikely, however, that this small amount of pre-tRNA could account for the great difference observed in the extents of aminoacylation. We conclude from the above data, therefore, that the observed differences in aminoacyl-tRNA formation reflect a genuine difference between the 4S RNA populations of liver and hepatoma 5123D.

In view of the differences found *in vitro* between the tRNA populations of liver and hepatoma, an effort was made to gain an indication of the nature of the tRNAs *in vivo*. The exposure of a mixture of acylated and unacylated tRNA molecules to sodium metaperiodate results in the selective oxidation of the 2′- and 3′-OH functions of the terminal adenosine moiety of the uncharged tRNAs, whereas the corresponding hydroxyl groups of acylated tRNAs are protected from such oxidation by aminoacyl-tRNA ester linkage. Therefore, following extractions of nucleic acids under conditions which maintain the aminoacyl-tRNA ester, one may expose the

TABLE I: Extent of Aminoacylation of Buffalo Rat Liver and Hepatoma 5123D tRNAs.[a]

Amino Acid	pmol of Amino Acid Incorp/A_{260} Unit of tRNA: Buffalo Rat Liver tRNA	Hepatoma 5123D tRNA	Ratio of 5123D/liver
Lysine	26.64	37.55	1.41
Phenylalanine	21.40	45.40	2.12
Serine	44.71	74.50	1.67
Tyrosine	12.40	21.18	1.72
Arginine	46.26	68.20	1.47
Histidine	17.24	24.31	1.41
Isoleucine	29.75	54.21	1.82
Methionine	43.69	60.41	1.38
Threonine	34.25	40.00	1.17
Glutamic acid	26.68	31.55	1.18
Glutamine	22.65	26.10	1.15
Glycine	33.73	32.10	0.95
Proline	20.00	20.00	1.00
Leucine	12.45	15.74	1.26
Tryptophan	24.30	23.85	0.98
Valine	45.31	47.70	1.05
[^{14}C]Protein hydrolysate	13,750[b]	20,100[b]	1.45

[a] Reactions were incubated for 20 min at 37°, and catalyzed by addition of a 0–80% ammonium sulfate saturated fraction from liver. Previous data indicated the extent of acylation to be the same whether liver or hepatoma enzymes were used to catalyzed the reaction (Ouellette, 1972). Radioactive aminoacyl-tRNA was determined by the filter disk assay. All reaction mixtures contained 20 μmol of sodium cacodylate (pH 7.4), 2 μmol of magnesium acetate, 1 μmol of KCl, 0.68 μmol of either liver or hepatoma total tRNA, and 2.0 μmol of ATP. The following amounts of amino acids were added to their respective reaction mixtures: 1.97 nmol of lysine, 1.07 nmol of phenylalanine, 4.0 nmol of serine, 1.125 nmol of tyrosine, 1.96 nmol of arginine, 1.96 nmol of histidine, 1.52 nmol of isoleucine, 1.25 nmol of methionine, 2.94 nmol of threonine, 2.47 nmol of glutamic acid, 2.29 nmol of glutamine, 5.72 nmol of glycine, 2.40 nmol of proline, 0.875 nmol of leucine, 0.97 nmol of tryptophan, and 1.00 nmol of valine. [b] Cpm/A_{260} unit of tRNA.

nucleic acids to periodate and estimate the level of intracellularly charged tRNA as a function of the degree of protection from periodate oxidation. This percentage of periodate protection was determined in liver and hepatoma 5123D, and, as shown in Table II, the ratios of protected tRNA:potentially acylatable tRNA are very similar. Therefore, while the degree of periodate protection may be the same in both tissues, the aminoacylation data clearly indicate that the amount of acylatable tRNA is greater, per microgram of 4S RNA, in the hepatoma.

TABLE II: Periodate Oxidation of Liver and Hepatoma 5123D Total tRNAa.[a]

tRNA Sample	pmol of Phe Incorp/A_{260} Unit	% Periodate Protection
Control liver tRNA	29.53	80.71
Oxidized liver tRNA	23.84	
Control hepatoma tRNA	50.24	76.05
Oxidized hepatoma tRNA	38.18	

[a] Thoroughly deproteinized total nucleic acids from both tissues were oxidized at pH 5.0 with 0.10 M sodium metaperiodate. After 40 min at 25°, the nucleic acids were washed and tRNAs were isolated by the procedure previously described (Ouellette, 1972). The maximal extent of phenylalanine incorporation into oxidized controls was then determined by the usual procedure.

Discussion

The experiments described above indicate that hepatoma 5123D contains significantly greater amounts of acylatable tRNA than does liver. For the 16 amino acids investigated, no instances were found in which the liver tRNAs were acylated to a greater extent. In addition several control experiments were conducted to determine the validity of these data and to insure that they did not result from some artifact. Since these control experiments failed to reveal any source of artifact, we conclude that our observations reflect a genuine difference between the tRNA populations of isogenic liver and the hepatoma.

Although efforts to find a trivial explanation for these data have failed, such a possibility remains open. Variability due to individual animals was eliminated by preparing tRNAs from the tissues of 5–8 animals at one time. In addition, all tissues were handled in parallel, thereby minimizing variability arising from the isolation procedure. Furthermore, the acylation data were found to be reproducible between individual batches of tRNA. A measure of this reproducibility may be seen in Table II, where each set of data (oxidized sample and unoxidized control) represents duplicate determinations on individual batches of tRNA.

Evidence from other laboratories suggests that the differences in extent of acylation which we have observed do not represent an isolated phenomenon. For example, tRNAs from the aging nematode, *Turbatrix aceti*, have been shown to be charged to only one-half the extent of tRNAs from young animals (Reitz and Sanadi, 1972). Similarly, evidence of a preliminary nature consonant with the data we have described above has been reported in a comparison of Novikoff hepatoma and rat liver (Ritter and Busch, 1971; E. Borek, personal communication).

Given the data we have described, the question becomes one of understanding their biological significance. If, indeed, the greater amounts of acylatable tRNA in the hepatoma resulted in or resulted from the increased growth rate of the tumor, then regenerating rat liver should exhibit a similar increase in amount of chargeable tRNA. Agarwal *et al.* (1970) examined the maximal extent of amino acid incorporation by tRNAs of regenerating rat liver and livers of sham-operated animals. In contrast to our data, no differences were detected in tRNA acceptor activity between the growing and nongrowing tissue. These data imply, therefore, (a) that an increase in the amount of acylatable tRNA is not required for increased growth, and (b) that the greater amount of acylatable tRNA in the hepatoma is not simply the result of the hepatomas' greater growth rate.

Considerable evidence is now being advanced which indicates that tRNA may have nontranslational regulatory functions such as in feedback inhibition (Blasi *et al.*, 1971), in feedback repression (Singer *et al.*, 1972), and in enzyme inhibition in *Drosophila* mutants (Jacobson, 1971). While findings of this nature have yet to be reported in mammalian systems, the possibility exists that mammalian tRNAs may have similar functional roles. If it were the case that more liver aminoacyl-tRNA acted in this fashion than in the hepatoma, a larger proportion of acylatable liver tRNA could be lost during the deproteinization step of the tRNA isolation procedure due to its association with protein. This possibility has been examined in our laboratory, and no acylatable tRNA has been recovered by repeated extraction of either the phenolic phase or the aqueous: phenolic interface (R. M. Kothari and M. W. Taylor, unpublished data). There are undoubtedly other aspects of cellular metabolism which would be affected by the situation these data describe, but until further evidence is brought to light, we conclude that a greater quantity of intracellular aminoacyl-tRNA may not be of great biological significance, *per se*, but rather indication of a fundamental alteration in a cell's macromolecular biosyntheses.

It is known from the data of Bernhardt and Darnell (1969), Burdon and Clason (1969), and Choe and Taylor (1972) that mammalian tRNAs are synthesized as 4–5S pre-tRNA molecules, and that these precursors are processed by unknown mechanisms to the active 4S configuration. In addition, a number of enzymes exist which modify specific nucleotides within tRNA molecules (Söll, 1971), and, although the function of these enzymes is not understood, they are known to utilize RNAs as substrates. Presumably, these tRNA modifying enzymes play a role in the maturation process. The most extensively studied enzymes in this category are the tRNA methyltransferases (Srinivasan and Borek, 1966), and studies from several systems indicate that the specific activities of these methylases are greater in tumors than in their control tissues (Craddock, 1969, 1970). More specifically, Kerr has shown that the 4S RNAs of hepatoma 5123D are methylated to a greater extent than those of liver and that the relative frequency of methylated bases differs between hepatoma 5123 and liver (S. Kerr, personal communication). In addition, Kerr has described an inhibitor of tRNA methylases (Kerr, 1972) which is present in normal cells but absent in tumors, including Morris hepatomas 5123D (S. Kerr, personal communication) and 5123C (Kerr, 1971). This is in concert with the "molecular correlation" concept of Weber and Lea (1966, 1967) which hypothesizes that tumors generally have derepressed biosynthetic capacities while the enzymes of catabolic pathways exhibit lower specific activities.

Interpreting our data on the different extents of aminoacylation in light of the observations on tRNA methylases and differences in methylation patterns we would suggest the following model: the processing and maturation of transfer RNA are tightly controlled in the liver, and that in the steady-state condition there is a constant amount of precursor tRNA relative to the amount of tRNA having acceptor activity. In the hepatoma, the maturation processes may be reregulated such that, in the steady state, there is much less precursor relative to the amount of fully processed tRNA. This prediction is consistent with the data of Weber and Lea (1966, 1967) and the observations on the tRNA methylases, and provides an explanation for the differences in extent of aminoacylation.

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