Modifications of Ribonucleic Acid by Chemical Carcinogens. II. *In Vivo* Reaction of *N*-2-Acetylaminofluorene with Rat Liver Ribonucleic Acid*

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ABSTRACT: The binding of *N*-2-acetylaminofluorene, a hepatic carcinogen, and its metabolite, *N*-hydroxy-2-acetylaminofluorene, to rat liver RNA was studied *in vivo*. Analyses of liver ribonucleic acids obtained shortly after administration of either of these ^{14}C-labeled compounds indicated that the transfer ribonucleic acid fraction had two to three times the specific activity of 5S, 18S, and 28S ribonucleic acid. The preferential binding to transfer ribonucleic acid was established by 1 M NaCl fractionation, chromatography on Sephadex G-100, and sucrose gradient centrifugation. Kinetic studies indicated that maximal binding to liver ribosomal ribonucleic acid and transfer ribonucleic acid was attained by 12 hr after intraperitoneal injection. Within the first 24 hr, transfer ribonucleic acid had a higher specific activity than ribosomal ribonucleic acid. By 24 to 48 hr, however, *N*-2-acetylaminofluorene-labeled transfer ribonucleic acid and ribosomal ribonucleic acid had comparable specific activities which declined with $T_{1/2}$ values of approximately 5 days. Chromatography on benzoylated DEAE-cellulose columns separated the small amounts of [^{14}C]*N*-2-acetylaminofluorene transfer nucleic acid formed *in vivo* from the bulk of the unmodified transfer ribonucleic acid. Further chromatography of the *N*-2-acetylaminofluorene transfer ribonucleic acid on methylated albumin kieselguhr columns suggested that the carcinogen had reacted with several types of transfer ribonucleic acid. Cochromatography on benzoylated DEAE-cellulose columns of transfer ribonucleic acids, obtained from rats fed either normal or *N*-2-acetylaminofluorene-containing diets and pulse labeled with [^{3}H]- or [^{14}C]orotic acid revealed that dietary *N*-2-acetylaminofluorene does not produce gross changes in the profile of newly synthetized liver transfer ribonucleic acids.

It is well established that several hepatic carcinogens bind *in vivo* to liver nucleic acids, proteins, and carbohydrates (Miller and Miller, 1967; Farber *et al.*, 1967). At the present time it is not known which of these reactions are critical to the induction of tumors, and which are merely side reactions. The binding of these agents to nucleic acids is of particular interest, due to the central role of DNA and RNA in replication and expression of genetic information. Binding of carcinogens to DNA and protein has been investigated in considerable detail, but there are relatively few studies which examine in detail the binding of carcinogens to cellular RNAs. Recent results from this laboratory (Weinstein, 1968), as well as earlier studies by Farber *et al.* (1967), suggest that liver tRNA may be a critical target during hepatic carcinogenesis. The hepatic carcinogen ethionine reacts preferentially with liver tRNA *in vivo* and produces a specific modification in the pattern of isoaccepting species for $tRNA^{Leu}$ (Axel *et al.*, 1967). The possibility that changes in the tRNA population may play an important role in cell regulation and differentiation is now widely recognized, and evidence is accumulating that the tRNA population of certain tumors may differ qualitatively from that of the related normal tissue (for a review of this subject, see Weinstein, 1968, 1969).

N-2-Acetylaminofluorene is a potent hepatocarcinogen which also binds to liver RNAs including tRNAs when administered *in vivo*. *In vitro* experiments, employing a highly reactive derivative of AAF,[1] *N*-acetoxy-AAF, indicated that the binding of AAF to *Escherichia coli* tRNA produces modifications in amino acid acceptance, codon recognition, and ribosomal binding, and chromatographic behavior of specific types of tRNA (Fink *et al.*, 1969). These model experiments indicate that AAF binding to tRNA can alter its biologic activity. The present studies were undertaken to examine in greater detail the binding of AAF to liver tRNA *in vivo*. Conclusive evidence is presented that following administration of 9-^{14}C-labeled AAF, or *N*-OH-AAF, radioactivity becomes firmly bound to the tRNA fraction, when the tRNA is purified by a variety of procedures. The kinetics of this binding have been compared with the binding of AAF to liver ribosomal RNA (rRNA). Chromatography on BD-cellulose and MAK columns resolved the small amounts of [^{14}C]AAF-tRNA, formed *in vivo*, from the bulk of the unmodified tRNA. The data indicate that AAF reacts with several amino acid specific types of tRNA and does not grossly alter the profile of newly synthesized liver tRNA.

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[1] The following abbreviations are used throughout this paper: AAF, *N*-2-acetylaminofluorene; *N*-OH-AAF, *N*-hydroxy-2-acetylaminofluorene; AAF-tRNA, tRNA previously reacted with AAF or *N*-OH-AAF *in vivo;* BD-cellulose, benzoylated DEAE-cellulose; MAK, methylated albumin kieselguhr.

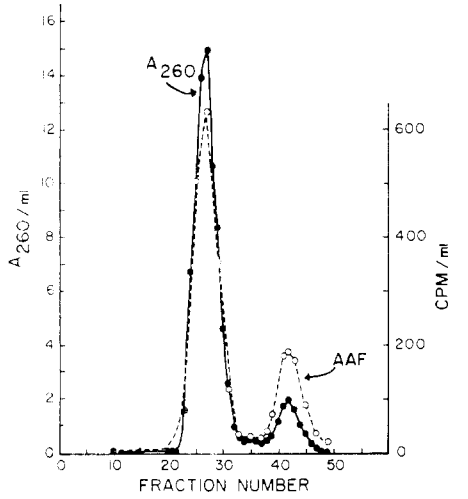

FIGURE 1: Fractionation of liver RNA by chromatography on Sephadex G-100 columns. A Sephadex G-100 column (0.8 × 130 cm) was equilibrated with 0.01 M Tris-HCl (pH 7.0) and 0.1 M NaCl. Liver RNA was obtained by phenol extraction from rats injected with [^{14}C]*N*-OH-AAF (33 μCi/150 g of body weight) 18 hr prior to sacrifice. RNA (16 mg in 1 ml of buffer) was applied to the column. Elution was carried out with the above buffer, according to the method of Kirtikar and Kaji (1968). Fractions (2 ml) were collected at a flow rate of 12 ml/hr and counted directly in Bray's solution.

A preliminary report of some of these results has been presented elsewhere (Agarwal and Weinstein, 1969).

Materials

[9-^{14}C]*N*-2-Acetylaminofluorene (3 mCi/mmole) and [9-^{14}C]-*N*-hydroxy-2-acetylaminofluorene were purchased from New England Nuclear Corp. In some studies, the latter compound (14 mCi/mmole) was a product of Tracerlab. Nonradioactive *N*-2-acetylaminofluorene was obtained from Eastman Kodak Chemicals. Benzoylated DEAE-cellulose (lot 6802), 20–50 mesh, was obtained from Schwarz BioResearch. Phenol (Mallinckrodt) was analytical reagent grade, and disodium ATP was a product of P-L Biochemicals. Sephadex G-100 was purchased from Pharmacia Fine Chemicals, Inc. Wistar male albino rats (150–200 g body weight) were employed throughout the present study.

Methods

Radioactive compounds were injected intraperitoneally in 1 ml of a suspension in gum acacia (7 g of gum acacia/100 ml of isotonic saline). The animals were killed by decapitation at the indicated time intervals.

The RNA was extracted with phenol as previously described (Fink *et al.*, 1968). Unless indicated otherwise, fractionation into tRNA and rRNA was carried out with 1 M NaCl, as described in the latter reference. Further resolution of RNAs was obtained by sucrose gradient fractionation (Henshaw and Hiatt, 1963), Sephadex G-100 column chromatography (Kirtikar and Kaji, 1968), polyacrylamide gel electrophoresis (Grossbach and Weinstein, 1968), benzoylated DEAE-cellulose column chromatography (Gillam *et al.*, 1967; Fink *et al.*, 1968), and chromatography on methylated albumin kieselguhr (Axel *et al.*, 1967). Additional details are given in the figure legends. RNA was quantitated by absorption at 260 mμ, assuming that 20 A_{260} units equal 1 mg/ml (Nishimura and Weinstein, 1969).

TABLE 1: Binding of Radioactive AAF and *N*-OH-AAF to Rat Liver RNA *in Vivo*.

Compound Injected	Binding ($\mu\mu$moles/mg) to: rRNA	tRNA
[^{14}C]AAF	52	81
[^{14}C]*N*-OH-AAF	65	160

Eighteen hours after the intraperitoneal injection of either compound, 33 μCi (11 μmoles) per 150 g of body weight, liver RNA was extracted by the phenol method and fractionated into tRNA and rRNA by 1 M NaCl extraction (see *Methods*). Radioactivity determinations were made on 5% trichloroacetic acid precipitable material. Additional details are described under Methods.

Radioactivity measurements were made with a Packard TriCarb liquid scintillation spectrometer. All samples were precipitated with 5% trichloroacetic acid and 500 μg of carrier DNA, collected on 24-mm membrane filters (0.45 μ pore size), washed with 5% cold trichloroacetic acid and 60% ethanol, and counted in 10 ml of Bray's solution, as previously described (Baliga *et al.*, 1969). In some experiments liquid samples were counted directly in Bray's solution.

Results

Distribution of AAF in tRNA and rRNA. As a first step in the study of binding of radioactive AAF or *N*-OH-AAF to liver tRNA, it was important to determine the relative distribution of the label in various types of RNA. Table I contains results obtained when liver tRNA and rRNA were separated by extraction with 1 M NaCl at 5°. With both AAF and *N*-OH-AAF, the tRNA had a higher specific activity than rRNA, when studied at 18 hr after injection. Furthermore, whereas binding of both compounds to rRNA was comparable, twice as much of the proximate carcinogen, *N*-OH-AAF, was bound to tRNA as the parent compound AAF. In other experiments, when guinea pigs were injected with 9-[^{14}C]AAF, less than 2 $\mu\mu$moles of AAF was bound per mg of tRNA. The lack of *in vivo* binding to guinea pig tRNA has also been observed by Drs. Elizabeth and James Miller (personal communication). This finding is of particular interest, since guinea pigs are resistant to hepatocarcinogenesis by AAF, presumably because they fail to activate the compound (Miller and Miller, 1967).

Other fractionation procedures were employed to examine the distribution of the labeled carcinogen in RNA. Rat liver RNA, extracted 18 hr after injecting [9-^{14}C]*N*-OH-AAF, was applied to a column of Sephadex G-100. The first peak in Figure 1 represents mainly 18S and 28S ribosomal RNA. Fractions 34–36 contain 5S RNA, and the last peak corresponds with 4S RNA, or tRNA. The 4S region had approx-

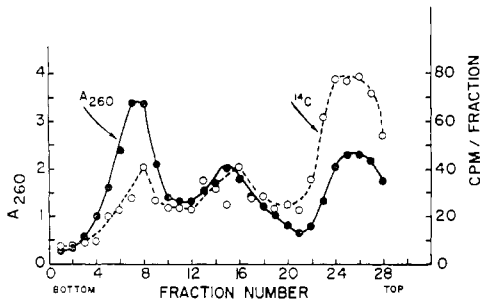

FIGURE 2: Sucrose gradient analysis of RNA from [^{14}C]*N*-OH-AAF injected rats. RNA (4 mg) obtained as described in the text was layered on top of 30 ml of 5–20% sucrose gradient, buffered with 0.02 M Na phosphate (pH 7), and centrifuged at 24,000 rpm for 18 hr in the SW 25.1 rotor of a Spinco ultracentrifuge, Model 12-65B. Fractions of 1 ml each were collected and, after determination of A_{260}, processed for determination of radioactivity by precipitation with 5% trichloracetic acid as described under Methods.

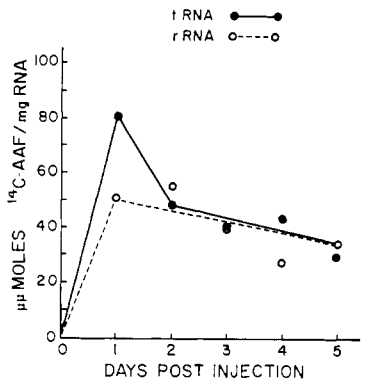

FIGURE 3: Kinetics of binding [^{14}C]*N*-OH-AAF to rat liver rRNA and tRNA *in vivo*. Rats were injected with [^{14}C]*N*-OH-AAF (33 μCi/150 g of body weight) and sacrificed at the indicated time points. For further details, see legend to Table I.

imately twice the specific activity of the 18S, 28S, and 5S regions. Identification of the 4S and 5S regions was confirmed by gel electrophoresis (Grossbach and Weinstein, 1968) of material reprecipitated from these fractions.

When rat liver RNA was fractionated on 5–20% sucrose gradients, the 4S RNA was again found to have approximately two to three times the specific activity of 18S and 28S RNA (Figure 2). Similar results have been described by Henshaw and Hiatt (1963) and by Irving *et al.* (1967).

Kinetics of Binding of AAF to tRNA and rRNA. Results described thus far leave little doubt that the carcinogen AAF, and its *N*-hydroxy metabolite, bind preferentially to rat liver tRNA, as compared with liver rRNA. It became important, therefore, to study the kinetics of binding of 9-[^{14}C]*N*-AAF to rat liver RNA. Ten rats were injected intraperitoneally with 9-[^{14}C]AAF (33 μCi/rat) and sacrificed 1, 2, 3, 4, and 5 days thereafter. Data in Figure 3 indicate that binding of the radioactive carcinogen to liver tRNA and rRNA was maximal by 24-hr postinjection. Earlier time points indicated that maximal binding was actually achieved by 12 hr. Within the first 24 hr tRNA had a higher specific activity than rRNA. (See also Table I and Figures 1 and 2.) Subsequently, the specific activity of tRNA declined rapidly so that, by 48-hr postinjection, tRNA and rRNA had comparable specific activities which declined with a $T_{1/2}$ of approximately 5 days. The latter value is comparable with previously reported data on the half-life of normal rat liver tRNA and rRNA (Darnell, 1968). These results suggest that, after modification with AAF, a fraction of the tRNA population is unstable but that the remaining fraction of the modified tRNA has a normal half-life. The possibility that our tRNA fraction was contaminated with a small amount of some other macromolecular substance, which complexes with AAF and has a rapid turnover, is unlikely in view of the results obtained below.

Separation of AAF tRNA by Column Chromatography. Even though AAF binds preferentially to tRNA *in vivo*, the total amount bound after a single injection is extremely small. It was important, therefore, to develop a technique which would separate those tRNAs which had reacted with AAF from those which did not, since this would permit further characterization of the reactive fraction. Chromatography on BD-cellulose columns was chosen for this purpose, since this resin has a high affinity for lipophilic substances and, therefore, would be expected to retain tRNAs bearing AAF residues. Indeed, previous studies from this laboratory had established that the BD-cellulose column did resolve *E. coli* tRNA, which had been modified with *N*-acetoxy-AAF *in vitro*, from the unmodified tRNA (Fink *et al.*, 1970). As shown by data in Figure 4, when rat liver tRNA was applied to a BD-cellulose column, 90–95% of the A_{260} material eluted from the column with the 0.3–1.5 M NaCl gradient (fractions numbers 1–65), the remaining 5–10% was eluted with a 5–20% ethanol gradient (fraction numbers 66–110). Previous studies indicate that most of the amino acid specific tRNAs in normal rat liver are eluted in the region of the NaCl gradient (Fink *et al.*, 1968; M. K. Agarwal and I. B. Weinstein, unpublished studies). An exception to this is tRNA^{Phe} which contains a

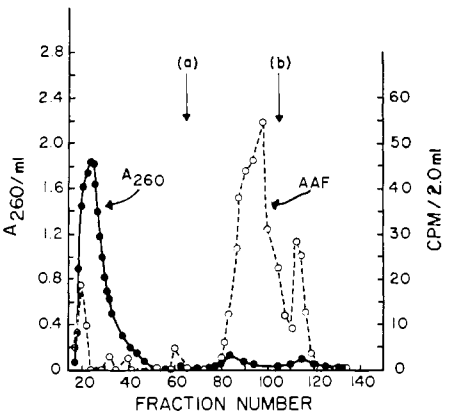

FIGURE 4: Chromatography of AAF-tRNA on a BD-cellulose columns. A BD-cellulose column (0.8 × 12 cm) was equilibrated with 0.01 M Na acetate (pH 4.5)–0.01 M $MgSO_4$ buffer containing 0.3 M NaCl. tRNA, 4 mg in 2 ml of buffer, prepared as described in the text, was applied to the column. A linear gradient elution was carried out, using 100 ml each of 0.3 and 1.5 M NaCl, both buffered as described above. The column was then washed [starting at (a)] with 200 ml of a linear 5–20% ethanol gradient, in 1.5 M NaCl, buffered as above. Finally, the column was washed with 30% ethanol and 1.5 M NaCl [starting at (b)]. Fractions of 4 ml each were collected at a flow rate of 30 ml/hr, and processed for determination of radioactivity, as described in the legend to Figure 2.

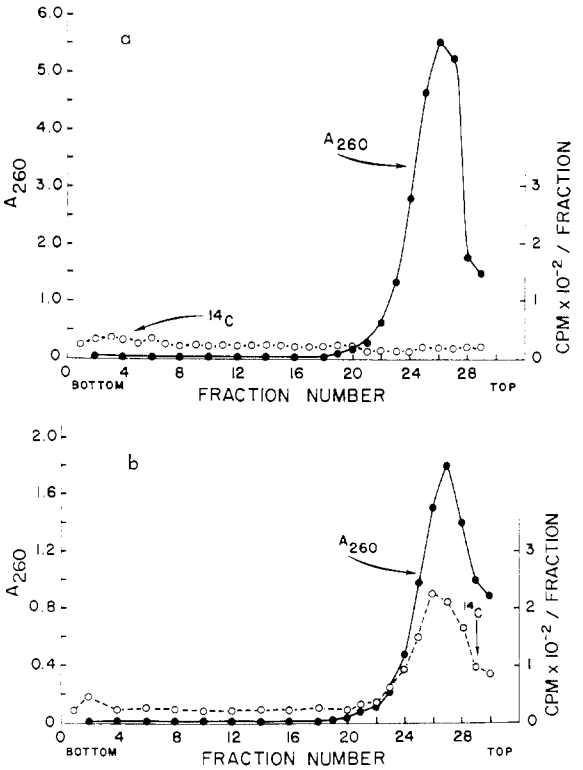

FIGURE 5: Sucrose gradient analyses of RNA precipitated from the salt and ethanol regions of a BD-cellulose column. Details of chromatography on a BD-cellulose column are described in the legend to Figure 4. The salt and the ethanol regions correspond with fractions 20–40 and 80–120 of Figure 4. The RNA from these regions was precipitated with Na acetate and ethanol, and applied to sucrose gradients, as described in the legend to Figure 2. Figure 5a corresponds to the salt region, and 5b to the ethanol region, respectively.

highly lipophilic residue and, therefore, elutes in the ethanol region. When liver tRNA extracted 18 hr after the injection of [9-^{14}C]*N*-2-OH-AAF was applied to this column, almost all of the radioactivity remained tightly bound to the column and was eluted only with 5–30% ethanol (Figure 4).

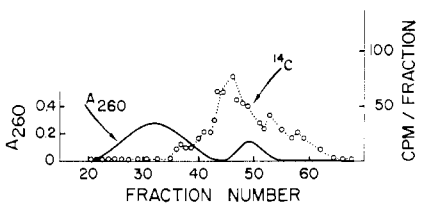

FIGURE 6: Chromatography of partially purified AAF-tRNA on a MAK column. A MAK column (3.0 × 3.5 cm) was prepared and equilibrated as described by Axel *et al.* (1967). Details for the preparation of AAF-tRNA from the ethanol region of a BD-cellulose column are described in the legend to Figure 5. This tRNA (0.5 mg) was mixed with 1.5 mg of unfractionated normal rat liver tRNA and applied to the column. A linear gradient elution was carried out with 100 ml each of 0.2 and 1.0 M NaCl in 0.05 M Na phosphate buffer (pH 6.7). Fractions of 2 ml were collected at a flow rate of 60–70 ml/hr, and processed for determination of 5% trichloroacetic acid precipitable radioactivity, as described under Methods.

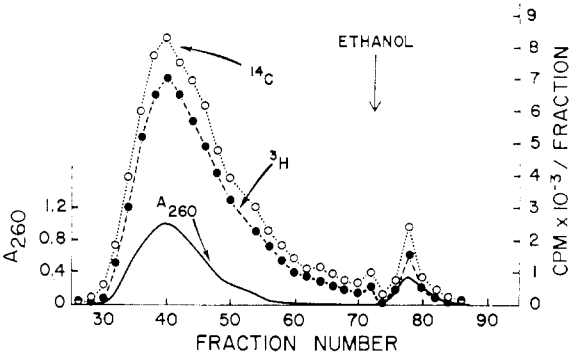

FIGURE 7: Cochromatography of tRNA obtained from rats fed either a control or an AAF-containing diet and labeled *in vivo* with [^{14}C]- and [^{3}H]orotic acid, respectively. Details for the preparation, equilibration and operation of the BD-cellulose column are described in the legend to Figure 4. tRNA (2 mg), obtained as described in the text, was chromatographed, and the radioactivity precipitable with 5% trichloroacetic acid was determined in each fraction.

The labeled material appeared later in the ethanol region than the normal position of tRNA^{Phe}. Similar results were obtained when tRNA was extracted from rats injected with [9-^{14}C]AAF in place of [9-^{14}C]*N*-2-OH-AAF.

To further determine the nature of the radioactive material eluting in the ethanol region of the aforementioned column, the BD-cellulose procedure was carried out as described above, except that 30% ethanol was used in place of the 5–20% ethanol gradient. This permitted elution of most of the radioactive material as a sharp peak contained in a small volume of eluate. The tRNA present in the NaCl gradient region was pooled, a separate pool was made of the radioactive material eluted with ethanol, and both materials were concentrated by ethanol precipitation. Assays of aliquots indicated that the RNA from the ethanol region had 12 times the specific activity of that of the NaCl region. Sucrose gradient analysis of these RNAs indicated that both contained predominantly 4S RNA, and that almost all of the radioactivity present in the ethanol region of the BD-cellulose column was associated with 4S RNA (Figure 5b). No significant radioactivity could be detected in the 4S peak obtained from the NaCl region of the BD-cellulose column (Figure 5a).

Further Characterization of AAF tRNA. The question to be considered next was whether AAF interacts *in vivo* with the tRNA specific for only a single amino acid or with several, and perhaps all, types of tRNA. For this purpose, radioactively labeled AAF-tRNA was obtained from the ethanol region of a BD-cellulose column, as described in the preceding section, and chromatographed on a MAK column in the presence of a threefold excess (on an A_{260} basis) of unfractionated control tRNA (Figure 6). The AAF-tRNA is represented by the radioactivity, whereas the A_{260} profile mainly reflects the pattern of normal tRNA. The elution of the AAF-tRNA was markedly delayed when compared with normal tRNA. In contrast to the sharp peaks obtained on the MAK column with rat liver tRNAs specific for individual amino acids (Axel *et al.*, 1967; Baliga *et al.*, 1969), the profile of AAF-tRNA revealed a broad, heterogenous peak. The latter result suggests that several types of tRNA had reacted with AAF *in vivo*.

Studies employing *in vivo* orotic acid labeling of tRNA were also performed to examine the effect of a carcinogenic AAF diet on the profile of newly synthesized tRNAs. Two groups of rats were used. The first was fed for 2 weeks on a diet containing AAF (0.25%) (Miller, 1968), and the second control group the same diet without AAF. Prior to sacrifice (18 hr) the first group received [^{3}H]orotic acid (1 mCi/150 g of body weight) and the other group [^{14}C]orotic acid (100 μCi/150 g of body weight), intraperitoneally. Equal weights of liver from each group were mixed and the tRNAs were extracted with phenol (see Methods). This material (2 mg) was chromatographed on a BD-cellulose column (Figure 7). Since the ^{14}C and ^{3}H elution profiles were essentially identical, it would seem that the AAF diet had not grossly affected the profile of tRNA synthesis in rat liver. It is of particular interest that no major differences were evident in the ethanol region. Since tRNAs bearing AAF mainly elute in this region (see Figure 4), this indicates that dietary AAF must bind to only a very small (and thus far undetectable) fraction of the newly synthesized tRNAs.

Discussion

The above studies clearly demonstrate that the hepatic carcinogen AAF and its metabolite, *N*-OH-AAF, complex with rat liver tRNA *in vivo*. That AAF is bound to tRNA, and not a contaminant, has been established by phenol extraction and 1 M NaCl fractionation, Sephadex G-100 chromatography, and sucrose gradient centrifugation. Our results are consistent with previous evidence that this carcinogen binds *in vivo* to liver nucleic acids, both DNA and RNA (Miller and Miller, 1967). It has also been established that the major radioactive derivative in hydrolysates of RNA obtained from animals previously injected with labeled AAF *in vivo*, as well as of RNA reacted *in vitro* with *N*-acetoxy-AAF, is 8-(*N*-2-fluorenylacetamide)guanosine (Miller *et al.*, 1966; Kriek *et al.*, 1967).

Analyses of liver RNAs obtained shortly after *in vivo* injection of the drug indicated that the tRNA fraction had two to three times the specific activity of 5S, 18S, and 28S RNA. This preferential labeling of tRNA is also apparent in previously published data (Henshaw and Hiatt, 1963; Irving *et al.*, 1967). The high specific activity of tRNA may reflect the fact that, in contrast to ribosomal RNA and cellular DNA, this nucleic acid exists *in vivo* free of proteins and, therefore, is readily susceptible to chemical modification. Additional factors such as differences between rRNA and tRNA in terms of pool sizes or relative rates of formation and release may also account for the higher specific activity of tRNA observed in these studies. Our results do not exclude the possibility that AAF also interacts *in vivo* with messenger RNAs or specific nuclear RNAs. Indeed, although the 4S region of our sucrose gradients contained most of the radioactive AAF, we consistently observed small amounts of radioactivity associated with more rapidly sedimenting material. In particular, the possible *in vivo* interaction with mRNA deserves further evaluation, since recent results indicate that *in vitro* attachment of AAF to guanine residues in synthetic mRNAs markedly inhibit their coding properties (Grunberger and Weinstein, 1969).

The ability of BD-cellulose to selectively retain tRNAs containing AAF provides a method for obtaining almost complete separation of the small amounts of tRNA which react with the carcinogen *in vivo* from the bulk of the unmodified tRNA. This method should be useful for purifying the AAF-tRNA for further characterization. The distribution of AAF-tRNA on the MAK column suggests that the drug binds to several rather than only one type of tRNA. This also appears to be the case in the *in vitro* reaction of low concentrations of *N*-acetoxy-AAF with *E. coli* tRNA (Fink *et al.*, 1968). Thus far we have not been able to accumulate a sufficient amount of the purified AAF-tRNA to assess its functional properties, due to the small amounts of AAF-tRNA formed *in vivo*.

Finally, we can only speculate about the possible causal relation of AAF binding to tRNA and the mechanism of carcinogenesis. As mentioned above, the binding to tRNA may merely reflect the fact that tRNA lies freely exposed in the cell to a variety of chemical modifications. On the other hand, the increasing evidence that tRNA may play an important role in cell regulation, as well as previous evidence from this laboratory (Fink *et al.*, 1968) that AAF modifications of tRNA *in vitro* can produce specific effects on its structure and function, support the hypothesis that tRNA may be a critical target during carcinogenesis.

References

Agarwal, M. K., and Weinstein, I. B. (1969), *Proc. Am. Assoc. Cancer Res. 10*, 4.

Axel, R., Weinstein, I. B., and Farber, E. (1967), *Proc. Natl. Acad. Sci. U. S. 58*, 1255.

Baliga, B. S., Borek, E., Weinstein, I. B., and Srinivasan, P. R. (1969), *Proc. Natl. Acad. Sci. U. S. 62*, 899.

Darnell, J. E., Jr. (1968), *Bacteriol. Rev. 32*, 262.

Farber, E., McConomy, J., Franzen, B., Marroquin, F., Stewart, G. A., and Magee, P. N. (1967), *Cancer. Res. 27*, 1761.

Fink, L. M., Goto, T., Frankel, F., and Weinstein, I. B. (1968), *Biochem. Biophys. Res. Commun. 32*, 963.

Fink, L. M., Nishimura, S., and Weinstein, I. B. (1970), *Biochemistry 9*, 496.

Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.

Grossbach, U., and Weinstein, I. B. (1968), *Anal. Biochem. 22*, 311.

Grunberger, D., and Weinstein, I. B. (1969), *Abstract IX, Intern. Cancer Congr.* (in press).

Henshaw, E. C., and Hiatt, H. H. (1963), *Proc. Am. Assoc. Cancer Res. 4*, 27.

Irving, C. C., Veazey, R. A., and Williard, R. F. (1967), *Cancer Res. 27*, 720.

Kirtikar, M. W. D., and Kaji, A. (1968), *J. Biol. Chem. 243*, 5345.

Kriek, E., Miller, J. A., Juhl, U., and Miller, E. C. (1967), *Biochemistry 6*, 177.

Miller, E. C., Juhl, U., and Miller, J. A. (1966), *Science 153*, 1125.

Miller, J. A. (1968), *Cancer Res. 28*, 1875.

Miller, J. A., and Miller, E. C. (1967), Carcinogenesis: A Broad Critique, Twentieth Annual Symposium on Fundamental Cancer Research, Baltimore, Md., Williams & Wilkins, p 397.

Nishimura, S., and Weinstein, I. B. (1969), *Biochemistry 8*, 832.
Weinstein, I. B. (1968), *Cancer Res. 28*, 1871.
Weinstein, I. B. (1969), Genetic Concepts and Neoplasia, Twenty-third Annual Symposium on Fundamental Cancer Research, Baltimore, Md., Williams & Wilkins (in press).

Peptide Chain Elongation: Indications for the Binding of an Amino Acid Polymerization Factor, Guanosine 5′-Triphosphate–Aminoacyl Transfer Ribonucleic Acid Complex to the Messenger–Ribosome Complex*

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ABSTRACT: The intermediate steps in peptide chain elongation were studied in a cell-free system in which polyuridylic acid directs the formation of polyphenylalanyl transfer ribonucleic acid from phenylalanyl transfer ribonucleic acid. The system consists of guanosine 5′-triphosphate, salts, *Escherichia coli* ribosomes, and three amino acid polymerization factors (S_1, S_2, and S_3) from *Bacillus stearothermophilus*. (The factors from *B. stearothermophilus* correspond to the following factors from *E. coli:* S_1 to T_s, S_2 to G, and S_3 to T_u.) The incubation of S_1, S_3, guanosine 5′-triphosphate, and phenylalanyl transfer ribonucleic acid results in the formation of a S_3–guanosine 5′-triphosphate–phenylalanyl transfer ribonucleic acid complex (complex II). The incubation of complex II with a ribosome–polyuridylic acid–peptidyl transfer ribonucleic acid complex (actually acetylphenylalanyl transfer ribonucleic acid was used instead of peptidyl transfer ribonucleic acid) leads to the cleavage of guanosine 5′-triphosphate and the formation of an S_3–guanosine 5′-diphosphate complex and inorganic phosphate, as well as the synthesis of acetyldiphenylalanyl transfer ribonucleic acid. 5′-Guanylylmethylenediphosphonate, an analog of guanosine 5′-triphosphate which cannot be cleaved to guanosine 5′-diphosphate and inorganic phosphate, was substituted for guanosine 5′-triphosphate in an incubation mixture also containing S_1, S_3, phenylalanyl transfer ribonucleic acid, and the ribosome–polyuridylic acid–acetylphenylalanyl transfer ribonucleic acid complex. Under these conditions, approximately equimolar amounts of S_3, 5′-guanylylmethylenediphosphonate, and phenylalanyl transfer ribonucleic acid became bound and remained attached to ribosomes, and acetyldiphenylalanyl transfer ribonucleic acid was not formed. These results may reflect the transient existence of a ribosome-bound S_3–guanosine 5′-triphosphate–phenylalanyl transfer ribonucleic acid complex as an intermediate preceding acetyldiphenylalanyl transfer ribonucleic acid synthesis. The short life span of this hypothetical intermediate may be due to the rapid cleavage of the guanosine 5′-triphosphate molecule in it, which results in the release of an S_3–guanosine 5′-diphosphate complex and inorganic phosphate from the ribosome and acetyldiphenylalanyl–transfer ribonucleic acid formation.

The progress in the elucidation of the intermediate steps in peptide chain elongation is the consequence mainly of two developments: the isolation of the amino acid polymerization factors involved in the process, and the use of simple messenger RNAs, *i.e.*, homopolynucleotides in cell-free amino acid incorporating systems (Lipmann, 1969; Lengyel and Söll, 1969). *Escherichia coli* and *Pseudomonas fluorescens* were the first microorganisms in which three amino acid polymerization factors (T_u, T_s, and G) were described (Lucas-Lenard and Lipmann, 1966). The reported lability of one of these factors, T_u, prompted us to attempt the isolation of similar components from a different source. We purified from a thermophilic microorganism, *Bacillus stearothermophilus*, three such factors: S_1 corresponding to T_s, S_2 corresponding to G, and S_3 corresponding to T_u (Skoultchi *et al.*, 1968). Two of these are partially purified preparations (S_1, 30-fold; S_2, 70-fold) while the third, S_3 (purified 100-fold), moves as a single band on acrylamide gel electrophoresis. In a cell-free system consisting of S_1, S_2, and S_3 from *B. st.*,[1] thoroughly washed ribosomes from *E. coli*, GTP, and salts, polyuridylic acid directs the formation of polyPhe-tRNA from Phe-tRNA. The incubation of S_1 and S_3 with GTP and Phe-tRNA resulted in the formation of an S_3–GTP–Phe-tRNA complex (complex II) (Skoultchi *et al.*, 1968;

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[1] Abbreviations used are: *B. st.*, *Bacillus stearothermophilus;* GMPPCP, 5′-guanylylmethylenediphosphonate; polyPhe-tRNA, polyphenylalanyl transfer ribonucleic acid; Phe-tRNA, phenylalanyl-tRNA; ac-Phe-tRNA, acetylphenylalanyl-tRNA; ac-diPhe-tRNA, acetylphenylalanylphenylalanyl-tRNA; ac-polyPhe-tRNA, acetylpolyphenylalanyl-tRNA; AA-tRNA, aminoacyl-tRNA; RPA complex, ribosome–poly U–acetyl-Phe-tRNA complex; complex II, S_3–GTP–Phe-tRNA complex.