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Chromatographic Comparison of the Transfer Ribonucleic Acids of Rat Livers and Morris Hepatomas*

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ABSTRACT: tRNAs from hepatomas 5123D and 3924A have been aminoacylated and compared with the corresponding aminoacyl-tRNA from normal rat liver by cochromatography on reversed-phase columns. The iso-accepting species of Leu-, Lys-, and Tyr-tRNA from both hepatomas cochromatographed at approximately the same salt concentrations as the corresponding species from normal liver. The Ser-, Phe-, and His-tRNAs from hepatoma 3924A also showed no deviation from normality. Hepatoma 5123D exhibited two more Ser-tRNA species, one more Phe-tRNA species, and two fewer His-tRNA species than Buffalo rat liver. These chromatographic alterations appeared to be due to new or altered species of tRNA. They were not due to differences in the aminoacyl-tRNA synthetases, nor were they aggregates of the tRNA. The altered tRNAs were a property of the malignant cells, and were not due to an alteration in the environment or to their increased growth rate.

Alterations in the tRNAs of systems undergoing, or having undergone, changes in metabolic control processes have been reported to occur in numerous systems. These alterations have recently been the subject of an excellent review (Sueoka and Kano-Sueoka, 1970). The tRNAs of neoplastic tissues have been examined by several workers. Axel *et al.* (1967) reported differences in hepatic tumor tRNAs induced by ethionine feeding. Taylor *et al.* (1967, 1968) studied the methylated albumin kieselguhr elution profiles of the tRNAs from a number of sources. They reported that most of the aminoacyl-tRNAs studied showed a remarkable similarity. However, differences in the profiles of a number of aminoacyl-tRNAs, particularly Tyr-tRNA, from a variety of tumors as compared with the corresponding normal tissues were observed. In addition to this, the Leu-tRNAs of mouse plasma cell tumors have been shown to elute differently from reversed-phase columns as compared with the Leu-tRNAs from liver cells (Mushinski and Potter, 1969). Further work with mouse plasma cell tumors (Yang and Novelli, 1968) has indicated that a tumor which produces immunoglobulin A contains an extra species of Ser-tRNA as compared with tumors producing immunoglobulin G. A comparison of the tRNAs from Novikoff hepatoma with the corresponding tRNAs from normal rat liver has been carried out by two groups. Baliga *et al.* (1969) compared the elution of eighteen aminoacyl-tRNAs from methylated albumin keiselguhr columns, and reported that new species of His-, Tyr-, and Asp-tRNA were present in the hepatoma. No differences were observed in the Phe-tRNA. However, Goldman *et al.* (1969) reported that, using reversed-phase chromatography, only two Phe-tRNA peaks were observed in the hepatoma, while the rat liver Phe-tRNA appeared to contain three iso-accepting species. Gonano and Chiarugi (1969) Gonano *et al.* (1971); examined several tRNAs from the Morris hepatoma 5123, and reported the presence of a new species of Phe-tRNA in the hepatoma as compared with normal rat liver.

In an attempt to distinguish essential changes in tRNAs which may be connected with the carcinogenic process from nonessential changes brought about in tRNAs as a result of carcinogenesis, we have initiated a comparison of the tRNAs of three rat hepatomas, namely, 9618A, 5123D, and 3924A. Hepatoma 9618A is an extremely slow-growing tumor which is morphologically and histologically very similar to normal hepatocytes, and which contains the normal complement

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of chromosomes; hepatoma 5123D, which has an intermediate growth rate, possesses 45 chromosomes, and is less well differentiated than hepatoma 9618A; hepatoma 3924A is a fast-growing, poorly differentiated tumor which possesses 73 chromosomes. Detailed descriptions of these tumors have been published elsewhere (Nowell *et al.*, 1967; Morris and Wagner, 1968). A comparison of six aminoacyl-tRNAs from hepatomas 5123D and 3924A with the corresponding aminoacyl-tRNAs from normal rat liver is described below.

Materials and Methods

Materials. Buffalo rats bearing hepatoma 5123D and ACI rats bearing hepatoma 3924A were kindly supplied by Dr. H. P. Morris, Howard University, Washington, D. C. The tumors were maintained by serial transplantation into rats of the appropriate strain (Microbiological Associates, Bethesda, Md.). When the hepatomas were required for experimental purposes, the rats were decapitated, the tumors were removed and, either used immediately, or stored at −70°. Hepatoma 3924A was removed after 25 days, and hepatoma 5123D after 40 days. Normal livers were removed from decapitated rats and treated in the same way as the tumors.

All radioactive amino acids were obtained from New England Nuclear Corp., Boston, Mass. They had the following specific activities: [^{14}C]L-histidine, 256 mCi/mmole; [^{3}H]L-histidine, 6.25 Ci/mmole; [^{14}C]L-leucine, 240 mCi/mmole; [^{3}H]L-leucine, 58.2 Ci/mmole; [^{14}C]L-lysine, 254 mCi/mmole; [^{3}H]L-lysine, 4.38 Ci/mmole; [^{14}C]L-phenylalanine, 375 mCi/mmole; [^{3}H]L-phenylalanine, 8.3 Ci/mmole; [^{14}C]L-serine, 125 mCi/mmole; [^{3}H]L-serine, 3.79 Ci/mmole; [^{14}C]L-tyrosine, 379 mCi/mmole; [^{3}H]L-tyrosine, 31.6 Ci/mmole. Acid-washed Chromosorb W (mesh size 100–120; Lot Number 306) was obtained from Johns Manville Products, Maryland Heights, Mo. Aliquat 336 was supplied by General Mills, Kankakee, Ill., while Freon 214 was purchased from Peninsular Chemresearch, Gainesville, Fla.

Preparation of tRNA and Aminoacyl-tRNA Synthetases. Transfer RNA was prepared by a slight modification of the method of Taylor *et al.* (1968). The minced tissues were homogenized in 0.01 M Tris-HCl buffer, pH 7.5. The homogenate was made 1% with respect to sodium dodecyl sulfate, and an equal volume of buffer-saturated phenol was added. The aqueous layer was separated from the phenolic layer by centrifugation, and reextracted with phenol. After being extracted with chloroform, the solution was supplemented with NaCl to a final concentration of 0.2 M. The nucleic acids were precipitated by the addition of 2.5 volumes of 95% ethanol, and the mixture was left overnight at −20°. The precipitate was redissolved in Tris-HCl buffer, pH 7.5, containing 0.01 M $MgCl_2$ and incubated with electrophoretically pure DNase (Sigma) for 1 hr at 37°, phenol extracted, and ethanol precipitated. The precipitate was stirred with 1 M NaCl for 2 hr at 4°. The tRNA was removed from the insoluble rRNA by centrifugation, ethanol precipitated, treated for 1 hr at 37° with 0.01 M Tris-HCl buffer, pH 8.0, to remove any amino acids attached to the tRNA, and again ethanol precipitated. The precipitate was dissolved in water and stored at −70°. Approximately 30–40 mg of tRNA were obtained per 100 g wet weight of the original tissue.

Crude aminoacyl-tRNA synthetase preparations were obtained from tissue homogenates by passing a 10^5g supernatant through a Sephadex G-100 column and collecting the void volume, as described previously (Taylor *et al.*, 1968).

Preparation of Aminoacyl-tRNAs. Labeled aminoacyl-tRNAs were prepared as follows. A reaction mixture containing 1.0 μmole of radioactive amino acid, 0.25 mg of tRNA, 20 μmoles of sodium cacodylate buffer, pH 7.4, 0.2 μmole of ATP, 1.0 μmole of KCl, 2.0 μmoles of magnesium acetate, and the aminoacyl-tRNA synthetase preparation (1.0 mg of protein) in a final volume of 0.2 ml, was incubated for 15 min at 37°. The reaction was terminated by the addition of 1.0 ml of sodium acetate buffer (0.05 M; pH 5.0) containing 0.14 M NaCl. After deproteinization with buffer-saturated phenol, the aminoacyl-tRNA was precipitated three times with 2.5 volumes of ethanol, and dissolved in standard buffer (0.01 M sodium acetate buffer, pH 4.5, containing 0.01 M magnesium acetate and 0.001 M EDTA) supplemented with 0.35 M NaCl.

Chromatographic Procedures. The column packings were prepared by coating Chromosorb W with a solution of Aliquat 336 in Freon 214 using the method described by Weiss and Kelmers (1967). The coated packing was made into a slurry with standard buffer containing 0.35 M NaCl and stored at −20°.

Columns (225 × 1 cm) of the coated Chromosorb W were poured and washed with 1.0 l. of standard buffer containing 0.35 M NaCl. The column was maintained at a temperature of 20°. Mixtures of the labeled aminoacyl-tRNAs were applied to the column and eluted with a gradient (2.0 l.) of 0.35–0.55 M NaCl in standard buffer. The elution rate was 1.0 ml/min, and 10-ml fractions were collected. Yeast tRNA (100 μg) was added to each fraction, and the tRNA was precipitated by the addition of 5 ml of cold 30% trichloroacetic acid. After standing in the cold for 30 min, the precipitates were collected on Millipore filters (Type HA, pore size 0.45 μ), washed with 10% trichloroacetic acid, dried for 30 min at 60°, and counted in a Beckmann scintillation counter to an error of 2% using a toluene-based 2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene cocktail.

Results

Transfer RNAs isolated from hepatoma 3924A or hepatoma 5123D were aminoacylated with [^{14}C]amino acids using homologous enzyme preparations, and cochromatographed using RPC2[1] with [^{3}H]aminoacyl-tRNAs from normal liver prepared in a similar manner.

Transfer RNAs specific for six amino acids were compared. The elution profiles of three of these, namely Leu-, Lys-, and Tyr-tRNA, which show little or no differences, are shown in Figure 1. The Leu-tRNAs (a and b) from all the sources show very similar patterns having one major species and two minor species. Two large peaks, and one small peak, of Lys-tRNA activity may be distinguished (c and d). The first Tyr-tRNA peak was sometimes rather badly defined, appearing as a shoulder in some cases. However, as can be seen in Figures 1e and f, it is present in all tissues examined, as are two other Tyr-tRNA species.

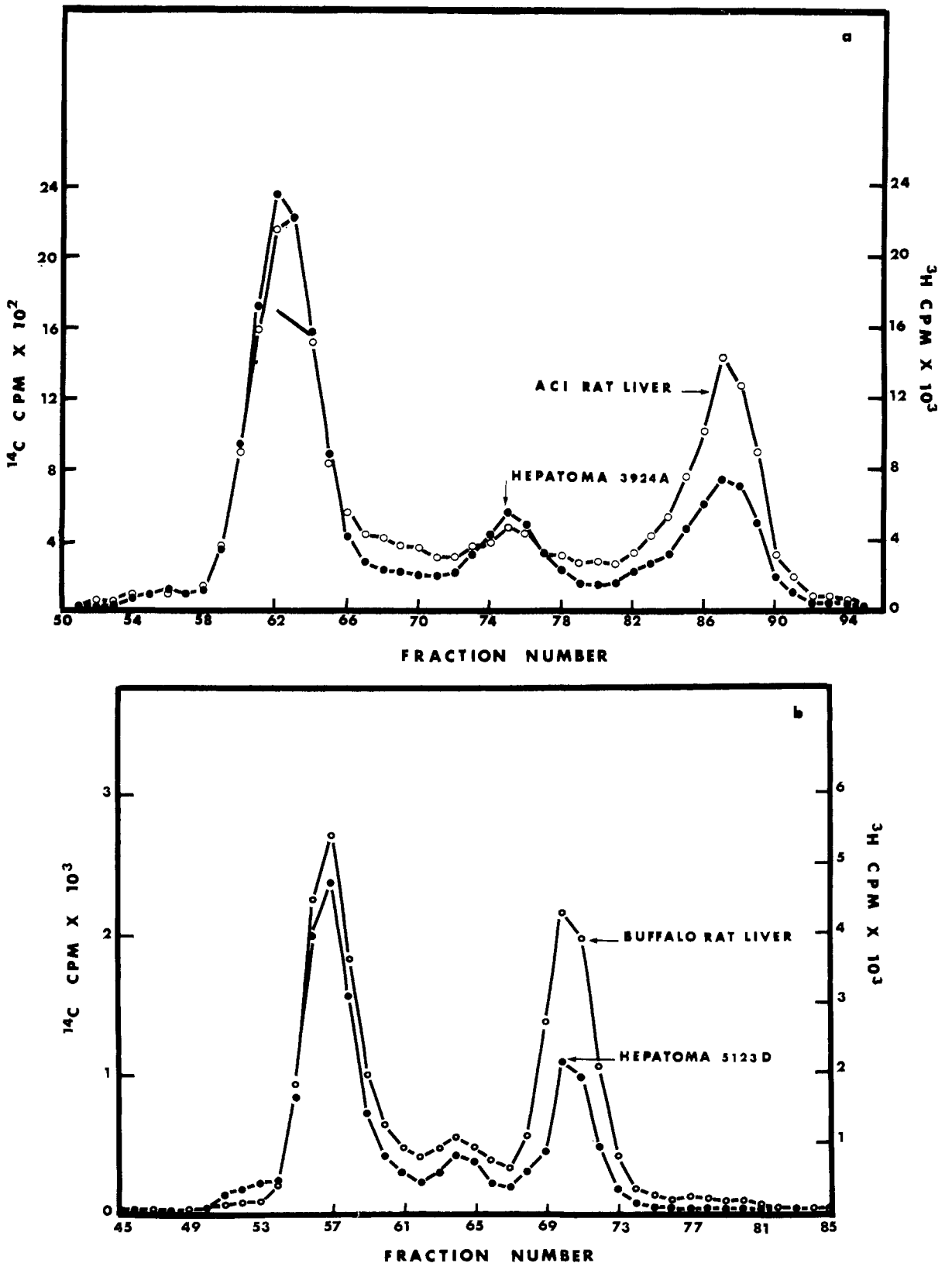
Figure 2 shows the chromatographic patterns of Ser-tRNA from a number of sources. It can be seen that Ser-$tRNA_{Buff}$ exhibits two iso-accepting species, which are present in roughly the same amounts. Ser-$tRNA_{5123D}$ also contains these

[1] Abbreviations used are: RPC 2, reversed-phase chromatography type 2; $tRNA_{Buff}$, $tRNA_{ACI}$, $tRNA_{5123D}$, $tRNA_{3924A}$, tRNA obtained from Buffalo rat liver, ACI rat liver, hepatoma 5123D, and hepatoma 3924A, respectively.

two species, although the Ser-tRNA eluting at the lower salt concentration is present in very small amounts. In addition to these two species, Ser-tRNA$_{5123D}$ shows two further peaks, one large and the other small. No differences can be observed in the elution patterns of Ser-tRNA$_{3924A}$ and Ser-tRNA$_{ACI}$, each tRNA exhibiting two iso-accepting species.

An extra species of the Phe-tRNA$_{5123D}$ as compared with Phe-tRNA$_{Buff}$ can be seen in Figure 3. Again, the Phe-tRNA$_{3924A}$ shows the same elution profile as the Phe-tRNA$_{ACI}$.

Figure 4 shows the chromatographic profiles of His-tRNA from all four sources. Two of the species exhibited by His-tRNA$_{Buff}$ appear to be missing, or present in reduced amounts,



(Figure 1, pp 490 to 492)

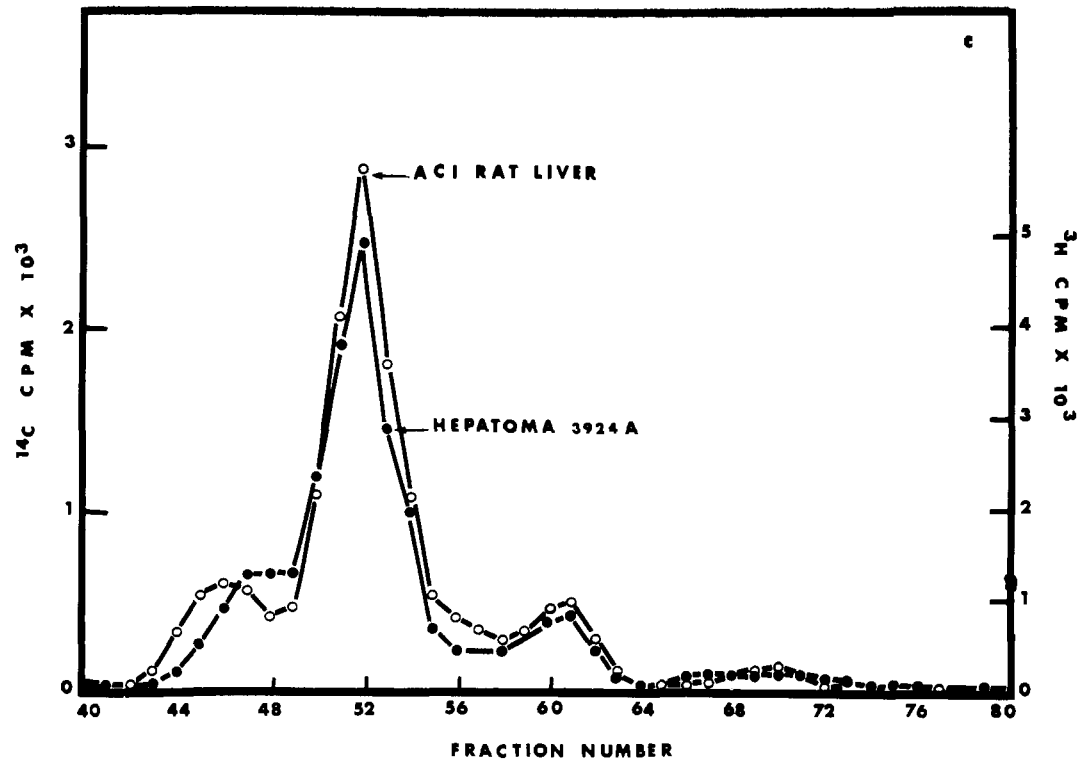
in the His-tRNA$_{5123D}$ profile. These species can still be observed in the profiles of His-tRNA$_{3924A}$ and His-tRNA$_{ACI}$.

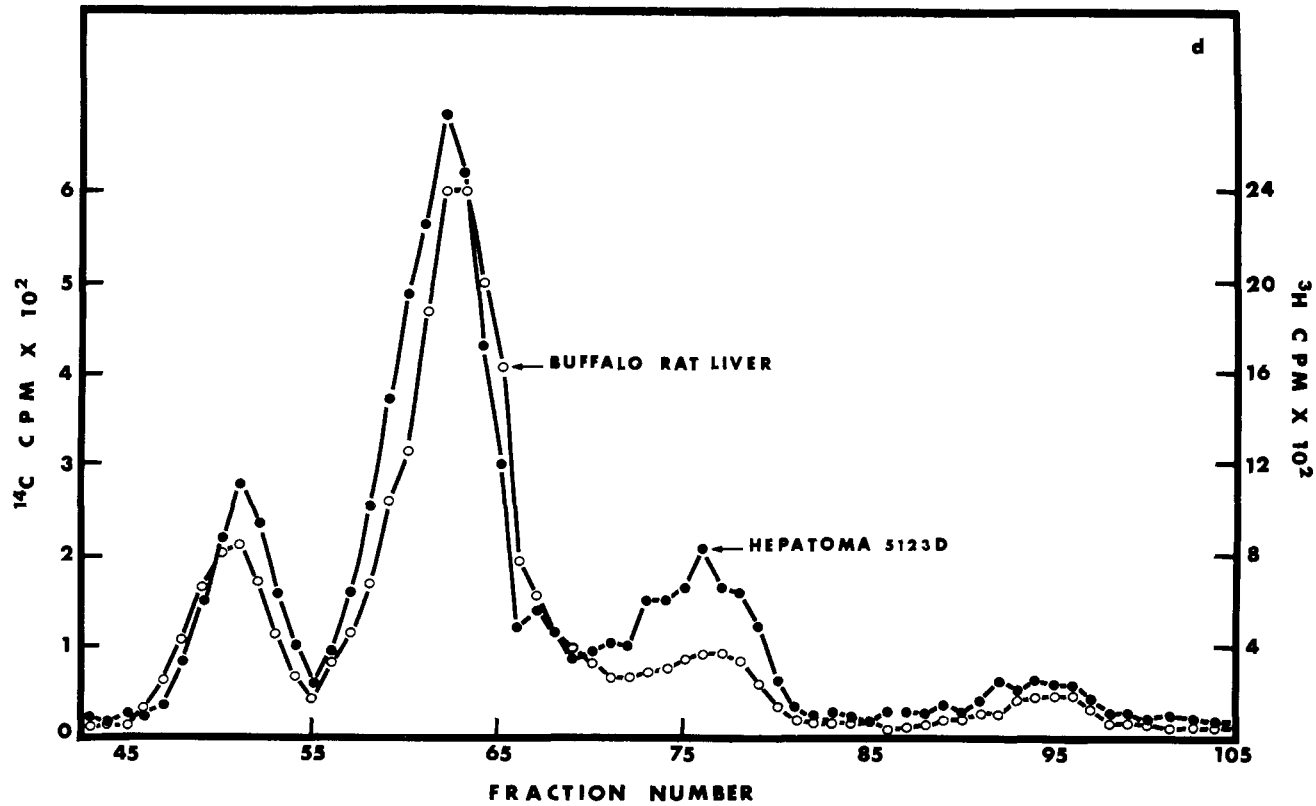
The numbers of iso-accepting species of tRNA observed in the two hepatomas as compared with those detected in rat liver are summarized in Table I.

Any differences which were observed have been authenticated by reversing the enzymes used to aminoacylate the tRNA, reversing the labeled amino acid, and by heating the tRNA to 80° for 1 min prior to aminoacylation. The latter procedure dissociates any tRNA aggregates which may have formed on standing (Adams and Zachau, 1968).

Discussion

The data presented in this communication indicate that multiple iso-accepting species of specific tRNAs exist in





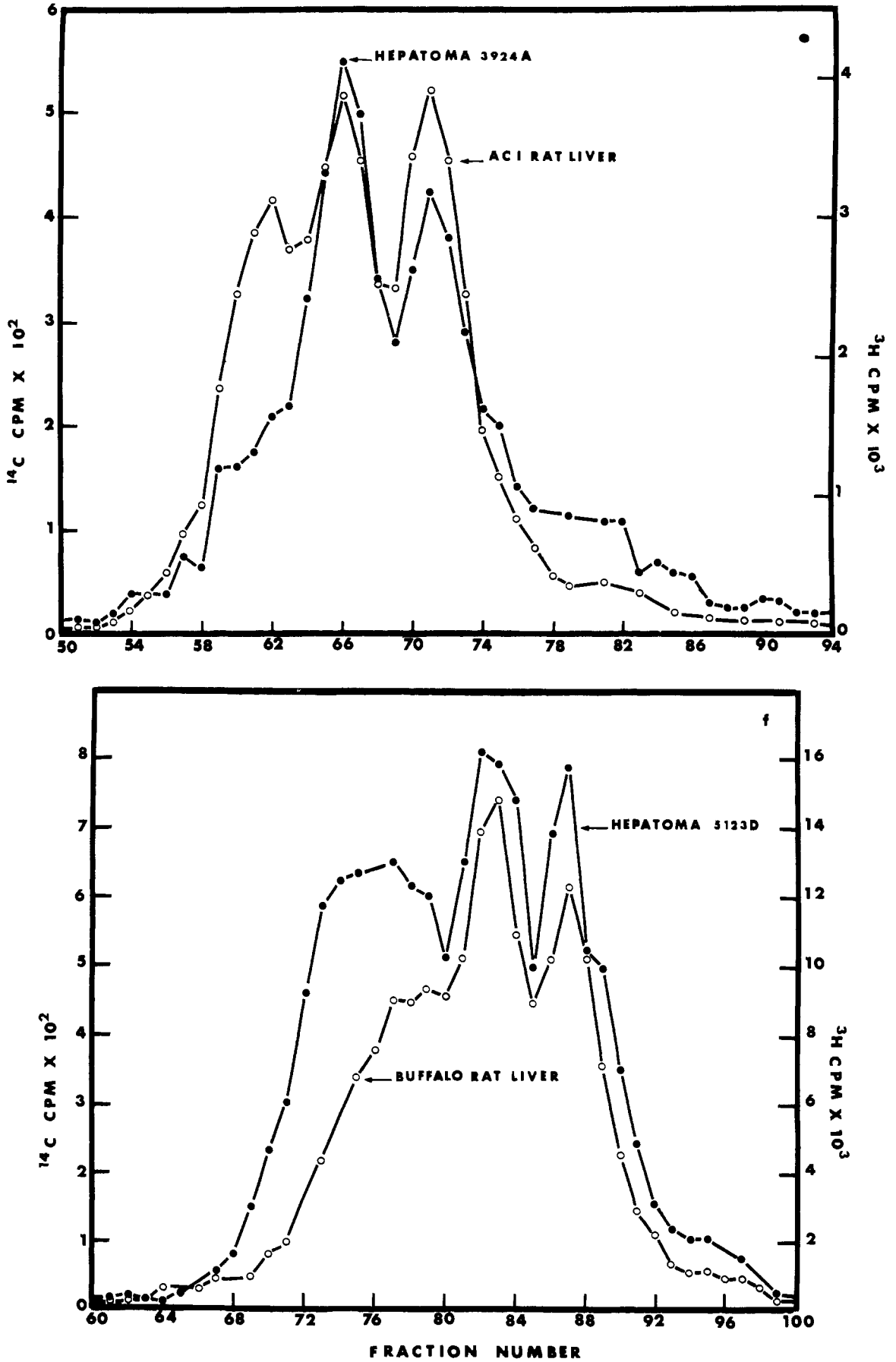


FIGURE 1: RPC 2 Cochromatography of three aminoacyl-tRNAs which show little or no differences between the hepatomas and the livers. In each case the hepatoma tRNA was aminoacylated with [^{14}C]amino acid, and the liver tRNA with [^{3}H]amino acid. The chromatographic conditions were as described in the text. Figures 1a and b show Leu-tRNA; c and d show Lys-tRNA; e and f show Tyr-tRNA.

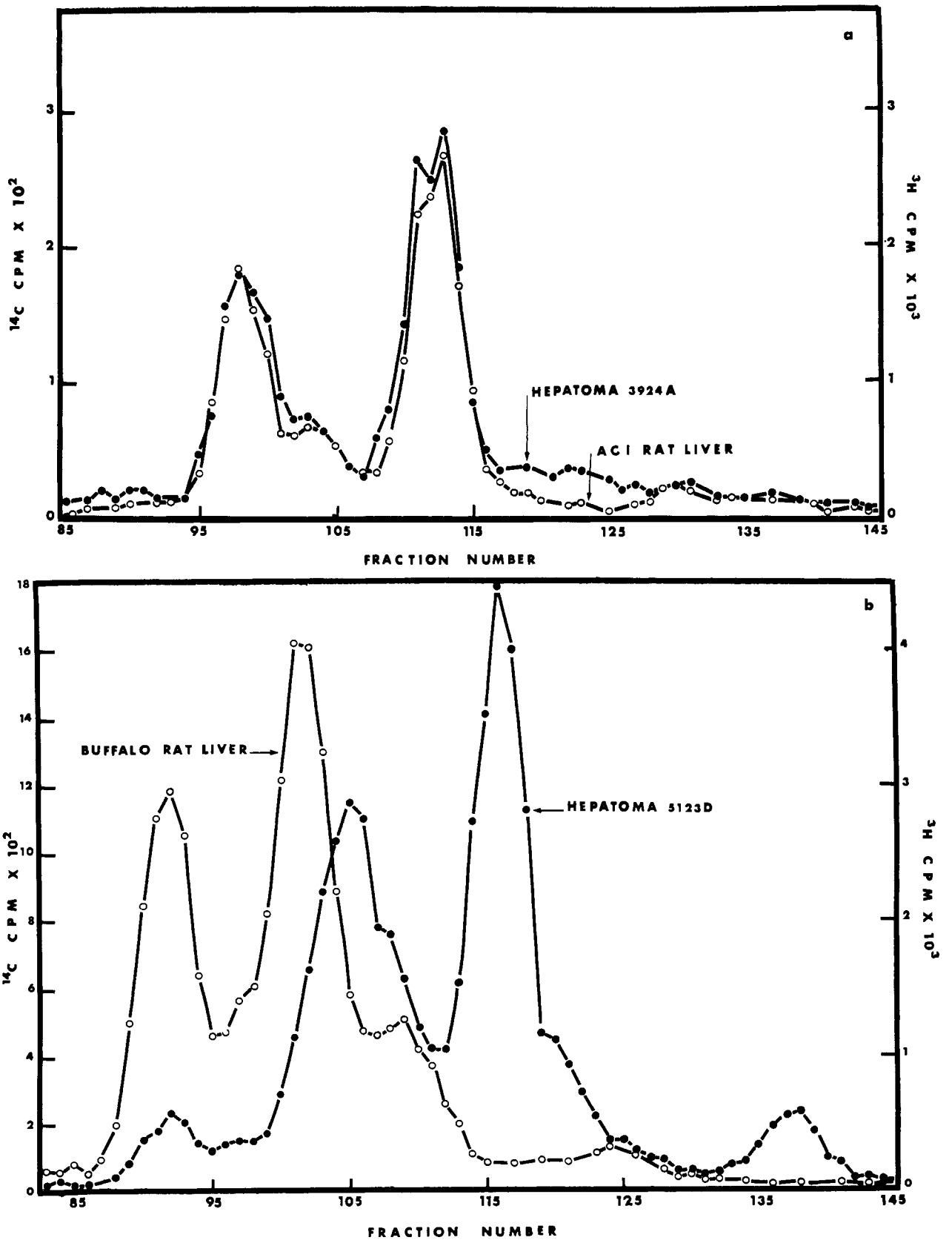


FIGURE 2: RPC 2 cochromatography of (a) [^{14}C]Ser-tRNA$_{3924A}$ and [^{3}H]Ser-tRNA$_{ACI}$ and (b) [^{14}C]Ser-tRNA$_{5123D}$ and [^{3}H]Ser-tRNA$_{Buff}$. The chromatographic conditions were as described in the text.

rat livers and hepatomas. This confirms the finding of other workers (Goldman *et al.*, 1969; Baliga *et al.*, 1969; Gonano and Chiarugi, 1969; Gonano *et al.*, 1971). However, our results do not agree with other published work as to the number of iso-accepting species present in rat liver. Taylor *et al.* (1968) suggested that the tRNAs of corresponding

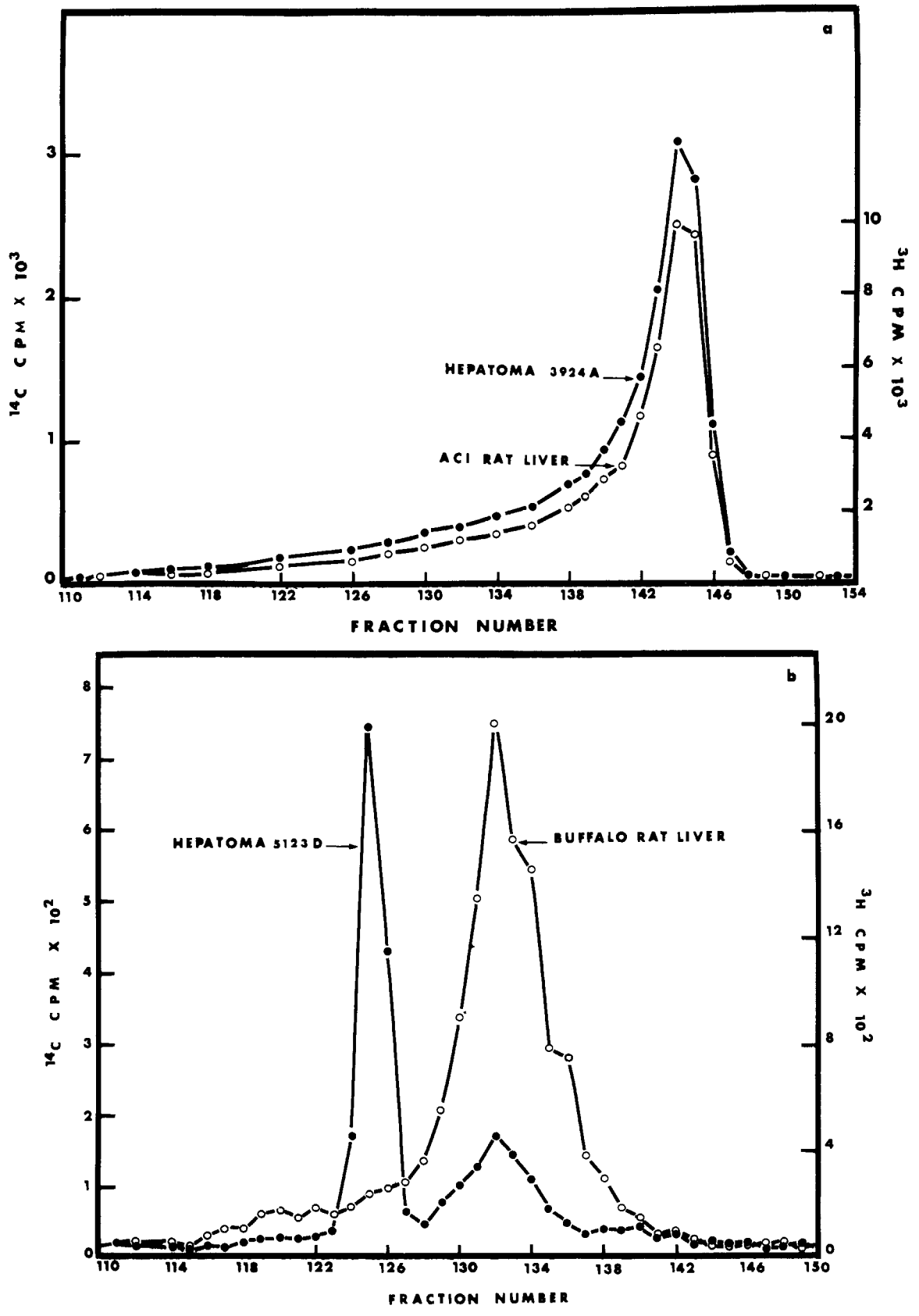


FIGURE 3: RPC 2 cochromatography of (a) [^{14}C]Phe-tRNA$_{3924A}$ and [^{3}H]Phe-tRNA$_{ACI}$ and (b) [^{14}C]Phe-tRNA$_{5123D}$ and [^{3}H]Phe-tRNA$_{Buff}$. The chromatographic conditions were as described in the text.

tissues from different mammals are very similar. It would, therefore, be expected that our rat liver aminoacyl-tRNAs would show similar chromatographic properties to those of other workers using the same chromatographic system We have consistently found three peaks for His-, Tyr-, Leu-, and Lys-tRNA, two for Ser-tRNA, and one for Phe-tRNA

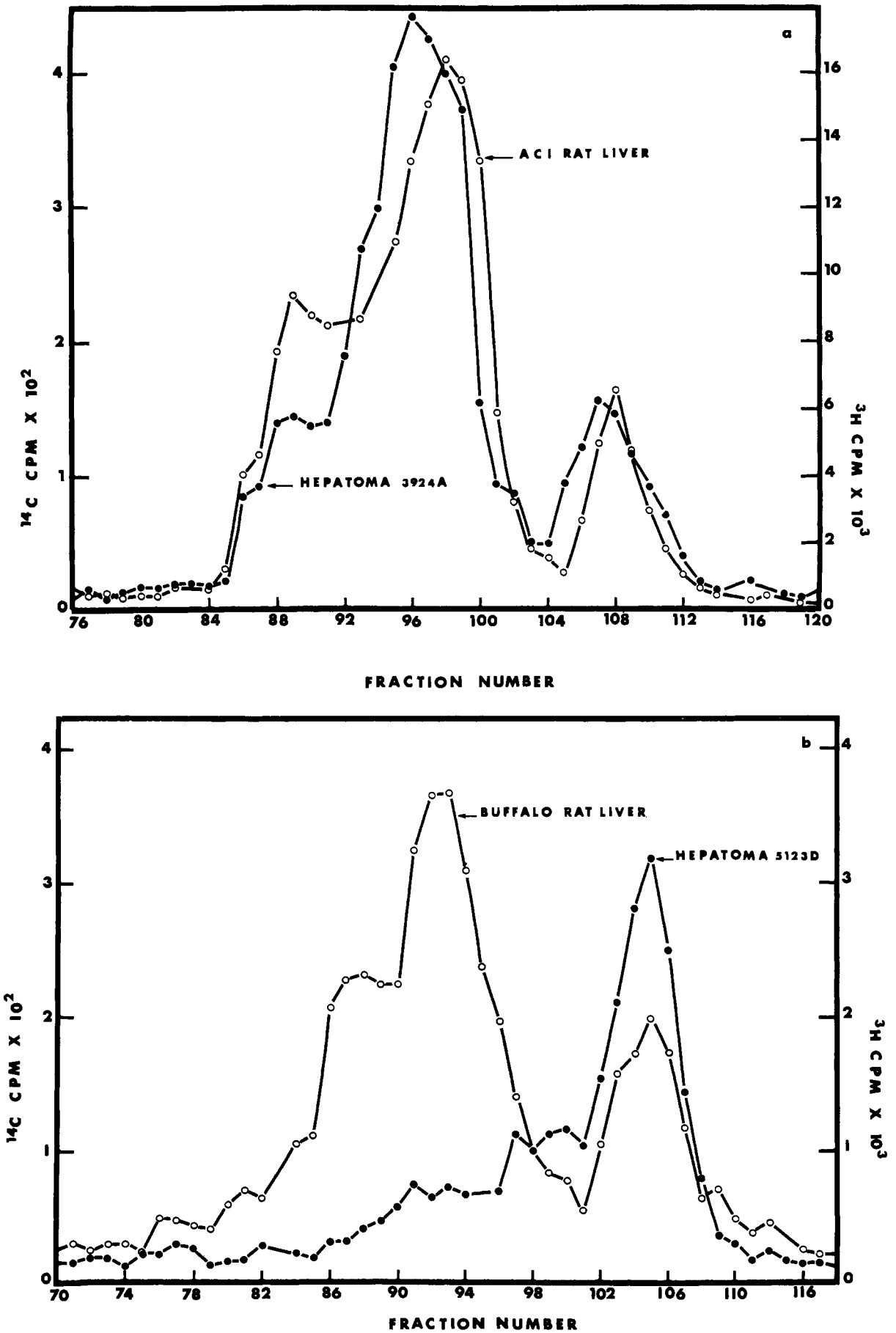


FIGURE 4: RPC 2 cochromatography of (a) [^{14}C]His-tRNA$_{3924A}$ and [^{3}H]His-tRNA$_{ACI}$ and (b) [^{14}C]His-tRNA$_{5123D}$ and [^{3}H]His-tRNA$_{Buff}$. The chromatographic conditions were as described in the text.

TABLE I: Number of Iso-Accepting Species of tRNA Observed in the Tissues Studied.

Amino Acid	Tissue		
	Rat Liver	Hepatoma 5123D	Hepatoma 3924A
Leucine	3	3	3
Lysine	3	3	3
Histidine	3	1	3
Phenylalanine	1	2	1
Serine	2	4	2
Tyrosine	3	3	3

from both Buffalo and ACI rat livers. Gonano and his coworkers (Gonano and Chiarugi, 1969; Gonano *et al.*, 1971) claimed that His-, Tyr-, and Leu-tRNAs showed two peaks on RPC 2 columns, while Ser-tRNA showed four and Phe-tRNA showed one peak. Goldman *et al.* (1969), using a similar system, claimed that the Phe-tRNA from random-bred rats showed three peaks. These discrepancies can, perhaps, be explained by our unpublished finding that different batches of Chromosorb W give widely varying results.

We have found two new species of Ser-tRNA and one new species of Phe-tRNA present in hepatoma 5123D, while we were unable to detect in the hepatoma two of the three His-tRNA species found in Buffalo rat liver. These alterations in elution profile are not due to an artifact introduced in the preparation of the tRNA, as they were repeatable with different batches of tRNA prepared from different generations of the hepatoma. Similarly, different batches of enzyme give the same result. They are not due to an alteration in the synthetase enzymes of the hepatoma as opposed to the liver, as aminoacylating $tRNA_{5123D}$ using liver synthetase, and *vice versa*, gives a similar profile. The extra peaks are not caused by aggregation of the tRNAs, as heating the tRNA to 80° for 1 min prior to aminoacylation (Adams and Zachau, 1968) caused no alteration in the elution pattern. The possibility that the peaks could be due to the aminoacylation of different amino acid specific tRNAs with the radioactive amino acid was eliminated by adding a mixture of the other 19 cold amino acids to the reaction mixture. Furthermore, these differences are an inherent property of these neoplastic cells, and not a general phenomenon caused by an increased growth rate. This is borne out by the fact that aminoacyl-tRNAs prepared from regenerating rat liver cochromatographed at the same point as the corresponding aminoacyl-tRNAs prepared from normal liver (S. A. S. Volkers and M. W. Taylor, unpublished observations). It could be also argued that these alterations may be due to the fact that the hepatoma has been removed from its "natural" environment (*i.e.*, the liver), and placed in an "unnatural" environment (the thigh muscle). Yang *et al.* (1969) have reported that tumors grown in culture show different aminoacyl-tRNA profiles from the same tumor grown in the host animal. This cannot be the case in the present study, as all the aminoacyl-tRNAs isolated from hepatoma 3924A which were studied showed the same elution patterns as the corresponding aminoacyl-tRNAs from normal liver.

We conclude, therefore, that the differences between the aminoacyl-tRNAs of hepatoma 5123D and normal Buffalo rat liver are due to the neoplastic nature of these cells. Gonano and his coworkers (Gonano and Chiarugi, 1969; Gonano *et al.*, 1971) reported the presence of a new species of Phe-tRNA in hepatoma 5123, but concluded that there were no differences between Ser- and His-tRNA from the hepatoma and normal liver. It is difficult to compare our results with theirs, as they described their hepatoma as "hepatoma 5123B". As there are at least four different sublines of hepatoma 5123 (Morris, 1965), the possibility exists that we may, in fact, be dealing with a different tumor.

These differences are certainly not general for all tumors, or even for all hepatomas, as we have detected no differences in the aminoacyl-tRNAs studied from the relatively undifferentiated hepatoma 3924A.

The cause of the chromatographic differences found in hepatoma 5123D is, as yet, unclear. They may be due to changes in secondary structure which either cause the tumor to elute differently or alter the ability of a certain species to recognize the corresponding aminoacyl-tRNA synthetase, thus causing an alteration in the size of a peak. A tRNA could perhaps be made to elute differently by the alteration of the amino acid moiety after aminoacylation, in a similar manner to the formylation of Met-tRNA in bacteria. There have recently been reports of the presence of *N*-acetyl-Ser-tRNA in regenerating rat liver (Liew *et al.*, 1970) and of pyrrolidonecarboxylyl-tRNA in rabbit liver (Rush and Starr, 1970). Finally, the extra peaks could be brought about by the presence of new species of tRNA, coded for by a gene which is derepressed during, or prior to, carcinogenesis.

These altered tRNAs could function in several ways. Ames and Hartman (1963) suggested that a tRNA recognizing a certain codon could play a part in the regulation of protein synthesis if it were present in rate-limiting amounts. It is possible that such a tRNA is present in liver in amounts too small to detect; increased synthesis of the tRNA in the hepatoma removes its rate-limiting properties, and also allows it to be detected by reversed-phase chromatography. A similar situation would arise if the recognition of the aminoacyl-tRNA synthetase were the rate-limiting step. There is also the possibility that two, or more, tRNAs possessing the same anticodon could not bind to ribosomes with the same efficiency, due to the presence of different bases next to the anticodon which interfere sterically with the binding. Evidence for such a system has been put forward by Gefter and Russell (1969) who isolated three types of $tRNA^{Tyr}$ from phage-infected *Escherichia coli*, all of which recognized the codon UAG, but with differing efficiency, due to the modification of the side chain on the base next to the anticodon. It is also possible that the aminoacyl-tRNA, or one iso-accepting species of the tRNA, may act as an inhibitor in amino acid, and, therefore, protein biosynthetic pathways. Duda *et al.* (1968) have reported that Phe-tRNA interferes with the biosynthesis of phenylalanine in *E. coli* K12, while DeLorenzo and Ames (1970) have suggested that a His-tRNA–His-tRNA synthetase complex may be involved in the regulation of histidine synthesis in *Salmonella typhimurium*.

We are in the process of purifying the new species of tRNA which we have found in hepatoma 5123D, and propose to examine some of these possibilities more closely.

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Interaction of Phenazines with Polydeoxyribonucleotides*

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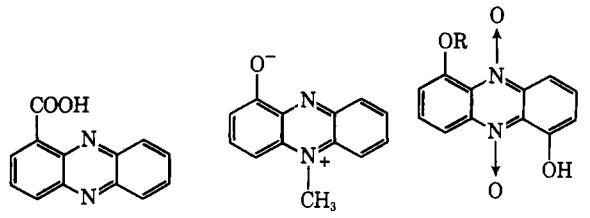
ABSTRACT: The interaction of several phenazine antibiotics (phenazine-1-carboxylic acid, phenazine-1-carboxamide, pyocyanin, 1,6-dimethoxyphenazine and its mono- and di-*N*-oxide, 1,6-dihydroxyphenazine, iodinin, and myxin) with DNA was studied by visible spectrophotometry. There were two types of binding for these derivatives: a strong binding (association constants 10^4–10^6 range) and a weaker binding (association constants 10^3–10^4 range). There was no base specificity for phenazine-1-carboxamide and pyocyanin; however, the binding strength decreased for phenazine-1-carboxamide if DNA was substituted by poly(d(A-T))·poly(d(A-T)) or poly(dC)·poly(dG). Binding was weakened by increasing ionic strength. No binding to single-stranded polydeoxyribonucleotides was detected. These results are in agreement with an intercalative model which has also been proposed for other tricyclic planar aromatic antibiotics. The phenazine derivatives inhibit DNA template-controlled RNA synthesis whereby iodinin approaches the inhibitory intensity of actinomycin. It is suggested that the inhibition, at least in part, is due to intercalation.

Phenazine derivatives have been known for many years from microbial as well as synthetic origins. Many of about 20 known microbial metabolites possess antibiotic activity (Miller, 1961). Antiviral and antitumor activity has been detected in numerous synthetic phenazine derivatives (Endo *et al.*, 1966; Katagiri *et al.*, 1967). Of particular interest is the recently discovered antibiotic myxin which has been reported to possess an unusually broad antimicrobial spectrum (Peterson *et al.*, 1966). A few representative phenazine derivatives are shown in Chart I. There is a striking similarity between the planar aromatic phenazine skeleton and that of the acridines, phenoxazines (actinomycins), and other planar tricyclic antibiotics such as chromomycin, daunomycin, and ethidium bromide. Studies of these antibiotics led to an intercalative model for a ligand–DNA complex (Ward *et al.*,

CHART I



phenazine-1-carboxylic acid | pyocyanin | R = H, iodinin; R = CH_3, myxin

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