Acknowledgments

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Abundance of tRNA^{Phe} Lacking the Peroxy Y-Base in Mouse Neuroblastoma†

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ABSTRACT: Affinity chromatography on anti-Y (Y is a tricyclic imidazopurine to which is attached a complex four-carbon side chain) antibody immobilized to Sepharose was used to determine the proportion of rat liver tRNA^{Phe} species containing the peroxy Y-nucleoside. Unfractionated mammalian tRNA was aminoacylated with labeled phenylalanine. The phenylalanyl-tRNA was then chemically acetylated to yield *N*-acetylphenylalanyl-tRNA. When this preparation was applied to the antibody column, between 6–10% of the radioactivity was not bound to the column, indicating a deficiency of peroxy Y-nucleoside in a minor isoaccepting tRNA^{Phe} species. In contrast to normal tissues (including embryonic tissue), about 85% of the tRNA^{Phe} from mouse neuroblastoma C-1300 or N-18 tumors lack the peroxy Y-base, a property which is not affected by tumor age. Rat liver labeled *N*-acetylphenylalanyl-tRNA preparations were resolved on Plaskon chromatography (RPC-5) into two minor peaks closely followed by a major component. A high proportion of the two minor tRNA^{Phe} species was unable to bind to anti-Y antibodies. Upon mild acid treatment, the minor and major tRNA^{Phe} species eluted simultaneously from Plaskon columns, at a much reduced salt concentration. These results would indicate that the two minor tRNA^{Phe} species from rat liver as well as the major component contain a tricyclic imidazopurine base that differs from each other in its side chain. About 85% of the *N*-acetylphenylalanyl-tRNA from neuroblastoma was resolved by Plaskon chromatography as an early eluting peak. The position of this major neuroblastoma tRNA^{Phe} species was not altered by mild acid treatment, and its elution position from the column almost coincides with that of acid-treated normal rat liver tRNA^{Phe}. The latter results would suggest that most of the tRNA^{Phe} chains from neuroblastoma lack the tricyclic imidazopurine of normal rat liver tRNA^{Phe}, but are very close if not identical in primary nucleotide sequence.

Mammalian tRNA^{Phe} contains a highly modified peroxy Y[1]-nucleoside located next to the 3′ end of the anticodon. The mammalian peroxy Y-nucleoside differs from the yeast Y-nucleoside by the presence of a hydroxy peroxide group on the β carbon of the side chain (Blobstein et al., 1973). It was previously shown that goat antibodies against yeast tRNA^{Phe} are specific to the Y-nucleoside (Fuchs et al., 1974) and cross-react with the peroxy Y-nucleoside from rat liver tRNA^{Phe} (Salomon et al., 1975). These antibodies when immobilized on Sepharose enabled us to determine the proportion of rat liver

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[1] Abbreviations used are: Y, tricyclic imidazopurine to which is attached a complex four-carbon side chain; Na_2EDTA, disodium ethylenediaminetetraacetate.

TABLE I: Percent of tRNA^{Phe} Deficient in Peroxy Y-Nucleoside from Various Sources.

Source of tRNA	Peroxy-Y-Deficient tRNA^{Phe} (%)[a]	tRNA^{Phe} (%)
Mouse embryo	6	94
Mouse spleen	10	90
Mouse neuroblastoma tumor (C-1300)	92	8
Mouse neuroblastoma tumor (N-18)	85	15
Rat liver	8	92
Rat liver, newborn	4	96
Rat brain	8	92
Hamster embryo	5	95
Rat Morris Hepatoma 7777 tumor	20	80
Subclone of mouse teratoma (OTT6050)	4	96
Human blood	8	92

[a] The percent of tRNA^{Phe} deficient in peroxy Y-nucleoside was determined by affinity chromatography on anti-Y-Sepharose column.

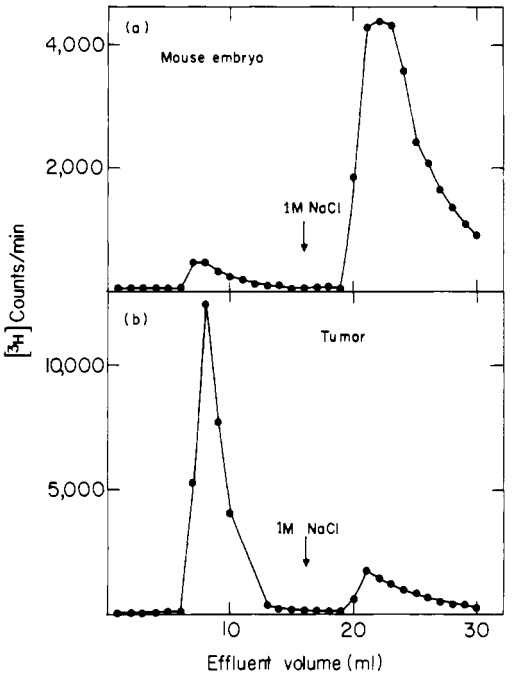

FIGURE 1: Separation of *N*-acetyl[^{3}H]phenylalanyl-tRNA by affinity chromatography on anti-Y Sepharose column. (a) tRNA from mouse embryo. (b) tRNA from mouse neuroblastoma tumor (N-18).

tRNA^{Phe} species containing peroxy Y-nucleoside. Using this method, between 6 and 10% of the rat liver tRNA^{Phe} was found to be deficient in the peroxy Y-nucleoside (Salomon et al., 1975). In the present report, affinity chromatography on anti-Y Sepharose in combination with Plaskon chromatography (RPC-5) enabled us to detect at least two minor rat liver tRNA^{Phe} species deficient in the peroxy Y-nucleoside. In addition, the present work reveals that about 85% of the tRNA^{Phe} from mouse neuroblastoma lacks the peroxy Y-nucleoside. However, this tRNA^{Phe} species of neuroblastoma appears to be different from peroxy-Y-nucleoside-deficient tRNA^{Phe} species found in normal tissues.

Experimental Section

Materials. L-[^{3}H]Phenylalanine (19.6 Ci/mmol) was obtained from Nuclear Research Center (Negev, Israel) and L-[^{14}C]phenylalanine (522 mCi/mmol) from Amersham. Proteinase K was purchased from Merck Darmstadt.

Methods. The preparations of tRNA, crude aminoacyl-tRNA synthetase, and affinity chromatography on anti-Y Sepharose columns were performed as previously described (Salomon et al., 1975).

The aminoacylation reaction was as described (Salomon et al., 1975), except that it also contained 19 unlabeled amino acids (2 μmol each) excluding phenylalanine. *N*-Acetylphenylalanyl-tRNA was prepared by acetylation of phenylalanyl-tRNA (Salomon and Littauer, 1974; Salomon et al., 1975; Heanni and Chapeville, 1966).

Plaskon chromatography (RPC-5) was carried out in a glass column 0.5 × 36 cm using a Milton Roy minor pump with maximum pressure of 1000 psig (Pearson et al., 1971; Roe et al., 1973). The tRNA^{Phe} was eluted with a 140-ml linear gradient of 0.6–0.7 M NaCl in 50 mM sodium acetate (pH 4.5), 1 mM $MgCl_2$, and 0.4 mM Na_2EDTA, and 2.5-ml fractions were collected (Grunberger et al., 1975). The only deviation from these procedures was the use of labeled *N*-acetylphenylalanyl-tRNA rather than phenylalanyl-tRNA (Salomon et al., 1975). The fractions were precipitated and filtered on Whatman GF/C filters as described (Salomon et al., 1975). Mouse neuroblastoma tumors C-1300 or N-18 were grown subcutaneously in A/J mice for periods of 3–16 days. Subclone of mouse teratoma (OTT6050) was a gift from Mr. H. Bustan.

Results

Analysis of N-Acetylphenylalanyl-tRNA on Anti-Y Antibody Columns. The percentage of tRNA^{Phe} deficient in the peroxy Y-nucleoside from various sources was determined by affinity chromatography on an anti-Y antibody column. Unfractionated tRNA was aminoacylated with labeled phenylalanine; the phenylalanyl-tRNA was then chemically acetylated to yield *N*-acetylphenylalanyl-tRNA. This was done since N-substituted aminoacyl-tRNA derivatives have been shown to be considerably more resistant to hydrolysis than the corresponding aminoacyl-tRNA molecules (cf. Daniel et al., 1970). When *N*-acetylphenylalanyl-tRNA preparations from normal tissues (embryonic or adult) were applied to the antibody column, between 6 and 10% of the radioactivity was not bound to the column, indicating a deficiency of peroxy Y-nucleoside in a minor isoaccepting tRNA^{Phe} species (Table I). In contrast to normal tissues, in mouse neuroblastoma over 85% of the tRNA^{Phe} was not bound to the antibody column and appears to be lacking the peroxy Y-nucleoside. An example of such an analysis is depicted in Figure 1 where the affinity chromatography profile of *N*-acetyl[^{3}H]phenylalanyl-tRNA from mouse embryo (Figure 1a) was compared with that from mouse neuroblastoma tumor (Figure 1b). On the other hand, another tumor, Morris hepatoma, lacks only 20% in this modification, which is in agreement with previous results (Grunberger et al., 1975), and mouse teratoma shows an entirely normal pattern. It seems that neuroblastoma is a unique tumor, both in the parent tumor (C-1300) and the subclone (N-18) (Table I). When tumors in various stages of development were analyzed using 3-day old specimens through 16-day tumors, no difference in the peroxy Y-nucleoside deficiency was noticed. Thus, it can be concluded that the occurrence of

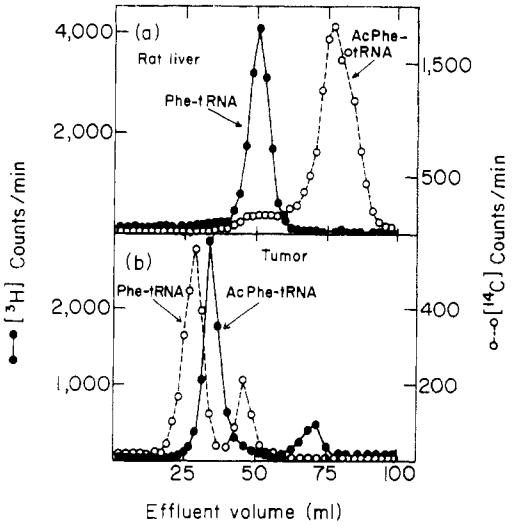

FIGURE 2: The elution profile of phenylalanyl-tRNA together with *N*-acetylphenylalanyl-tRNA from a RPC-5 column. (a) Rat liver tRNA: (● -- ●) [^{3}H]phenylalanyl-tRNA; (O - - - O) *N*-acetyl[^{14}C]phenylalanyl-tRNA. (b) Mouse neuroblastoma tumor (N-18) tRNA: (O - - - O) [^{14}C]phenylalanyl-tRNA; (● — ●) *N*-acetyl[^{3}H]phenylalanyl-tRNA.

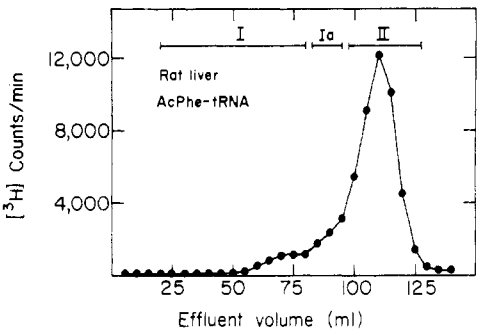

FIGURE 3: Large scale fractionation of rat liver *N*-acetyl[^{3}H]phenylalanyl-tRNA. The incubation mixture contained 0.5 mg of rat liver tRNA that was aminoacylated with L-[^{3}H]phenylalanine and 0.25 ml of crude rat liver phenylalanyl-tRNA synthetase. The [^{3}H]phenylalanyl-tRNA (2×10^6 counts/min) was acetylated and fractionated on a RPC-5 column. Aliquots of 0.2 ml were removed from each fraction and their radioactivity was determined. The pooled fractions, I, Ia, and II, are noted on the figure.

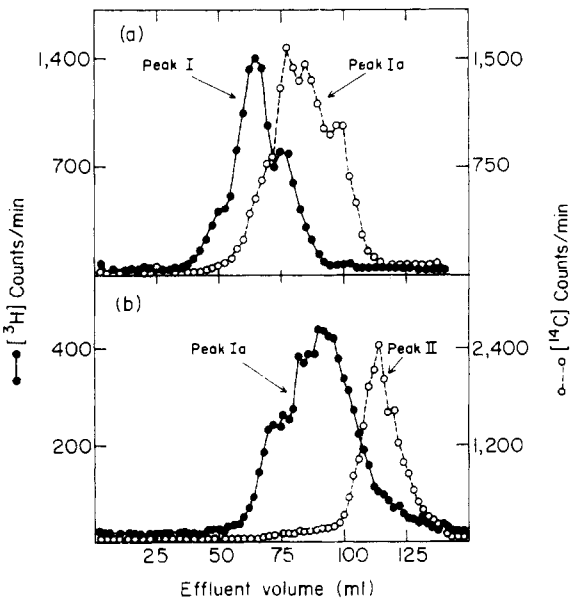

FIGURE 4: Rechromatography of the pooled rat liver tRNA fractions on RPC-5 column. (a) Elution profile of (● — ●) peak I, *N*-acetyl-[^{3}H]phenylalanyl-tRNA, together with (O - - - O) peak Ia, *N*-acetyl[^{14}C]phenylalanyl-tRNA. (b) Elution profile of (● — ●) peak Ia, *N*-acetyl[^{3}H]phenylalanyl-tRNA, together with (O - - - O) peak II, *N*-acetyl[^{14}C]phenylalanyl-tRNA.

tRNA^{Phe} lacking the peroxy Y-nucleoside is not age dependent.

Fractionation on N-Acetylphenylalanyl-tRNA on Plaskon Columns. We have observed that the acetylation of phenylalanyl-tRNA to yield *N*-acetylphenylalanyl-tRNA improves the resolution on RPC-5 columns. Figure 2a shows an RPC-5 elution profile of *N*-acetyl[^{14}C]phenylalanyl-tRNA from rat liver which was cochromatographed with unmodified [^{3}H]phenylalanyl-tRNA from the same source. The unmodified [^{3}H]phenylalanyl-tRNA was resolved into a major peak and a minor shoulder eluting slightly ahead of the main fraction. The acetylated derivative is more hydrophobic than the unmodified phenylalanyl-tRNA and was eluted from the column at a higher salt concentration. Moreover, there was a better resolution of the material comprising the minor early eluting shoulder from the main species. Similar results were obtained with tRNA^{Phe} from mouse liver. Figure 2b shows an RPC-5 elution profile of *N*-acetyl[^{3}H]phenylalanyl-tRNA from neuroblastoma which was cochromatographed with unmodified [^{14}C]phenylalanyl-tRNA from the same source. In both cases the neuroblastoma tRNA^{Phe} resolved into two well-separated peaks. In this case too, acetylation of phenylalanyl-tRNA to *N*-acetylphenylalanyl-tRNA improved the resolution of the two neuroblastoma tRNA^{Phe} species. The acetylation of phenylalanyl-tRNA, thus, offers two advantages, an easy-to-handle stable compound and better resolution on RPC-5 column. In all the experiments to be described we have, therefore, used labeled *N*-acetylphenylalanyl-tRNA.

It should be noted that neuroblastoma *N*-acetylphenylalanyl-tRNA showed a striking difference in its elution profile as compared with that obtained from rat liver. About 90% of the tRNA^{Phe} from neuroblastoma emerged from the column as an early peak ahead of the minor shoulder from normal rat liver tRNA^{Phe} (Figure 2). On the other hand, only 10% of the neuroblastoma tRNA^{Phe} species eluted at a higher salt concentration at a position which coincided with the major tRNA^{Phe} species from rat liver.

The reversal of radioactive labels on *N*-acetylphenylalanyl-tRNA from rat liver and neuroblastoma had no effect on their separation on RPC-5 columns. Since a small amount of contamination in the labeled phenylalanine may result in the appearance of external aminoacylated-tRNA peaks upon chromatography, 19 unlabeled amino acids, excluding phenylalanine, were always included in the aminoacylation reactions. Indeed, when unlabeled amino acids were not used, the reverse-phase chromatograpby revealed traces of radioactive peaks eluting at low salt concentration.

Chromatographic Analysis of Rat Liver tRNA^{Phe} Species. The early eluting shoulder of rat liver tRNA^{Phe} from RPC-5 was further characterized by rechromatography on similar columns. In order to obtain large quantities of this variety of rat liver tRNA^{Phe}, preparative chromatography was undertaken. The various fractions were collected and pooled as indicated in Figure 3. The *N*-acetyl[^{3}H]phenylalanyl-tRNA was then recovered by precipitation with 2 volumes of ethanol, with 1 mg of *Escherichia coli* tRNA serving as carrier. A similar procedure was also applied to *N*-acetyl[^{14}C]phenylalanyl-

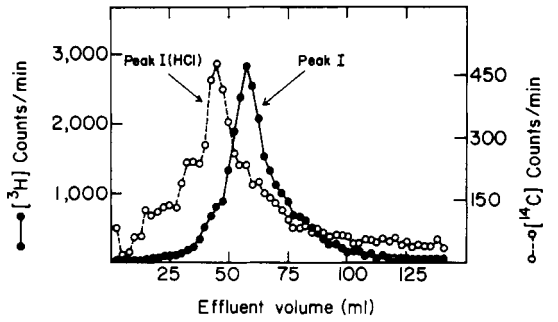

FIGURE 5: RPC-5 elution profile of normal and acid-treated peak I, *N*-acetylphenylalanyl-tRNA from rat liver. (● — ●) Peak I, *N*-acetyl[^{3}H]phenylalanyl-tRNA; (○ - - - ○) acid-treated peak I, *N*-acetyl[^{14}C]phenylalanyl-tRNA.

tRNA. The various pooled fractions from the RPC-5 chromatography were designated peaks I, Ia, and II. Samples from RPC-5 peaks I, Ia, and II were then analyzed by rechromatography on RPC-5 columns. Rechromatography of peak I together with peak Ia reveals that these fractions are distinct in their elution profile, although some cross-contamination still persists (Figure 4a). Peak Ia may be composed of more than one component and eluted earlier than peak II which represents the major fraction of rat liver tRNA^{Phe} (Figure 4b). Thus there are at least two minor rat liver tRNA^{Phe} species which can be separated on RPC-5 columns.

In order to determine the presence of a tricyclic imidazopurine in the minor tRNA^{Phe} species, the following experiments were performed. Peaks I and Ia were subjected to mild acid treatment that can selectively excise such a modified base from tRNA without breaking the polynucleotide chain (Thiebe and Zachau, 1968). Figure 5 shows that following acid treatment the material comprising peak I eluted at a reduced salt concentration at a position earlier than the untreated preparation. This result would suggest that the minor peak I tRNA^{Phe} contains an acid-sensitive tricyclic imidazopurine base which may have some further modifications. Similar results were obtained upon acid treatment of peaks Ia and II (data not shown). Moreover, acid-treated peaks I, Ia, and II eluted at the same position from the Plaskon columns. Therefore, it appears that the three tRNA^{Phe} species all contain a tricyclic imidazopurine base and are similar or identical in their primary nucleotide sequence.

The various rat liver tRNA^{Phe} species obtained after fractionation on RPC-5 columns were further analyzed by affinity chromatography on anti-Y Sepharose columns, as shown in Figure 6. Only trace amounts of materials that bind to anti-Y antibodies were in peak I (Figure 6a). On the other hand, all of the tRNA^{Phe} in peak II was retained on the antibody column eluting with 1 M NaCl (Figure 6b). Peak Ia resolved into two fractions: about 58% were not bound to the column, while the rest of the material showed affinity toward the antibody (Figure 6c). Since all the rat liver tRNA species were shown to contain an acid-excisable base, it is possible that the differences in affinity toward the antibody reflect various modifications in the side chain of the tricyclic imidazopurine base.

Chromatographic Analysis of tRNA^{Phe} Species from Mouse Neuroblastoma Tumors. It has already been shown in Figure 2 that about 90% of the neuroblastoma tRNA^{Phe} emerges at relatively low salt concentration from Plaskon columns. This major neuroblastoma tRNA^{Phe} species was not bound to the antibody column as opposed to the minor neuroblastoma tRNA^{Phe} fraction (data not shown). The major

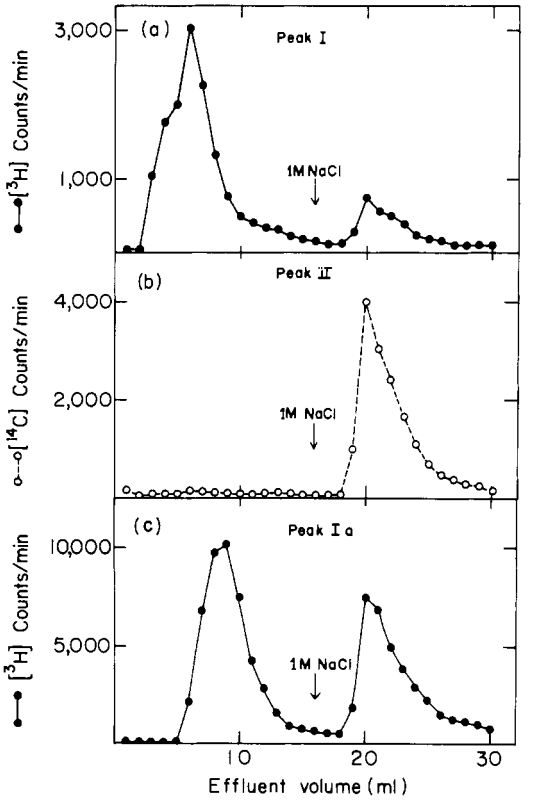

FIGURE 6: Separation of the pooled rat liver tRNA fractions on anti-Y Sepharose columns. (a) peak I: (● — ●) *N*-acetyl[^{3}H]phenylalanyl-tRNA. (b) Peak II: (○ - - - ○) N-acetyl[^{14}C]phenylalanyl-tRNA. (c) Peak Ia: (● — ●) *N*-acetyl[^{3}H]phenylalanyl-tRNA.

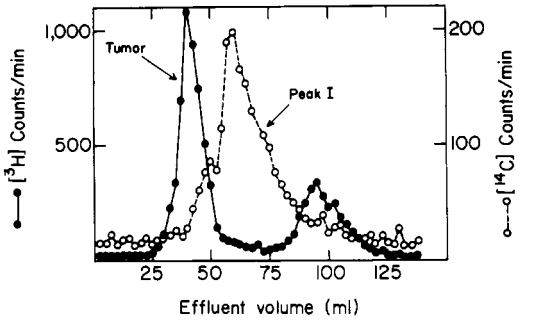

FIGURE 7: RPC-5 elution profile of neuroblastoma and rat liver peak I, *N*-acetylphenylalanyl-tRNA. (● — ●) *N*-Acetyl[^{3}H]phenylalanyl-tRNA from neuroblastoma (N-18) tumor; (○ - - - ○) peak I, *N*-acetyl[^{14}C]phenylalanyl-tRNA from rat liver.

neuroblastoma tRNA^{Phe} species, however, differed from rat liver peak I tRNA^{Phe} species in its elution position from Plaskon columns (Figure 7). Cochromatography of neuroblastoma *N*-acetyl^{3}H]phenylalanyl-tRNA with acid-treated peak I *N*-acetyl[^{14}C]phenylalanyl-tRNA from rat liver reveals that the two preparations have a similar but not identical elution position (Figure 8). In contrast to rat liver tRNA^{Phe}, mild acid treatment of the major neuroblastoma tRNA^{Phe} species did not alter its elution position from RPC-5 columns, suggesting the lack of an excisable tricyclic imidazopurine base.

Discussion

Rat liver tRNA^{Phe} contains at least two minor tRNA^{Phe} species that were resolved on rechromatography on Plaskon columns (Figure 4). The elution pattern of peak Ia exhibits

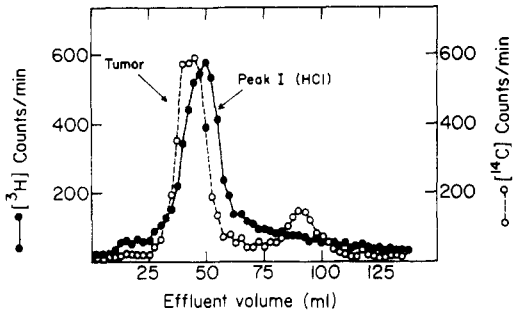

FIGURE 8: RPC-5 elution profile of neuroblastoma and acid-treated rat liver peak I, *N*-acetylphenylalanyl-tRNA; (● — ●) acid-treated peak I, *N*-acetyl[^{3}H]phenylalanyl-tRNA from rat liver; (○ - - - ○) neuroblastoma (N-18) tumor *N*-acetyl[^{14}C]phenylalanyl-tRNA.

several shoulders which may indicate that it is heterogeneous. Unlike the material comprising peak II, the major tRNA^{Phe} component, peak I material, does not bind to the antibody column. About 40% of peak Ia material was bound to the antibody column. The relatively high proportion of binding of peak Ia to the antibody is more than expected from cross-contamination with peak II as revealed in Figure 4b and may suggest that it is composed of more than one component.

It was shown in previous studies that the affinity of the antibodies is much weaker toward the heterologous peroxy Y-nucleoside from rat liver than toward the homologous yeast Y-nucleoside (Salomon et al., 1975), although the difference between the modified nucleosides is only at the second and third carbons of the side chain (Li et al., 1973; Blobstein et al., 1973). It is, therefore, plausible that the major antigenic determinant resides in the side chain of the Y-nucleoside and that any difference in the four-carbon side chain may either weaken or abolish the binding to the antibody. Thus, the rat liver tRNA^{Phe} species that are not bound to the antibody could contain a derivative of peroxy Y-base or may lack it altogether. Our finding that mild acid treatment causes a change in the RPC-5 mobility of the minor rat liver tRNA species would suggest the presence of an acid-excisable tricyclic imidazopurine in these preparations. However, the inability of peak I and most of peak Ia to bind to the antibody suggests that these preparations contain tricyclic imidazopurine derivatives that differ from the peroxy Y-nucleoside in its side chain. It is possible that the minor tRNA^{Phe} species from rat liver are immature forms of newly synthesized molecules representing various degrees of modification toward the formation of the peroxy Y-nucleoside and that these modifications, very likely, take place in the side chain. The present data do not exclude possible differences in the primary sequences of these tRNA^{Phe} species. Although, if these differences exist, they must be rather small since, upon acid treatment, all the rat liver tRNA^{Phe} species had an identical mobility on Plaskon column chromatography. In this respect tRNA^{Phe} species from rat liver differ from those of bovine lens, where, upon differentiation into lens fiber, an additional tRNA^{Phe} species appears with different primary sequence. However, acid treatment of the two lens tRNA^{Phe} species still shows two distinct tRNA^{Phe} peaks on RPC-5 columns (Ortwerth et al., 1975).

Grumberger et al. (1975) have shown that 20% of the tRNA^{Phe} from Morris hepatoma 7777 tumor is deficient in the peroxy Y-base. However, mouse neuroblastoma tumor tRNA^{Phe} has an unusual structure, i.e., over 85% of the tRNA^{Phe} is lacking the tricyclic imidazopurine. This is in contrast to all normal tissues examined which contain less than 10% of tRNA^{Phe} deficient in the peroxy Y-nucleoside. Moreover, these minor tRNA^{Phe} species still contained the tricyclic imidazopurine. The evidence for this conclusion rests on the findings that 85% of the neuroblastoma tRNA^{Phe} was not bound to the anti-Y antibody column and was resolved by Plaskon chromatography as an early eluting peak. In addition, the position of this major neuroblastoma tRNA^{Phe} species was not altered by mild acid treatment, indicating the absence of a tricyclic imidazopurine. It should be noted that after acid treatment the rat liver tRNA^{Phe} eluted from the RPC-5 column in a position similar but slightly behind the major neuroblastoma peak (Figure 8). This slight difference may be due to the absence of a base in the position adjacent to the anticodon in the excised rat liver tRNA^{Phe}. It remains to be determined if the neuroblastoma tRNA^{Phe} lacking the peroxy Y-base has a nucleotide sequence identical with that of the rat liver tRNA^{Phe} or is a product of a different tRNA^{Phe} gene.

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