Uptake of Exogenous tRNA by Novikoff Hepatoma Ascites Cells* (35678)

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Recently, interest in RNA uptake by mammalian cells has been stimulated by studies on infectivity of viral RNA and interferon production (1–5). In addition, a number of types of polycations have been shown to enhance infectivity and interferon production (4–17). Studies on viral RNA infectivity and interferon production were aided by biological amplification, but in the demonstration of cellular uptake of RNA or oligonucleotides which may not be amplified, more precise biochemical assays are necessary. Because the possibility exists that macromolecules or macromolecular segments may become useful in chemotherapy (18), the present study was designed to determine whether intact tRNA could be taken up by tumor cells as a preliminary step in the delineation of the mechanisms of uptake, the intracellular distribution, and possible effects of uptake of exogenous RNA or oligonucleotides. Homologous tRNA was chosen because of its relatively small size, ready obtainability and ease of purification. In addition, tRNA from Novikoff hepatoma ascites cells (NHAC[1]) can be highly labeled. During the course of these studies, Herrera *et al.* (19) demonstrated that functional *E. coli* tRNA became associated with normal and tumor cells after short incubation. This finding has been confirmed, but in the present studies, it was found that DEAE-D[1] was necessary for protection of tRNA and to enhance its uptake into cells.

Materials and Methods. Novikoff hepatoma ascites cells (NHAC). The tumor cells were maintained in adult male rats from the Holtzman Company (Madison, Wisconsin). Six days after transplantation, the ascites cells were harvested by abdominal drainage.

Preparation of tRNA. Approximately 50 mg of unlabeled tRNA (Fig. 1) were extracted by the hot phenol–SDS[1] method (20–22) from the 105,000*g* supernatant from 600 g of NHAC. After ethanol precipitation, the tRNA was centrifuged through a linear 5–40 sucrose density gradient at 28,000 rpm in a B-15 rotor of a Spinco zonal ultracentrifuge

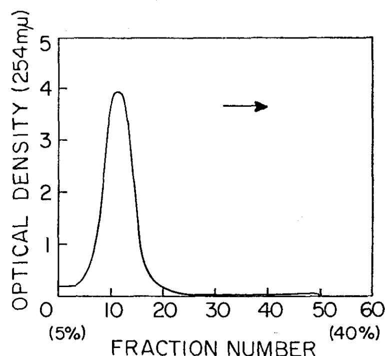

FIG. 1. Purification of NHAC tRNA by zonal ultracentrifugation: Approximately 50 mg of tRNA extracted by the hot phenol–SDS method from the 105,000*g* supernatant of NHAC (ca. 600 g) were centrifuged through a linear 5–40% sucrose density gradient in a B-15 rotor of a Spinco zonal ultracentrifuge for 16 hr at 28,000 rpm. (arrow) The direction of sedimentation.

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[1] The abbreviations used are: NHAC = Novikoff hepatoma ascites cells; DEAE-D = diethylaminoethyl-dextran; SDS solution = 0.3% sodium dodecylsulfate, 0.14 *M* sodium chloride, in 0.05 *M* sodium acetate buffer, pH 5.1; NKM = 0.13 *M* sodium chloride, 0.005 *M* potassium chloride, and 0.008 *M* magnesium chloride; XDC = a mixture of 2.5-diphenyloxazole, 35 g; 1,4-bis[2-(5-phenyloxazolyl)]-benzene, 1.75 g; naphthalene, 280 g; xylene, 500 ml; 1,4-dioxane, 1500 ml; and ethylene glycol monomethyl ether, 1500 ml.

for 16 hr (22). ^{32}P-labeled tRNA was obtained by the same procedure from the 105, 000*g* supernatant from 80 g of NHAC which had been incubated as previously described (21) with 500 mCi of carrier-free ^{32}P-orthophosphate. Approximately 9 mg of ^{32}P-tRNA (1.4×10^9 cpm/mg) were prepared by this method. In each case, the RNA preparation was shown by gel electrophoresis to contain a single band of 4S RNA (22).

Incubation and treatment of cells. All glassware used for incubations was acid-washed, siliconized, and steam sterilized to avoid bacterial and RNase contamination. Cells for incubation were purified, by the method of Mauritzen *et al.* (21), and suspended at a final concentration of 3×10^6 cells/ml in modified Eagle's medium from which calf serum was omitted[2] (21). In all cases, the cells were preincubated for 30 min at 37° prior to addition of labeled tRNA. After the incubations, they were pelleted by centrifugation for 5 min at 850*g* in a Sorvall refrigerated RC-3 centrifuge. An aliquot of the supernatant was taken to determine extracellular radioactivity. The cells were then washed three times with ice-cold NKM solution.[1] To determine cell-associated radioactivity, the cells were resuspended in 0.5 ml of NKM and the entire suspension was placed in 10 ml of scintillation fluor (XDC)[1] and counted in a Packard Tri-Carb liquid scintillation spectrometer (Model 3375). When sucrose density gradient centrifugation was to be performed to determine the degree of integrity of the tRNA, the cells were resuspended in SDS solution (1 g of cells/15 ml) and whole cellular RNA extracted by the hot phenol–SDS method (20). In such studies, an aliquot of the phenol–SDS homogenate of the cells was taken to determine cell-associated radioactivity.

When DEAE-cellulose column chromatography was employed to determine the integrity of the ^{32}P-tRNA in the cytoplasm, approximately 10 g of unlabeled cells were added to the labeled cells as carrier; the cells were then resuspended in 6 vol of 5% citric acid, an aliquot was taken to determine cell-associated radioactivity, and nuclei were isolated by the citric acid procedure (22). The pooled citric acid–sucrose supernatants contained the cytoplasmic fraction.

Spectral change of tRNA in the presence of DEAE-D. An aliquot of tRNA solution (50 μg/ml) was mixed with an equal volume of DEAE-D (Pharmacia, Uppsala, Sweden; mol wt. ca. 2×10^6; N content, ca. 3.2%) solutions of various concentrations. The absorbance at 260 mμ of each solution was measured in a Zeiss spectrophotometer, Model PMQ II, with a reference of each concentration of DEAE-D solution. The relative absorbance is the ratio of absorbance of the tRNA solutions containing DEAE-D to those tRNA solutions (25 μg/ml) which did not.

Sucrose density gradient centrifugation. For analytical runs, approximately 1 mg of RNA (containing ^{32}P-RNA) was layered on linear sucrose density gradients (5 to 40%) containing 0.1 *M* sodium chloride; 0.001 *M* EDTA; and 0.01 *M* sodium acetate buffer, pH 5.1. After centrifugation at 25,000 rpm for 18 hr in an SW-27 rotor of the Spinco Model L2 ultracentrifuge, the gradients were fractionated with the aid of an ISCO density gradient fractionator. The radioactivity of each fraction was then determined (20–22).

Rapid adsorption of tRNA to cells. ^{32}P-tRNA and DEAE-D at final concentrations of 0.05 mg/ml and 0.1 mg/ml, respectively, were added to the incubation mixture (3×10^6 cells/ml of modified Eagle's medium) which had been preincubated for 30 min at 37° and chilled to 0°. This mixture was immediately filtered through Whatman No. 2 filter paper (24-mm diam) with a Millipore apparatus. The filter paper was then washed three times with 1 ml of ice-cold NKM solution. The radioactivity on the filter and that of the filtrate were determined as described.

RNase treatment. The cell pellet obtained after incubation was washed three times with 1 ml of ice-cold phosphate buffer (0.05 *M* potassium phosphate, 0.14 *M* sodium chloride, pH 7.5), then resuspended in 2 ml of the

[2] The composition of modified Eagle's medium is a mixture of 22 amino acids, 0.5 m*M* each; L-glutamine, 2 m*M*; a mixture of 6 vitamins, 1 mg/liter each; riboflavin, 0.1 mg/liter; inositol, 2 mg/liter; penicillin G, 50 mg/liter; streptomycin sulfate 50 mg/liter; D-glucose, 18 g/liter; Earle's salts (no phosphate), pH 7.4.

TABLE I. Effect of DEAE-D on Cell Association of tRNA.[a]

Conc of DEAE-D (mg/ml)	Percentage of total radioactivity		
	Extracellular	Cell-associated	recovery (%)
0	82.7 ± 2.4	9.4 ± 0.8	92.1 ± 2.5
0.01	73.8 ± 2.9	13.4 ± 1.9	87.2 ± 3.5
0.03	68.2 ± 3.1	19.2 ± 2.2	87.4 ± 3.8
0.1	63.1 ± 2.4	25.2 ± 3.0	88.3 ± 3.8
0.3	76.3 ± 5.9	15.1 ± 2.1	91.4 ± 6.3
1.0	87.8 ± 2.7	10.0 ± 1.4	97.8 ± 3.0

[a] NHAC (3×10^6 cells/ml of modified Eagle's medium) were incubated for 120 min at 37° with ^{32}P-tRNA (0.05 mg/ml) and DEAE-D. The values are means of four experiments. The standard deviations were calculated by the following relationship: $SD = (\Sigma x^2 - \Sigma \bar{x}^2)^{1/2}/(n-1)$.

same buffer. An aliquot was taken to determine the cell-associated radioactivity, and 20 μg of bovine pancreatic RNase A (Worthington, Freehold, N.J.) were added. The cells were then incubated for 10 min at 37°, chilled, and immediately centrifuged at 850*g* in a Sorvall refrigerated RC-3 centrifuge. The cells were washed three times with 2 ml of the phosphate buffer, resuspended in 0.5 ml of the same buffer, and the radioactivity of the entire suspension was determined. In some experiments, whole cellular RNA was extracted, subjected to sucrose density gradient centrifugation, and the fractions obtained were analyzed for distribution of ^{32}P.

DEAE-cellulose column chromatography. Ion exchange chromatography was carried out on DEAE-cellulose (Whatman DE-52) prepared as described by Tener (23). Columns, 0.5 × 30 cm, were packed with DEAE-cellulose in starting buffer. Elution was carried out in 0.05 *M* sodium acetate buffer; 7 M urea, pH 5.4; with a linear sodium chloride gradient from 0 to 0.3 *M*. Residual radioactivity was eluted with 2 *M* sodium chloride. The total gradient volume was 300 ml. Fractions of 3 ml were collected, the absorbance at 260 mμ was analyzed with a Zeiss spectrophotometer, and the radioactivity was determined. Approximately 1.5 mg of torula RNA (Calbiochem, Los Angeles, Calif.) digested with bovine pancreatic RNase A were added as unlabeled carrier.

Results. Effect of DEAE-D on the cellular association of tRNA. Approximately 8% of the radioactivity of added ^{32}P-tRNA was cell-associated in the absence of any enhancing agent (Table I, Fig. 2). To determine whether this amount could be increased, several substances were investigated for "enhancing activity." Of these,[3] DEAE-D was most effective in a concentration of 0.1 mg/ml. In the experiments on the enhancing activity of DEAE-D, the concentration of ^{32}P-tRNA was 0.05 mg/ml (1 OD_{260} unit/ml). At higher concentrations of ^{32}P-tRNA, it was precipitated by DEAE-D in a concentration of 0.1 mg/ml; at lower concentrations of tRNA, less tRNA was cell associated.

At the optimal concentration of DEAE-D, the ^{32}P found in the cellular fraction at 120 min was approximately 24% of the total added, *i.e.*, approximately 3 times that of the control experiments in which DEAE-D was absent (Fig. 2).

Figure 2 shows the time course of cellular association of ^{32}P-tRNA in the presence of DEAE-D (0.1 mg/ml). The cell-associated radioactivity of ^{32}P-tRNA reached a plateau (23% at 60 min). The increase in uptake in the presence of DEAE-D at 37° is highly significant ($p<0.001$; $n = 17$). At 0°, the cell-associated radioactivity was 6.3% of the total in the presence of DEAE-D at zero time and 9.0% at 120 min. Thus, approximately 15% more tRNA is cell associated after incubation at 37° for 120 min than at 0°.

Complex formation between tRNA and

[3] A number of other potential enhancers were tested as enhancers but were found to be ineffective including fetal calf serum (10%), albumin (bovine fraction V) and γ-globulin (bovine fraction II) in concentrations of 0.1 to 10.0 mg/ml and Fetuin in concentrations of 0.1 to 1.0 mg/ml.

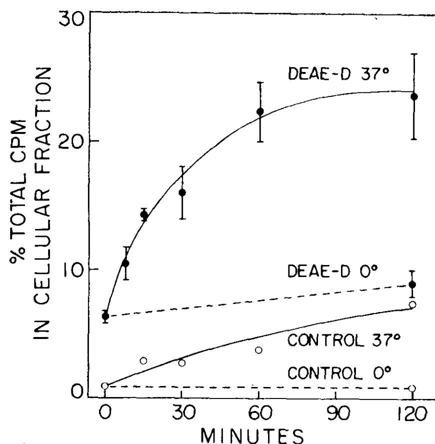

FIG. 2. Time course of cellular association of tRNA in the presence of DEAE-D (0.1 mg/ml): NHAC (3×10^6 cells/ml of modified Eagle's medium) were incubated under conditions shown. The values for DEAE-D are means and standard deviations from five experiments. The values for the controls are averages of two experiments.

DEAE-D. Since DEAE-D was found to be an effective enhancer of cell association of tRNA, it seemed possible that a tRNA–DEAE-D complex was formed. When a ^{32}P-tRNA solution (5 mg/ml) was mixed with an equal volume of DEAE-D solution (1 mg/ml), 92% of the radioactivity was found in the supernatant after the addition of 2 vol of ethanol containing 2% potassium acetate. In the absence of DEAE-D, only 1% of the tRNA was found in the supernatant. This result confirms the conclusion of Maes *et al.* (24) that a complex is formed. In addition, in the presence of DEAE-D (Fig. 3), 12–15% hyperchromicity of tRNA at 260 mμ was found when the weight ratio of DEAE-D:tRNA was 0.5–400:1. In the concentration used in the incubations shown in Fig. 2, the hyperchromicity of the DEAE-D–tRNA complex was about 13%.

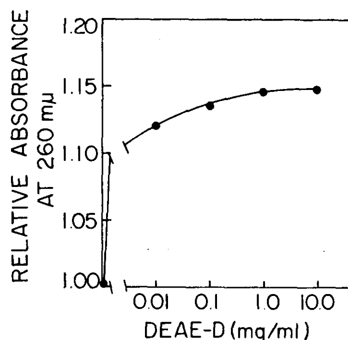

FIG. 3. Hyperchromicity of tRNA in the presence of DEAE-D: An aliquot of a tRNA solution (50 μg/ml) was mixed with an equal volume of a DEAE-D solution of the concentrations shown. The absorbance at 260 mμ of each solution was determined in a Zeiss spectrophotometer, model PMQ II. The relative absorbance is the ratio of absorbancy of the tRNA solutions containing DEAE-D to those tRNA solutions (25 μg/ml) which did not.

Another type of evidence for complex formation is presented in Fig. 4 which shows the change in the sucrose density gradient sedimentation pattern for ^{32}P-tRNA in the presence of the same relative concentration of DEAE-D employed in Fig. 2. In contrast to the control tRNA which had a sedimentation coefficient of 4S, the label remained at the top of the gradient in the presence of DEAE-D. Since DEAE-D had no RNase activity,[4] this shift presumably results from complex formation with tRNA.

Cellular adsorption and intracellular uptake of tRNA. In these studies, "cell-associated tRNA" is defined as the total tRNA which cosedimented with the cells. Some of the tRNA may simply have been "adsorbed" onto the cell surface, but some was apparently "taken up," *i.e.*, it was intracellular. To estimate the percentage of the cell-associated ^{32}P-tRNA that was "adsorbed," three methods were employed, namely, rapid filtration, 0° incubations, and RNase treatment. To avoid artifacts resulting from the duration of centrifugation, the rapid filtration method was employed to determine cell-associated radioactivity at zero time. Table II shows that 1.3 and 8.2% of total radioactivity were rapidly cell associ-

[4] Fetal calf serum, albumin, γ-globulin and DEAE-D were tested for RNase contamination by incubating them with ^{32}P-tRNA at 37° for 120 min. Degradation of tRNA was determined by ethanol precipitation or Amicon ultrafiltration. Calf serum and the serum proteins were highly contaminated with RNase, *e.g.*, calf serum produced 20.5 times as much degradation as in the control experiments. DEAE-D was not contaminated with RNase, *i.e.*, the degradation was only 1.1 times the control.

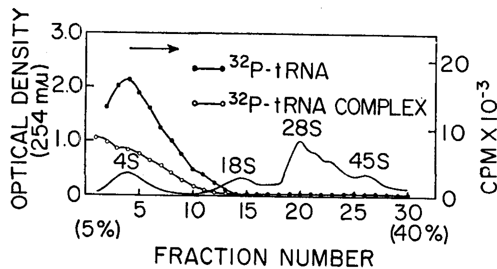

FIG. 4. Change in sucrose density gradient profile of tRNA in the presence of DEAE-D: ^{32}P-tRNA (0.05 mg) or its DEAE-D (0.1 mg) complex was layered on linear sucrose density gradients (5–40%) containing 0.1 M NaCl, 0.001 M EDTA; and 0.01 M Na-acetate buffer (pH 5.1); and certrifuged at 25,000 rpm for 18 hr in an SW-27 rotor of the Spinco Model L2 ultracentrifuge. The optical density profile is that of whole nuclear RNA (1 mg). (arrow) The direction of sedimentation; and (nos. above the peaks) the approximate sedimentation coefficients of the RNA.

ated in the control and DEAE-D experiments, respectively. These results agree well with the values obtained at 0° (Fig. 2). The lower recovery in the experiments with DEAE-D appears to be related to some adherence of the DEAE-D–tRNA complex to glass (v.i.).

In another procedure, the incubated cells were treated with pancreatic RNase at a concentration of 10 μg/ml at 37° for 10 min,

TABLE II. Rapid Adsorption of tRNA to Cells.[a]

Sample	Percentage of total radioactivity: In filtrate	Percentage of total radioactivity: On filter	Recovery (%)
Control	100.2	1.4	101.6
	97.0	1.2	98.2
DEAE-D, 0.1 mg/ml	74.0	7.7	81.7
	79.7	8.6	88.3

[a] ^{32}P-tRNA and DEAE-D at final concentrations of 0.05 and 0.1 mg/ml, respectively, were added to the incubation mixture (3 × 10^6 cells/ml of modified Eagle's medium) at 0°. This mixture was immediately filtered through Whatman No. 2 filter paper, which was washed three times with 1 ml of ice-cold NKM solution and then analyzed for radioactivity. The results of two experiments are shown.

since, at this enzyme to substrate ratio (1:5), tRNA is degraded (17, 25). Figure 5 shows that at the optimum DEAE-D concentration for cell association, the amount of RNase-resistant cell-associated radioactivity is also maximal. Furthermore, the similarity of the shapes of the curves indicates that cell association and uptake of the DEAE-D–tRNA complex are closely related. After RNase treatment of NHAC incubated with the ^{32}P-tRNA in the presence of DEAE-D (0.1 mg/ml), 40% of the cell-associated ^{32}P was RNase resistant, *i.e.*, the RNase-resistant radioactivity at this concentration was 9.6 ± 1.8% of the total (Fig. 5). This estimate of uptake by RNase treatment is not significantly different from that calculated from 0° and rapid adsorption experiments.

Integrity of cell-associated tRNA. To determine whether the cell-associated ^{32}P-tRNA was intact, DEAE-cellulose chromatography and sucrose density gradient centrifugation were employed. Figure 6B shows that tRNA recovered from cells incubated with DEAE-D–tRNA complex was not degraded to oligonucleotides. In Fig. 6, the peaks of absor-

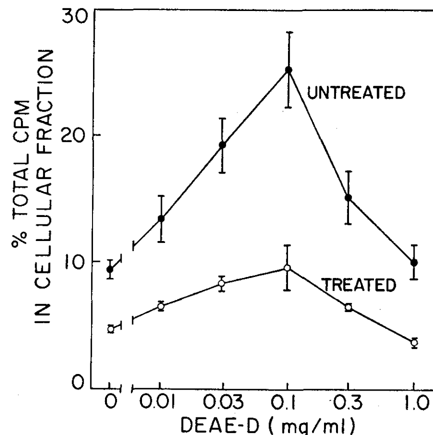

FIG. 5. Effect of pancreatic RNase on the cell-associated tRNA: The cells were incubated with the DEAE-D–tRNA complex under the condtions described for Table I. The cells were treated with pancreatic RNase (10 μg/ml) for 10 min at 37°. (●) The percentage of total radioactivity which was cell-associated before RNase treatment; and (○) the percentage found after RNase treatment. The values are means and standard deviations from four experiments.

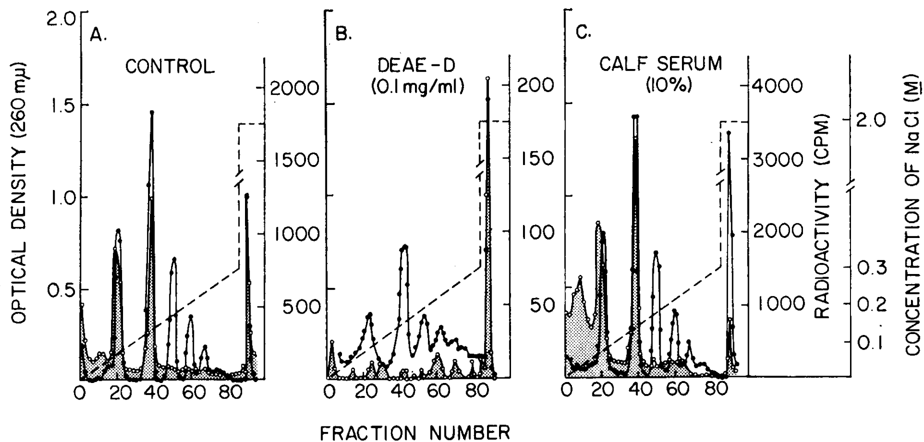

FIG. 6. Chromatography of cell-associated ^{32}P-tRNA or its degradation products on DEAE-cellulose (chloride form) columns: Cells were incubated with ^{32}P-tRNA (0.05 mg/ml) in the absence of enhancers (A); in the presence of DEAE-D (0.1 mg/ml) (B); in the presence of fetal calf serum (10%) (C). An aliquot (2 ml) of cytoplasmic fraction, *i.e.*, the supernatant of the 5% citric acid homogenate of cells, was chromatographed on DEAE-cellulose columns with approximately 1.5 mg of pancreatic RNase-digested torula RNA as carrier. Elution was carried out with a linear NaCl gradient followed by a 2 *M* NaCl wash. (●) The absorbance at 260 mμ; (shaded portions) the radioactivity (cpm); (- - -) the molarity of NaCl in the eluting buffer (7 *M* urea; 0.05 *M* sodium acetate buffer, pH 5.4). Because the samples contained cytoplasmic proteins and other components, the columns were washed with 15 ml of buffer before the gradient was started. In the chromatographic system used, intact tRNA elutes at approximately 0.7 *M* NaCl.

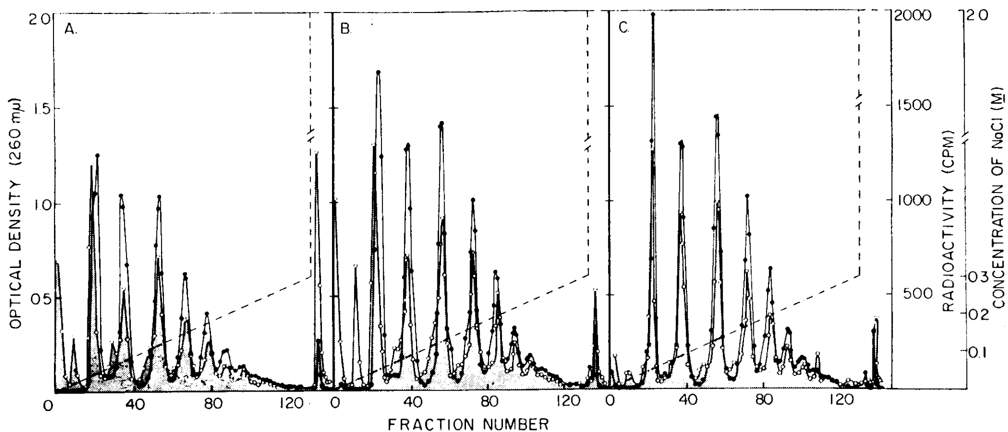

FIG. 7. Effect of DEAE-D on DEAE-cellulose (chloride form) column chromatography of a pancreatic digest of ^{32}P-tRNA: ^{32}P-tRNA (25 μg containing 1.3 × 10^4 cpm) was digested with pancreatic RNase (enzyme:substrate ratio, 1:20) for 120 min at 37° in 0.001 *M* EDTA, 0.01 *M* Tris–HCl (pH 7.4), in the absence (A,B); or the presence (C) of DEAE-D (0.1 mg/ml); and then chromatographed on DEAE-cellulose columns by methods described for Fig. 6. (●) The absorbance at 260 mμ; (shaded portions) the radioactivity (cpm); (- - -) the molarity of NaCl in eluting buffer (7 *M* urea, 0.05 *M* sodium acetate buffer, pH 5.4); (A) ^{32}P-tRNA digested with pancreatic RNase; (B) the ^{32}P-RNA was digested with pancreatic RNase, then DEAE-D was added; (C) the ^{32}P-tRNA–DEAE-D complex was digested with pancreatic RNase. The peaks eluted with 2 *M* NaCl contain either residual undigested tRNA or oligonucleotides longer than nonanucleotides. The height of the late peak in the control (A) is negligible compared to the overall degradation observed; and the amount of degradation is not significantly different in A, B, and C.

bance at 260 mμ represent oligonucleotides produced by pancreatic RNase digestion of torula RNA. However, when ^{32}P-tRNA was incubated with NHAC in the absence of DEAE-D or the presence of calf serum, the tRNA was markedly degraded after 120 min incubation (Fig. 6A, C), as shown by the high isotope content in the early peaks. Chromatography on DEAE-cellulose was unaffected by DEAE-dextran (Fig. 7). DEAE-D did not produce aggregation of oligonucleotides, *i.e.*, no more isotope of degraded RNA was found in the late peaks in the presence of DEAE-D (Fig. 7B, C) than in its absence (Fig. 7A). Moreover, Fig. 7C shows that addition of DEAE-D to the digestion mixture did not protect the tRNA against high concentrations of pancreatic RNase.

Sucrose density gradient centrifugation also showed that after incubation in the presence of DEAE-D, the reisolated ^{32}P-tRNA was undegraded as evidenced by the peak in the 4S region (Fig. 8D, E). However, in the absence of DEAE-D (Fig. 8A, B), there was degradation of the tRNA as shown by the shift of radioactivity to the top of the gradient. Under these conditions, pancreatic RNase treatment (10 μg/ml, 37°, 10 min) of the whole cells had no marked effect on the sucrose density gradient centrifugation pattern of whole cellular RNA (Fig. 8C, F).

Energy dependence. Both cell association and uptake of ^{32}P-tRNA were energy dependent as shown by the inhibition by iodoacetate (Fig. 9) and azide (Fig. 10). The 50% inhibitory concentrations for cell association and uptake of ^{32}P-tRNA were 2×10^{-2} *M* iodoacetate and 8×10^{-2} *M* for azide for both control and DEAE-D-incubated cells.

Discussion. These studies, which show that intact ^{32}P-tRNA can be taken up into NHAC, support and extend earlier studies on uptake of macromolecules into mammalian cells (1–5, 26–27). Although the enhancement by DEAE-D (0.1 mg/ml) of cell association of the isotope of ^{32}P-tRNA was approximately three times that found in control experiments, the enhancement of uptake of intact tRNA is considerably greater since only 20% of the ^{32}P of the labeled tRNA was eluted with 2 *M* NaCl (Fig. 6A) after incubation in the absence of DEAE-D. Thus, in

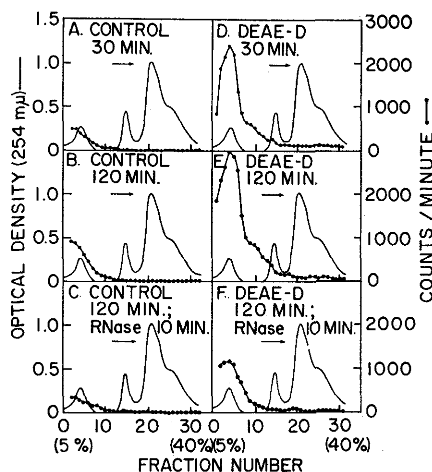

FIG. 8. Sucrose density gradient profiles of cell-associated ^{32}P-tRNA and its digestion products: Cells were incubated with ^{32}P-tRNA (0.05 mg/ml) in the absence or presence of DEAE-D (0.1 mg/ml) at 37°. Whole cellular RNA was extracted by the hot phenol–SDS method and applied to a linear (5–40%) sucrose density gradient containing 0.1 *M* NaCl; 0.001 *M* EDTA; and 0.01 *M* sodium acetate buffer, pH 5.1. After centrifugation at 25,000 rpm for 18 hr in an SW-27 rotor of the Spinco model L2 ultracentrifuge, the gradients were fractionated with the aid of an ISCO density gradient fractionator. (A, B) Control (without DEAE-D) incubated for 30 and 120 min, respectively; (C) same as (B), but then treated with pancreatic RNase; (D, E) cells were indubated for 30 and 120 min with DEAE-D-tRNA complex; (F) same as (E), but then treated with pancreatic RNase. The pancreatic RNase treatment was carried out at an enzyme concentration of 10 μg/ml for 10 min at 37°. (arrows) The direction of sedimentation.

the presence of DEAE-D, the amount of intact tRNA in the cells at 120 min was at least 15 times that of the control. In recent experiments, other polycations such as protamine and methylated bovine serum albumin have been found to have similar activity.

The evidence from DEAE-cellulose column chromatography and sucrose density gradient centrifugation analysis indicates that in the presence of DEAE-D, most, if not all, of the cell-associated radioactivity was in intact tRNA. The radioactive peak found in the 4S region could not have resulted from breakdown and reutilization of nucleotides of the ^{32}P-tRNA since, in NHAC, the synthetic rate for 45S RNA is higher than that of

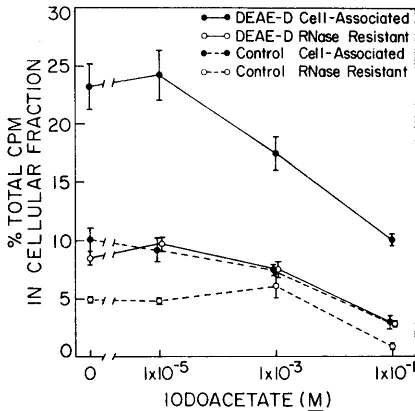

FIG. 9. Inhibition of cellular association and uptake of ^{32}P-tRNA by iodoacetate: The cells were incubated with ^{32}P-tRNA (0.05 mg/ml) and increasing concentrations of sodium iodoacetate in the presence and absence of DEAE-D (0.1 mg/ml) for 120 min at 37°. After an aliquot was taken for determination of cell-associated radioactivity (●); the cells were treated with pancreatic RNase (10 μg/ml) for 10 min at 37°, under conditions employed in Fig. 5. The values are averages and standard deviations from four experiments.

tRNA (22), and no isotope was found in 45S RNA. Even when the ^{32}P-tRNA was extensively degraded in the absence of DEAE-D, little isotope was found in the high molecular weight RNA region. The effects of temperature and inhibitors of energy metabolism demonstrate that the uptake processes represent active cellular functions. After 120

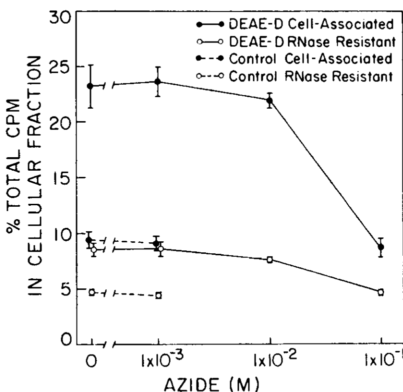

FIG. 10. Inhibition of cellular association and uptake of ^{32}P-tRNA by azide: The experiment was performed in the same manner as that shown in Fig. 9.

min of incubation in the presence of DEAE-D, 40% of the cell-associated tRNA was resistant to pancreatic RNase and therefore was presumably intracellular. Fairly good agreement was found between the estimates of adsorption at 0° and zero time and the results obtained with RNase. Calculations based on these estimates indicate that 10–14% of the isotope was taken up into the cells.

There may have been a number of factors involved in the enhancing effect of DEAE-D, including changes in the charge density of tRNA complexed with DEAE-D; adsorption of the complex, which is probably closely related to uptake of the tRNA; and the possibility that differing cell mechanisms are involved in the uptake of the complex as compared to the tRNA alone. As shown by the zero time and 0° studies, and the RNase treatment technique, DEAE-D increased the adsorption of tRNA to cells. Other evidence for an effect of DEAE-D on adsorption of tRNA was provided by experiments in which DEAE-D and tRNA (0.1 and 0.5 mg/ml, respectively) were mixed in a test tube; and over 90% of the tRNA was adsorbed to the glass.

It is interesting that DEAE-D protected tRNA from intracellular or membrane-associated RNase but did not protect against high concentrations of pancreatic RNase. This finding supports the proposal of Tovell and Colter (5) that the complex, rather than dissociated tRNA, is taken up. Although it is possible that the intracellular RNase concentration is lower than that used in cell-free digestions and DEAE-D is capable of protecting against lower concentrations, it is also possible that the intracellular compartmentalization of the DEAE-D–tRNA complex molecules differs from that of uncomplexed tRNA, and accordingly there is less exposure to intracellular RNases.

On the basis of these values, approximately 5 μg of tRNA were taken up/3 $\times$ 10^6 cells in the presence of DEAE-D. Assuming a molecular weight of 30,000 for tRNA, 4 $\times$ 10^7 molecules/cell were taken up. Assuming there are 1 $\times$ 10^8 molecules of tRNA/cell [an estimate obtained from our calculations and those of Weinberg and Penman (28) for

HeLa cells], 40% of the normal complement was taken up (28, 29). While these studies were in progress, Herrera *et al.* (19) reported that exogenous *E. coli* tRNA equivalent to 30% of the total endogenous tRNA was taken up by L1210 cells. Their result agrees well with those reported here. Since their tRNA was unprotected, it was not unexpected that approximately 80% was degraded in 30 min. This finding is also in agreement with the present study, which shows that at least 80% of unprotected cell-associated tRNA was degraded at 120 min. They studied unenhanced cell association of tRNA at short time intervals under conditions comparable to control experiments at zero time in the present study. At short incubation times, no significant different was found between cell association of tRNA at 0 or 37° (Fig. 2), in agreement with their data, which suggested that the cell association they studied was not energy dependent. However, it seems likely that there are at least two types of phenomena involved in cell association: (a) adsorption, which is partially energy dependent; and (b) intracellular uptake, which requires energy-linked transport. Some of the discrepancies between the results of the studies of Herrera *et al.* (19) and those of the present study may be related to the cell types employed and to the duration of the experiments.

The uptake of intact tRNA by Novikoff hepatoma ascites cells provides an opportunity for further analysis of mechanisms of macromolecular uptake by cells, and the disposition of these molecules as potential functional constituents in the nucleus, the nucleolus, and cytoplasmic elements. Moreover, the uptake of intact tRNA by cells suggests that it may be possible for cells to take up intact natural or synthetic oligonucleotides of defined structure. If these can be inserted into the genome, localized to special regions of the nucleus, nucleolus, or specifically utilized as components of messenger RNA, it is possible that specific genetic information can be added to functional cells and thereby provide a basis for "genetic engineering."

Summary. Studies with ^{32}P-labeled tRNA indicate that intact tRNA is efficiently taken up into Novikoff hepatoma ascites cells in the presence of DEAE-dextran (0.1 mg/ml). The ^{32}P-tRNA was taken up intact as demonstrated by DEAE-cellulose column chromatography and sucrose density gradient centrifugation. The formation of a DEAE-dextran–tRNA complex was demonstrated by hyperchromicity, sucrose density gradient centrifugation, and the lack of precipitation of the tRNA in the complex with ethanol. Although DEAE-dextran protects tRNA from intracellular RNases, it does not protect tRNA against high concentrations of pancreatic RNase. Inhibition of uptake of tRNA by the cells with either iodoacetate ($IC_{50} = 2 \times 10^{-2}\,M$) or azide ($IC_{50} = 8 \times 10^{-2}\,M$) demonstrated that both cell association and uptake of tRNA are energy dependent. To demonstrate that tRNA was intracellular and not simply adsorbed, control studies were made at 0° and zero time and by treatment of the cells with pancreatic RNase after the incubations. On the basis of these studies, approximately 25% of the total tRNA in the incubation mixture was cell associated at 120 min in the presence of DEAE-D. Of the cell associated tRNA, approximately 40% was intracellular.

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