(1972) may be involved in nucleic acid recognition. Further work is presently in progress to determine the specificity of binding of the above-mentioned systems to DNA.

References

Anderson, G. W., Zimmerman, J. E., and Callahan, F. M. (1967), *J. Amer. Chem. Soc. 89*, 5012.

Bekhor, I., Kung, G. M., and Bonner, J. (1969), *J. Mol. Biol. 39*, 351.

Clark, R. J., and Felsenfeld, G. (1971), *Nature (London), New Biol. 229*, 101.

Cohen, G., and Eisenberg, H. (1969), *Biopolymers 35*, 251.

Drummond, N. J., Pritchard, N. J., Simpson-Gildmeister, V. F. W., and Peacocke, A. R. (1966), *Biopolymers 4*, 971.

Gabbay, E. J. (1969), *J. Amer. Chem. Soc. 91*, 5136.

Gabbay, E. J., and DePaolis, A. (1971), *J. Amer. Chem. Soc. 93*, 562.

Gabbay, E. J., DeStefano, R., and Sanford, K. (1972), *Biochem. Biophys. Res. Commun. 46*, 155.

Gabbay, E. J., Gaffney, G., and Glaser, R. (1970), *Ann. N. Y. Acad. Sci. 171*, 810.

Gabbay, E. J., Gaffney, B., Glaser, R., and Denney, D. Z. (1969), *Chem. Commun.*, 1507.

Gabbay, E. J., and Glaser, R. (1971), *Biochemistry 10*, 1665.

Gabbay, E. J., and Kleinman, R. (1970), *Biochem. J. 117*, 247.

Haynes, M., Garret, R. A., and Gratzer, W. B. (1970), *Biochemistry 9*, 4410.

Helene, C., Dimicoli, J.-L., and Brun, F. (1971), *Biochemistry 10*, 3802.

Jardetsky, O., and Jardetsky, C. D. (1962), *Methods Biochem. Anal. 9*, 235.

Lerman, L. S. (1961), *J. Mol. Biol. 3*, 18.

Müller, W., and Crothers, D. M. (1968), *J. Mol. Biol. 35*, 251.

Passero, F., Gabbay, E. J., Gaffney, B., and Kurucsev, T. (1970), *Macromolecules 3*, 158.

Pople, J. A., Schneider, W. G., and Bernstein, H. J. (1959), High-Resolution Nuclear Magnetic Resonance, New York, N. Y., McGraw-Hill.

Shapiro, J. T., Leng, M., and Felsenfeld, G. (1969a), *Biochemistry 8*, 3219.

Shapiro, J. T., Stannard, B. S., and Felsenfeld, G. (1969b), *Biochemistry 8*, 3233.

Shih, T. Y., and Bonner, J. (1970), *J. Mol. Biol. 48*, 469.

Simpson, R. J. (1970), *Biochemistry 9*, 4814.

Sober, H. A., Schlossman, S., Yaron, A., Latt, S. A., and Rushizky, G. W. (1966), *Biochemistry 5*, 3608.

Yarus, M. (1969), *Annu. Rev. Biochem. 38*, 841.

Acylation of Transfer Ribonucleic Acid with the *N*-Hydroxysuccinimide Ester of Phenoxyacetic Acid†

Stanley Friedman

ABSTRACT: Uncharged rat liver tRNA can be eluted from BD-cellulose columns with 0.8 M sodium chloride, with the exception of $tRNA^{Phe}$, which can only be eluted with 0.8 M sodium chloride containing ethanol. When the uncharged rat liver tRNA is reacted with the *N*-hydroxysuccinimide ester of phenoxyacetic acid, there is an increase in the amount of tRNA that is eluted in the presence of ethanol. Greater than 50% of the tRNAs for tyrosine, threonine, isoleucine, cysteine, and asparagine can now be eluted only in the presence of ethanol. Only a small fraction of the tRNA from *Escherichia coli* and yeast shifted into the ethanol fraction after reaction with phenoxyacetoxysuccinimide. The rate of acylation of rat liver and *E. coli* tRNA with [^{14}C]phenoxyacetoxysuccinimide was greater than that with tRNA isolated from yeast. Furthermore, all the incorporated radioactivity could be hydrolyzed from the yeast tRNA by incubation in 0.34 M ammonia, whereas only a fraction of the radioactivity incorporated into *E. coli* and rat liver tRNA could be made acid soluble by this procedure. Only one acylated nucleoside could be isolated from an enzymic digest of rat liver tRNA. A compound with identical chromatographic, electrophoretic, and ultraviolet spectral properties was isolated from *E. coli* tRNA.

There have been several reports of the presence of certain bases in transfer ribonucleic acid that can undergo acylation reactions. However, the nature of these bases has not been fully characterized, nor has it been determined whether these are rare bases, or one or more of the four major bases, that are especially reactive because of their conformation in tRNA. With the advent of BD-cellulose[1] columns, which have affinity for nucleic acids with additional hydrophobic groups attached, and particularly for single-stranded nucleic acids, a method for the separation of acylated from unacylated nucleic acids became available (Gillam *et al.*, 1967).

Several groups have used the esters of *N*-hydroxysuccinimide to acylate specific aminoacyl-tRNAs (deGroot *et al.*, 1966; Gillam *et al.*, 1968; Schofield *et al.*, 1970). In the process of preparing acylaminoacyl-tRNAs, Gillam *et al.* (1968)

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[1] Abbreviations used are: BD-cellulose, benzoylated DEAE-cellulose; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; A_{260} unit, that quantity of material which, when dissolved in 1 ml of 0.01 N Tris-HCl (pH 8.9), has an absorbance at 260 nm of 1.0; ethanol fraction, that fraction of tRNA which is eluted from BD-cellulose with solutions containing ethanol and not with 0.8 M sodium chloride.

and Schofield *et al.* (1970) described the acylation of tRNA itself. Other investigators, using more potent acylating agents, have only described the extensive acylation of 3′-hydroxyl groups of ribose but negligible acylation of bases in tRNA (Stuart and Khorana, 1964; Knorre *et al.*, 1965).

In this paper I report on the specific tRNA species that can undergo extensive acylation, and describe some characteristics of the acylated base that can be isolated from digests of the tRNA. The extent of reaction of specific tRNA species was studied by acylating the tRNA with an aromatic compound, phenoxyacetoxysuccinimide, and following the elution pattern of the tRNA from BD-cellulose. Buffers that contained ethanol were used to elute those tRNAs that had an increased affinity for the column due to the incorporation of an aromatic phenoxyacetyl moiety into one or more nucleotide residues of the tRNA.

Experimental Section

Materials. BD-cellulose was obtained from Schwarz Biochemical. DEAE-cellulose was obtained from Sigma Chemical Co. Brewer's yeast and *Escherichia coli* tRNA were commercial preparations from Sigma Chemical Co. and Mann Research Laboratories, respectively. Tritiated and [^{14}C]-labeled amino acids were obtained from New England Nuclear Corp., and stated to be of greater than 99% purity.

Methods. Liver tRNA was prepared from fresh livers excised from 200- to 300-g Wistar strain male rats and frozen in liquid nitrogen. The tRNA was extracted according to the method of Brunngraber (1962). The tRNA was purified by isopropanol precipitation (Zubay, 1962) prior to DEAE-cellulose chromatography (Brunngraber, 1962). The tRNA was deacylated by incubation in 0.2 M Tris-HCl buffer (pH 8.8) at 37° for 3 hr.

Preparation of Aminoacyl-tRNA Synthetases. Employing a glass homogenizer with a motor-driven Teflon pestle, freshly excised rat liver was homogenized in 2 volumes of 0.01 M Tris-HCl buffer (pH 7.5), containing 0.01 M mercaptoethanol, 10% sucrose, 0.1 mM EDTA, and 0.4 M KCl. After centrifugation of the homogenate for 10 min at 15,000*g*, the resultant supernatant was centrifuged at 105,000*g* for 1 hr. The final supernatant thus collected from 10 g of liver was then passed through a 2.5 × 5 cm DEAE-cellulose column previously washed with the same buffer. The synthetases pass through the column and are concentrated by dialysis against 0.01 M Tris-HCl buffer (pH 7.5), containing 0.01 M mercaptoethanol and 50% sucrose. The enzyme extract was adjusted to a protein concentration of 60 mg/ml, divided into small aliquots, and stored at −20°. All purification steps were carried out at 4°. *E. coli* synthetases were contained in a crude supernatant fraction prepared according to the method of Nishizuka and Lipmann (1955).

Assays for Amino Acid Acceptor Activity. The tRNA was assayed in a volume of 0.1 ml containing NaATP (5 mM), magnesium chloride (10 mM), Hepes (pH 7.5, (0.1 M), albumin (10 μg), EDTA (0.5 mM), glutathione (5 mM), 0.01 mmole of each of 19 cold amino acids, and between 2.8 and 20 μM radioactive amino acid (0.2 μCi of [^{14}C] or 1 μCi of [^{3}H]amino acid). Cystine was reduced with 0.012 M dithiothreitol for 15 hr at 24° before use (Caskey *et al.*, 1968). The reaction was started by adding 0.01 ml of enzyme, and incubations were performed at 37°. After 20 min, 0.075 ml of the reaction mixture was pipetted onto a Whatman No. 3MM paper disk, 2.3 cm in diameter, and placed in ice-cold 10% trichloroacetic acid. The disks were then washed three times with 3% perchloric acid, once each in ethanol and ethanol–ether (50:50), and dried after a final ether wash. The disks were then counted in a Packard scintillation counter in 5 ml of a toluene-based scintillation mixture. The counter was calibrated with samples of radioactive amino acids dried on filter paper.

Acylation of tRNA. Modifications were made in the procedure reported by Gillman *et al.* (1968). tRNA (11 mg) was dissolved in 4 ml of 0.1 M triethanolamine-HCl (pH 4.7), containing 0.01 M magnesium chloride. The solution was cooled in an ice bath and stirred continuously. A solution (0.5 ml) containing the *N*-hydroxysuccinimide ester of phenoxyacetic acid (18 mg) in dry tetrahydrofuran (1 ml) was added. An aliquot of 1.5 ml was removed and placed in 0.1 ml of 20% potassium acetate (pH 5) and treated as a control. The experimental solution was then brought to pH 8 with 1 M sodium hydroxide. Another aliquot was removed at 30 min and placed in potassium acetate. At this time, another 0.5 ml of acylating agent was added. After 1 hr, 1 M acetic acid was used to adjust the pH to 5. The RNA was precipitated with 2 volumes of ethanol. The precipitate was washed twice by solution in 2% potassium acetate, and precipitated with 2 volumes of ethanol. The precipitates were then dissolved in 10 ml of 0.01 M sodium acetate buffer (pH 4.5), containing 0.3 M sodium chloride and 0.01 M magnesium chloride (solution A), and chromatographed on BD-cellulose. Elution buffers contained, in addition to 0.01 M sodium acetate (pH 4.5) and 0.01 M magnesium chloride, either 0.8 M sodium chloride (solution B), 0.8 M sodium chloride and 20% ethanol (solution C), or 2 M sodium chloride and 20% ethanol (solution D).

Chromatography on BD-cellulose. The columns were packed and washed as described by Gillam *et al.* (1967), except that all procedures were performed at 4°. Less than 100 A_{260} units of phenoxyacetylated tRNA were applied to a 1 × 8 cm column of BD-cellulose equilibrated with solution A. The column was washed with the same buffer until the A_{260} of the eluate was less than 0.025 unit (usually 20 ml). The column was then developed with solution B until the A_{260} fell below 0.05 unit. Approximately 70–100 ml of buffer was required. The column was finally washed with solution C unless otherwise noted. The flow rate was 0.7 ml/min and 6-ml fractions were collected. The combined fractions were either dialyzed extensively against water and lyophilized or, when large amounts of RNA were processed, concentrated by ultrafiltration in the Diaflo apparatus (Amicon Corp., Cambridge, Mass), using a type UM 10 filter and precipitated with ethanol. The residue was finally dissolved in water for assay.

Preparation of [^{14}C]Phenoxyacetoxysuccinimide. The method of Hayes and Branch (1943) was used for the preparation of radioactive phenoxyacetic acid. [1-^{14}C]Monochloroacetic acid (12 mg, 1 mCi), phenol (12.6 mg), and sodium hydroxide (0.1 ml of a 10% solution) were mixed together in a test tube which was then sealed. The reaction mixture was heated in a boiling-water bath for 5 hr. The tube was then opened, 1 ml of 1.0 N hydrochloric acid was added, and the solution extracted three times with 2 ml of diethyl ether. After evaporation of the ether, the residue was applied to two 20 × 20 cm silica gel thin-layer plates, which had previously been activated by heating at 105° for 30 min. The plates were developed two times in chloroform. The phenoxyacetic acid remained near the origin (R_F 0.03) and was scraped off the plates and eluted from the silica gel with ether. Ten per cent of radioactivity was recovered as phenoxyacetic acid, as determined by cochromatography with authentic phenoxyacetic acid on silica gel in benzene–acetic acid–methanol (45:4:8), R_F 0.68; chloroform–ethyl

acetate–acetic acid (50:40:10), R_F 0.65; and benzene–butanol–acetic acid (82:15:5, Kagawa *et al.*, 1963), R_F 0.71. The radioactivity cocrystallized at constant specific activity with authentic phenoxyacetic acid. The phenoxyacetic acid was dissolved in 0.5 ml of ether, and 0.25 ml (0.05 mCi) was transferred to an 18 × 150 mm test tube. The ether was removed by blowing dry nitrogen into the tube. After adding 70 mg of phenoxyacetoxysuccinimide and 1 ml of dry dioxane, the tube was sealed and heated at 100° for 24 hr. The reaction mixture was lyophilized to dryness and the product crystallized twice from hot 1-propanol; the yield was 55.2 mg (specific activity 0.1 μCi/mg). All but 3–5% of the radioactivity cochromatographed with phenoxyacetoxysuccinimide in benzene–acetic acid–methanol (45:4:8), R_F 0.79; chloroform–ethyl acetate–acetic acid (50:40:10), R_F 0.73; and benzene–butanol–acetic acid (82:15:5), R_F 0.83. The remainder of the radioactivity chromatographed with phenoxyacetic acid. Upon hydrolysis with base, all the radioactivity cochromatographed with phenoxyacetic acid.

Isolation of Phenoxyacetylated Bases from Rat Liver tRNA. Rat liver tRNA (161 A_{260} unit, the alcohol fraction) containing 9980 cpm of phenoxyacetylated tRNA was dissolved in 1.5 ml of water; and snake venom phosphodiesterase (0.05 unit), *E. coli* alkaline phosphatase (0.07 unit), and magnesium chloride (4 μmoles) were added. The mixture was incubated at 37° for 3 hr, and during this period the pH was adjusted to 8.6 every 0.5 hr with 0.1 N potassium hydroxide. The addition of enzymes and magesium chloride was repeated, and the incubation was continued for 5 more hr. The pH was then adjusted to 4.4 with formic acid, and the mixture was heated at 60° for 30 min. The precipitate was removed by centrifugation and washed twice with 0.5 ml of 0.3 M ammonium formate buffer (pH 4.4) and the washings were combined. The digest and washings were applied to a 0.9 × 47 cm Dowex 50 column that was equilibrated with the same formate buffer at 40° (Uziel *et al.*, 1968). The column was developed with the ammonium formate buffer at 0.9 ml/min, and 4.4-ml fractions were collected. Radioactivity was detected by counting 0.25 ml of the eluate fractions in 10 ml of Bray's solution. The radioactive fractions were pooled, concentrated by rotary evaporation, and finally lyophilized several times to remove remaining ammonium formate. The residue was dissolved in water and chromatographed on Whatman No. 3MM paper which had been washed with 0.1 M hydrochloric acid. The paper was developed with isobutyric acid–0.5 M ammonia–0.1 M EDTA (100:60:1.6) (Magasanik *et al.*, 1950).

The radioactivity, which coincided with an ultraviolet-absorbing spot, was eluted and rechromatographed in butanol–water (5:2, upper phase). Other solvents used were isopropyl alcohol–hydrochloric acid–water (68:17.6:14.4) (Wyatt, 1951) and 1-propanol–ammonia–water (60:30:10) Hanes and Isherwood, 1949). Thin-layer electrophoresis was performed on Avicel plates (No. 1440, Schleicher and Schuell, 20 × 20 cm). Spectra were taken on a Bausch and Lomb Spectronic 505 recording spectrophotometer. Inorganic phosphate was measured by the method of Fiske and Subbarow (1925).

Results

Elution Profile of Phenoxyacetylated tRNA. When rat liver tRNA is chromatographed on BD-cellulose, virtually all of the tRNA is eluted with solution B. However, there is a small amount of tRNA for each amino acid that can be eluted with solution C. The major exception is $tRNA^{Phe}$, which is known

TABLE I: Distribution of Acceptance Activity of Rat Liver tRNA on BD-cellulose Chromatography before and after Treatment with Phenoxyacetoxysuccinimide.

Amino Acid Assayed	Time of Acylation (min)	Act. Eluted from BD-cellulose with Soln C (% of Total)	Total Acceptor Act. Eluted with Solns B and C (pmoles/A_{260} Unit)
Alanine	0	5	21
	60	17	17
Arginine	0	7	69
	30	15	78
Asparagine	0	7	75
	60	80	52
Aspartic acid	0	6	89
	60	8	82
Cysteine	0	1	5
	60	60	7
Glutamic acid	0	15	2
	60	15	2
Glutamine	0	10	6
	60	11	6
Glycine	0	13	4
	30	19	3
Histidine	0	2	19
	30	9	22
Isoleucine	0	7	365
	60	55	293
Leucine	0	18	8
	60	33	8
Lysine	0	5	41
	30	15	47
Methionine	0	15	14
	60	20	10
Phenylalanine	0	58	6
	30	55	7
Proline	60	5	8
Serine	0	16	34
	30	29	44
Threonine	0	8	83
	30	49	66
	60	53	61
	30[a]	4	27
	30[b]	8	59
Tryptophan	0	10	25
	60	9	24
Tyrosine	0	11	27
	30	70	21
	60	76	18
	30[a]	5	9
	30[b]	11	19
Valine	0	4	177
	60	7	183

[a] Treated with phenoxyacetoxysuccinimide at pH 5.1. [b] Treated with phenoxyacetic acid and *N*-hydroxysuccinimide (pH 8.0).

TABLE II: Distribution of Acceptance Activity of Yeast tRNA on BD-cellulose Chromatography before and after Treatment with Phenoxyacetoxysuccinimide.

Amino Acid Assayed	Time of Acylation (min)	Act. Eluted from BD-cellulose with Soln C[a] (% of Total)	Total Acceptor Act. Eluted with Solns B and C[a] (pmoles/A_{260} Unit)
Leucine	0	2	1
	30	4	1
Lysine	0	5	2
	30	10	2
Serine	0	18	2
	30	23	3
Threonine	0	0	4
	30	1	4
Tyrosine	0	22	6
	30	36	6

[a] Assays were performed with rat liver enzyme.

TABLE III: Distribution of Acceptance Activity of *E. coli* tRNA on BD-cellulose Chromatography before and after Treatment with Phenoxyacetoxysuccinimide.

Amino Acid Assayed	Time of Acylation (min)	Act. Eluted from BD-cellulose with Soln C (% of Total)	Total Acceptor Act. Eluted with Solns B and C (pmoles/A_{260} Unit)
Alanine	0	4	19
	60	12	21
Arginine	0	3	37
	60	12	38
Asparagine	0	2	4
	60	10	4
Aspartic acid	0	3	7
	60	16	5
Cysteine	0	14	7
	60	18	6
Glutamic acid	60	11	9
Glutamine	60	10	12
Glycine	0	1	12
	60	13	13
Histidine	0	7	12
	60	21	18
Isoleucine	60	12	79
Leucine	0	14	12
	60	21	13
Lysine	0	3	7
	60	18	9
Methionine	60	11	36
Phenylalanine	0	5	10
	60	34	8
Proline	0	1	9
	60	7	10
Serine	0	31	90
	60	25	83
Threonine	0	2	17
	60	10	22
Tryptophan	0	72	10
	60	63	15
Tyrosine	0	70	4
	60	90	5
Valine	60	8	58

to have a high affinity for BD-cellulose (Fink *et al.*, 1968; Gillam *et al.*, 1967; for exception, see Griffiths, 1970). When phenoxyacetylated tRNA is chromatographed (Table I), there is an increase in the number of tRNA species eluted with buffers containing ethanol. Virtually all the amino acids have species of tRNA that have shifted into the ethanol fraction. This shift is a real one and not due to loss of activity of some tRNA species due to the acylation procedure, since the specific activity (pmoles/A_{260} unit) remains the same before and after acylation. Where the specific activity does change, the change is not great enough to account for large shifts in elution pattern of the tRNA species, nor is the change in specific activity consistent in different experiments. The acylation reaction is pH dependent, since there is no shift in the elution pattern if the acylation reaction is carried out at pH 5. Some of the tRNAs react to a much greater degree than others. The major reactive species are $tRNA^{Tyr}$, $tRNA^{Thr}$, $tRNA^{Ile}$, $tRNA^{Cys}$, and $tRNA^{Asn}$.

The same species in other organisms are not reactive. In neither yeast nor *E. coli* were any of the tRNAs tested extensively shifted into the ethanol fraction (Tables II and III). The *E. coli* $tRNA^{Tyr}$ and $tRNA^{Trp}$ are primarily eluted in the ethanol fraction, an observation previously reported by Roy and Söll (1968).

Those tRNA species that react extensively with the acylating agent do so in 30 min. There is no further reaction after another 30-min incubation with the reagent (Table I, $tRNA^{Tyr}$).

Methylated Albumin Kieselguhr Chromatography. From the batch elution experiments it is unclear whether several new species are formed as a result of multiple reactions of the acylating reagent with tRNA. The $tRNA^{Tyr}$ eluted with solutions B and C was therefore aminoacylated with ^{14}C- and tritium-labeled tyrosine, respectively, mixed, and chromatographed with a linear gradient of sodium chloride on an methylated albumin kieselguhr column (Figure 1A). The phenoxyacylated tyrosyl-$tRNA^{Tyr}$ eluted as a single peak at a higher salt concentration than the species that had not reacted with phenoxyacetoxysuccinimide. The identical shape of the two peaks suggests that the same number of phenoxyacetic acid molecules has been incorporated into each $tRNA^{Tyr}$, causing a shift in peak position but no change in peak shape. This shift in position of the phenoxyacetylated tRNA was not solely due to the incorporation of one additional aromatic group into $tRNA^{Tyr}$, since tyrosyl-$tRNA^{Tyr}$ elutes at the same position as $tRNA^{Tyr}$ that is not aminoacylated (Figure 1B).

Effect of Oxidation of tRNA with I_2. Schofield *et al.* (1970) have demonstrated that under some conditions the thiol groups in tRNA will react with acylating agents. To prove that thiol groups were not being acylated, tRNA was first

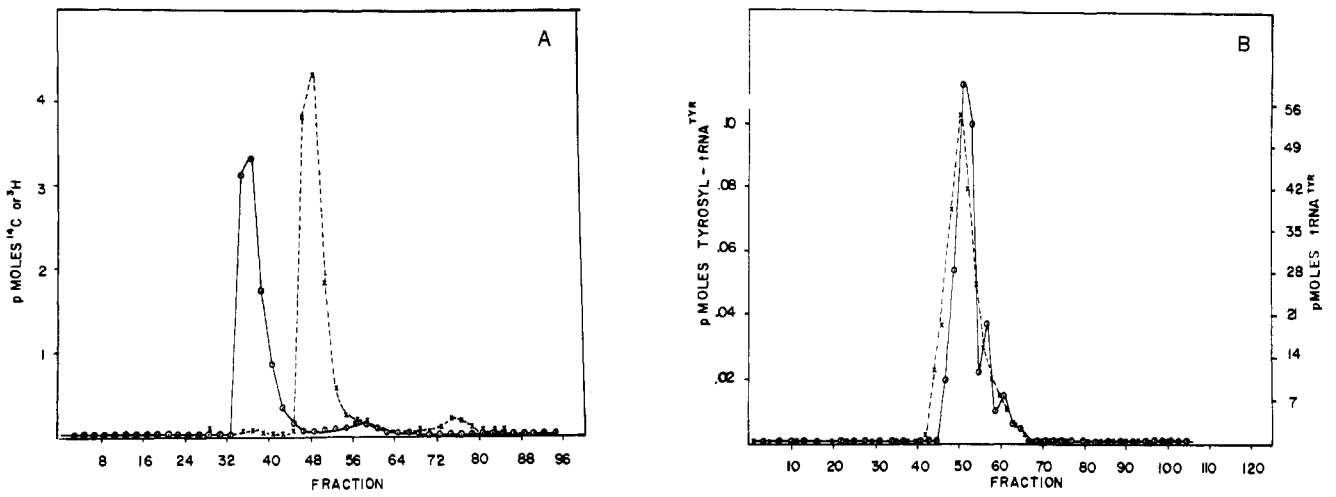

FIGURE 1: Methylated albumin kieselguhr chromatography of rat liver tRNA. (A) An aliquot of phenoxyacetylated tRNA eluted from BD-cellulose with solution B was charged with [^{14}C]tyrosine, and an aliquot of phenoxyacetylated tRNA eluted with solution C was charged with [^{3}H]tyrosine. The two reaction mixtures were combined, and 2 mg of beef tRNA was added as carrier and extracted with 1 volume of water-saturated phenol. The tRNA was then precipitated with ethanol and chromatographed on a methylated albumin kieselguhr column by the method of Sueoka and Yamane (1967). A gradient of 0.2–1.0 M sodium chloride was used. Fractions of 2 ml were collected at a flow rate of 1 ml/min. Yeast tRNA (0.2 mg) and 20% trichloroacetic acid (2 ml) were added to the fractions; the precipitate was collected on a Whatman glass fiber filter and counted in a scintillation counter. (——) ^{14}C, (----) ^{3}H. (B) 2000 cpm of tRNA charged with [^{14}C]tyrosine was mixed with 1.7 mg of tRNA and chromatographed on a methylated albumin kieselguhr column as above, except the gradient was 0.2–0.7 M sodium chloride. Alternate fractions were precipitated and counted as above, or 0.1-ml aliquots were assayed as in Methods. (——) Tyrosyl-tRNA^{Tyr} and (----) tRNA^{Tyr}.

oxidized with I_2 in the presence of potassium iodide and mercaptoethanol (Carbon *et al.*, 1965) before being phenoxyacetylated. There was no decrease in the amount of tRNA eluted with ethanol; if anything, there was an increase in the shift of specific tRNAs into the ethanol fraction (Table IV). I_2 oxidation by itself did not alter the elution pattern of the liver tRNA from the BD-cellulose column.

4-Thiouridine has an ultraviolet absorption maximum near 330 nm (Fox *et al.*, 1959). When the base is oxidized with iodine (Lipsett, 1966) or acetylated (Schofield *et al.*, 1970), there is a marked decrease in the intensity of this peak. When 48 A_{260} units of *E. coli* tRNA were phenoxyacetylated there was no change in the spectrum at 330 nm, although iodine oxidation caused the disappearance of this peak. With rat liver tRNA, no change in ultraviolet absorbance at 330 nm could be detected upon oxidation with iodine. This is presumably due to the low thiouridine content of rat liver tRNA (Carbon *et al.*, 1965).

Studies on [^{14}C]Phenoxyacetyl-tRNA. When rat liver tRNA, from which the ethanol fraction had been removed, was treated with [^{14}C]phenoxyacetoxysuccinimide, the radioactivity was incorporated into an acid-insoluble material. This substance labeled with ^{14}C had a greater affinity for BD-cellulose than the unlabeled tRNA. Only 4% of the labeled material was eluted with solution B. In some experiments as much as 10% of the radioactivity was eluted in this fraction. Ethanol-containing solutions were necessary to elute the bulk of radioactivity from the column. Even solution C could not elute all of the radioactivity (Table V). More radioactive material could be eluted with buffer containing 2 M sodium chloride in addition to ethanol. There was always a greater recovery of A_{260} units than of radioactivity after chromatography. This may be due either to slow hydrolysis of some of the [^{14}C]phenoxyacetyl-tRNA during chromatography, or to the presence of noncovalently bound [^{14}C]-phenoxyacetate that is not removed by repeated ethanol precipitation. The experiment demonstrates that (1) the tRNA is phenoxyacetylated; (2) virtually all the rat liver tRNA that is acylated is eluted with solutions C and D and very little is eluted with 0.8 M sodium chloride; and (3) approximately 1 mole of phenoxyacetic acid is incorporated per mole of reactive tRNA.

When [^{14}C]phenoxyacetoxysuccinimide was used to acylate yeast and *E. coli* tRNA under the conditions used for rat liver tRNA (legend to Table V), the results were different from those found with rat liver tRNA. Whereas rat liver

TABLE IV: Effect of Iodine Oxidation on the Subsequent Acylation of Rat Liver tRNA.[a]

Amino Acid Assayed	Time of Acylation (min)	Act. Eluted from BD-cellulose with Soln C (% of Total)	Total Acceptor Act. Eluted with Solns B and C (pmoles/ A_{260} Unit)
Isoleucine	0	5	201
	60	87	216
Lysine	60	19	20
Serine	60	30	31
Threonine	0	0	31
	60	82	30
Tyrosine	0	11	34
	60	93	34

[a] tRNA was oxidized with iodine in the presence of potassium iodide and mercaptoethanol, as described by Carbon *et al.* (1965). A portion of the tRNA was then acylated with phenoxyacetoxysuccinimide and chromatographed on BD-cellulose.

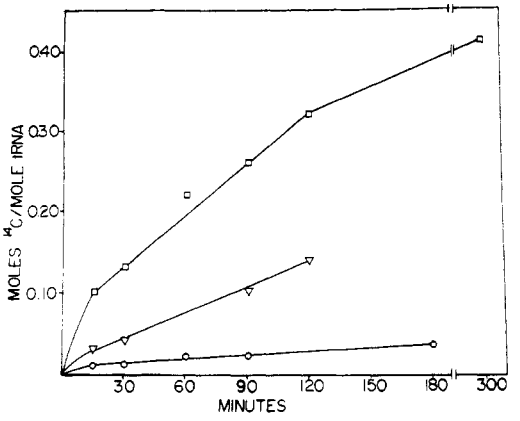

FIGURE 2: The effect of pH on the rate of acylation of tRNA. Incubation mixtures contained 120 A_{260} units of rat liver tRNA in 0.6 ml of 20% tetrahydrofuran containing 5 μmoles of magnesium chloride. Aliquots of [^{14}C]phenoxyacetoxysuccinimide, 5 μl (specific activity 76 μCi/mmole, 15.4 mg/ml of tetrahydrofuran), were added to start the reaction, and also at 30 and 60 min, and every 60 min thereafter. The pH was adjusted by adding 1 N sodium hydroxide at 15-min intervals. Aliquots (50 μl) were removed at the appropriate times and treated as described in Methods. After the second ethanol precipitation they were dissolved in 1 ml of water and dialyzed against three changes of 1 l. of 0.02 M potassium acetate (pH 5) for 16 hr. An aliquot of the tRNA was used to determine the A_{260} units, and the tRNA in a second aliquot was precipitated with an equal volume of 20% trichloroacetic acid, filtered through a glass fiber filter, washed two times with 10% trichloroacetic acid, twice with ethanol, dried, and counted. (□) pH 8.0, 50 μmoles of triethanolamine-HCl; (▽) pH 7.0, 25 μmoles of sodium phosphate; (○) pH 6.0, 50 μmoles of sodium phosphate.

tRNA incorporated 0.22 mole of [^{14}C]phenoxyacetic acid/mole of tRNA and *E. coli* tRNA incorporated 0.26 mole of ^{14}C/mole of tRNA, yeast tRNA was acylated only to the extent of 0.09 mole of ^{14}C/mole of tRNA in a period of one hour.

When the acylated yeast and *E. coli* tRNAs were subjected to BD-cellulose chromatography, most of the [^{14}C]phenoxyacetylated tRNA was eluted with solution B. Only 40 and 36% of the ^{14}C incorporated into yeast and *E. coli* tRNA, respectively, were eluted with solution C.

The rate of the reaction is very dependent upon pH (Figure 2). Under the conditions used, the phenoxyacetoxysuccinimide precipitates when added to the reaction mixture. As the reaction proceeds, the reagent disappears, faster at higher pH's, due to the base-catalyzed hydrolysis of the reagent. The reaction mixture was therefore kept saturated with reagent by adding it at fixed intervals, and the pH was readjusted every 15 min with 1 N sodium hydroxide. The kinetics of the reaction are complex; however, at pH 8, there is a rapid phase of acylation, followed by a slower reaction of the reagent with tRNA that remains linear for at least 90 min.

Stability of the Phenoxyacetylated tRNA. If [^{14}C]phenoxyacetylated tRNA is dissolved in 0.34 M ammonia (pH 9.6) and incubated at 37°, a fraction of the radioactivity becomes acid soluble within 15 min. The remainder of the radioactivity is stable to hydrolysis at this pH for as long as 1 hr. The per cent of the total radioactivity incorporated into tRNA that is hydrolyzed by dilute ammonia depends upon the initial extent of acylation. If rat liver tRNA that has been acylated to the extent of 0.22, and 0.32 mole of ^{14}C/mole of tRNA is subjected to hydrolysis with NH_3, 22 and 32% of the radioactivity, respectively, is made acid soluble. Acylation, resistant to base hydrolysis, therefore reaches a maximum within one hour and amounts to approximately 0.2 mole/mole of tRNA.

TABLE V: Recovery of Absorbance and Radioactivity from BD-cellulose Chromatography of [^{14}C]Phenoxyacetylated Rat Liver tRNA.[a]

Elution Buffer	A_{260} Units Eluted	Cpm Eluted	Sp Act. (Moles of ^{14}C/Mole of tRNA[b])
Buffer B	963	481	0.007
Buffer C	173	10,000	0.72
Buffer D	37	3,018	1.03
	1,173	13,499	

[a] 2000 A_{260} units of tRNA from which the ethanol fraction had been removed was acylated in 5 ml of buffer, as described in Methods. A total of 10 mg of [^{14}C]phenoxyacetoxysuccinimide in 1 ml of tetrahydrofuran was added in two portions. The reaction was allowed to proceed for 1 hr. The tRNA was precipitated with ethanol and washed three times. It was then chromatographed on a 2 × 10 cm BD-cellulose column. A total of 1696 A_{260} units and 23,000 cpm were applied to the column. [b] Calculated using an assumed value of 77 nucleotide residues/molecule of tRNA (2000 pmoles/A_{260} unit, Maxwell *et al.*, 1968).

E. coli tRNA reacts in a similar manner. When acylated to the extent of 0.26 mole of ^{14}C/mole of tRNA, 29% of the radioactivity is hydrolyzed by dilute NH_3. Yeast tRNA reacts differently under these conditions. All the [^{14}C]phenoxyacetate that is incorporated into the tRNA is made acid soluble.

When phenoxyacetylated rat liver tRNA is treated with ammonia (Table VI), there is a shift of tRNA species from the alcohol fraction into the fraction eluted with solution B. This effect is greatest on those tRNAs that are acylated to only a small extent: $tRNA^{Gly}$, $tRNA^{Lys}$, and $tRNA^{Ser}$ in Table VI. The tRNAs that undergo extensive acylation are little affected by this treatment.

Isolation of the Radioactive Nucleoside. The phenoxyacetylated tRNA, purified by chromatography on BD-cellulose, was digested with snake venom phosphodiesterase and *E. coli* alkaline phosphatase. On the basis of liberated phosphate, the digestion went to 61% of completion in 8 hr. The digest was chromatographed on a Dowex 50 column (Figure 3); 32% radioactivity was excluded from the column and eluted ahead of uridine. The remaining 68% radioactivity was included in the column and eluted slightly in front of, and overlapping, the uridine peak. Of the applied radioactivity 75% was recovered in the eluates. The major peak was pooled and chromatographed on paper in isobutyric acid–ammonia–EDTA. Two radioactive spots were detected. A minor peak had an R_F of 0.87, identical with that of phenoxyacetic acid, and did not correspond to any ultraviolet-absorbing spot. The only other radioactive peak, comprising 70% of the applied radioactivity, coincided with an ultraviolet-absorbing spot and had an R_F of 0.73. This material was eluted and chromatographed in water-saturated butanol. It consisted of one ultraviolet-absorbing band with an R_F of 0.09. All the radioactivity coincided with this band, which was eluted with water and used for the experiments described below. The uv data for this compound in water are 264 nm

(ε 13,300) and 238 nm (ε 4000). Both the ultraviolet absorption and the radioactivity coincided on chromatography on Whatman No. 1 paper in isopropyl alcohol–water–ammonia (7:2:1) and water adjusted to pH 10 with ammonia with R_F's of 0.65 and 0.60, respectively. It did not migrate on thin-layer electrophoresis in 1 M formic acid or in 0.025 M ammonium formate (pH 9.2). However, it moved toward the anode in 0.025 M sodium tetraborate (pH 9.2) at a mobility of 0.75 compared to picric acid.

A radioactive compound with identical spectral, chromatographic and electrophoretic properties was isolated from *E. coli* tRNA. This compound was hydrolyzed in 1 M hydrochloric acid at 100° for 2 hr, to yield two uv-absorbing products. One had the mobility and spectrum of phenoxyacetic acid. The uv spectral data on the second was λ_{max} 264, λ_{min} 237 nm in water.

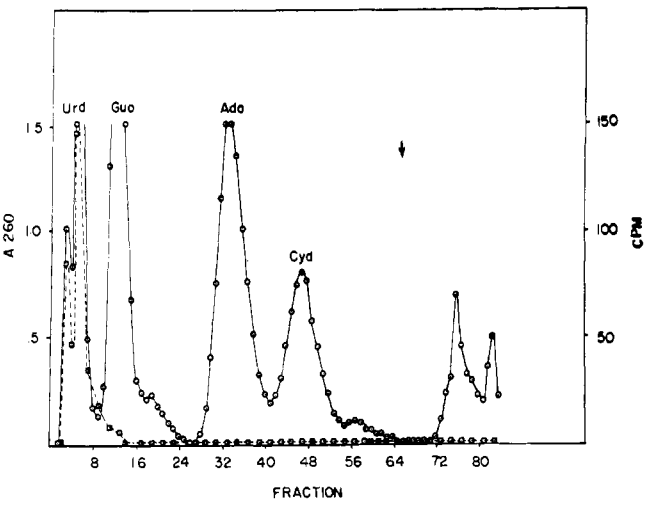

FIGURE 3: Dowex 50 (NH_4^+) chromatography of enzymic digest of ^{14}C-phenoxyacetylated tRNA. Radioactive tRNA was prepared as described in Table V. The ethanol fractions were combined, digested, and chromatographed as described in Methods. (----) cpm, (——) A_{260}. The elution buffer was 0.3 M ammonium formate. At the arrow, the elution buffer was changed to 4 M hydrochloric acid.

Discussion

Gillam *et al.* (1968) developed the method of purifying specific tRNAs by preparing their phenoxyacetylaminoacyl derivatives, which caused a large shift in the position of elution of the charged tRNA from BD-cellulose. These workers recognized that a small amount of ultraviolet-absorbing material from tRNA preparations, stripped of their amino acids, reacted with the acylating reagent. This material could also be separated from the bulk of the tRNA by BD-cellulose chromatography. The acylated material had a higher affinity for BD-cellulose, and was eluted with buffers containing ethanol after the bulk of the tRNA had been eluted with buffers containing sodium chloride. The higher affinity of phenoxyacetylated tRNA or phenoxyacetylaminoacyl-tRNA for BD-cellulose was due to the high affinity this ion exchanger has for aromatic groups. The results of the present study demonstrate that, in rat liver tRNA, there are some species that react rapidly and extensively with the phenoxyacetic acid ester of *N*-hydroxysuccinimide. These tRNA species could be separated from the unreacted tRNA by BD-cellulose chromatography.

Griffiths (1970) has reported that the introduction of an aromatic group into tRNA does not necessarily cause a shift of the species into the ethanol fraction on BD-cellulose chromatography. Gillam *et al.* (1968) also noted that a significant fraction of some phenoxyacetylaminoacyl-tRNAs could be eluted from BD-cellulose by extensive washing with a saline buffer.

In the present work similar findings were obtained when *E. coli* and yeast tRNA, acylated with [^{14}C]phenoxyacetoxysuccinimide, were studied. Although the acylated tRNA was preferentially eluted in the ethanol fraction (the specific activities for *E. coli* tRNA were 0.18 mole of ^{14}C/mole of tRNA eluted with solution B, and 0.63 mole of ^{14}C/mole of tRNA eluted with solution C), most of the radioactivity was eluted with saline buffer. Rat liver tRNA responded differently to these procedures. Only a small fraction of the rat liver ^{14}C-phenoxyacetylated tRNA was eluted with saline buffers (solution B Table V). This was unexpected, since rat liver tyrosyl-tRNA^{Tyr} is eluted from BD-cellulose with solution B.[2] Tyrosyl-tRNA^{Tyr} also elutes at the same position on methylated albumin kieselguhr chromatography as tRNA^{Tyr}, yet phenoxyacetylated tyrosyl-tRNA^{Tyr} elutes at a different position than tyrosyl-tRNA^{Tyr}. An explanation for the different behavior of phenoxyacetylated tRNA^{Tyr} and tyrosyl-tRNA^{Tyr} is that the tyrosine group is not exposed to solvent, and the phenoxyacetyl group is so exposed. An alternative explanation is that phenoxyacetylation causes some structural alteration in tRNA^{Tyr}. Both BD-cellulose (Sedat *et al.*, 1967) and methylated albumin kieselguhr (Mandell and Hershey, 1960) have a greater affinity for single-stranded nucleic acids than for nucleic acids that have significant base pairing. The greater affinity of these columns for phenoxyacetylated tRNA could be due to structural alterations, resulting in an increase in the number of unpaired bases in these molecules.

This would be in agreement with the findings of Stern *et al.* (1969), who blocked the amino group of phenylalanyl-tRNA^{Phe} and methionyl-tRNA^{Met} with various aliphatic groups, and found that this increases the hydrodynamic size of the tRNA as determined by polyacrylamide gel chromatography and retards the elution from methylated albumin silicic acid columns, as compared to the unblocked species. Although the changes in conformation may be due to the loss of the free amino group in the work of Stern *et al.* (1969), this cannot explain the chromatographic changes observed in the present work, since both tyrosyl-tRNA^{Tyr} and phenoxyacetylated tyrosyl-tRNA^{Tyr} have free tyrosyl amino groups.

The magnitude of the change in elution position on phenoxyacetylation would therefore be caused not just by the incorporation of the group into tRNA, but also by the effect this had on tRNA structure, which would not be the same in all tRNAs. This may explain the large differences in elution patterns of *E. coli*, yeast, and rat liver tRNA. Although the structure of the tRNA may be altered by acylation, there is no significant loss of activity of the tRNA with respect to its ability to accept amino acids, since the recoveries of tRNA before and after acylation were equivalent (Table I).

Since [^{14}C]phenoxyacetoxysuccinimide acylates the tRNA, and a sham procedure carried out at pH 8 with phenoxyacetic acid and *N*-hydroxysuccinimide does not alter the chromatographic properties of the tRNA, the change in these properties cannot be explained solely by a conformational change in the tRNA caused by the manipulations involved. However, the nature of the base, or bases, being acylated has

[2] Unpublished observations.

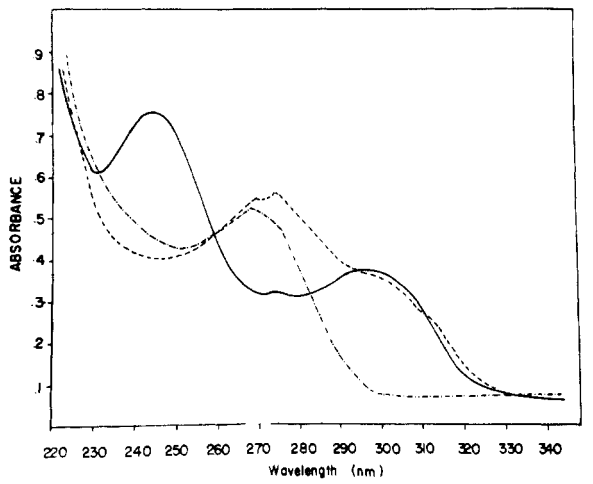

FIGURE 4: Ultraviolet spectra of the compound isolated from the reaction of cytidine with phenoxyacetoxysuccinimide. (——) pH 1, (– – – –) pH 12, (– · –), after standing at pH 12 for 10 min at room temperature.

not been completely elucidated. Although it has been suggested that thiol bases may be involved (Schofield *et al.*, 1970), this has been ruled out in the present work in two ways. First, there is no change in the absorbance of *E. coli* tRNA at 330 nm after acylation; and secondly, iodine oxidation of rat liver tRNA does not affect the acylation procedure (Table IV).

Akashi *et al.* (1965) have described the isolation from yeast-soluble RNA of modified guanosine residues containing amino acids in ester linkage to the 2′-hydroxyl group of the ribose moiety. The acylated nucleoside isolated in the present study differs from the expected acylated product of such a modified guanosine residue by its behavior on Dowex 50, its electrophoretic behavior in 1 M formic acid and sodium tetraborate, as well as by its spectrum.

Chladek *et al.* (1969) have been able to acetylate cytidine with acetoxysuccinimide in dimethylformamide, and Schofield *et al.* (1970) have been able to demonstrate a slight reaction of the *N*-hydroxysuccinimide ester of 2,2,5,5-tetramethyl-3-carboxylpyrrolin-1-oxyl with poly(C). Treatment of the four major nucleosides with phenoxyacetoxysuccinimide under the conditions used in this study, followed by examination of the reaction mixture by electrophoresis and chromatography, failed to reveal any new ultraviolet-absorbing components. However, when the reaction was carried out with cytidine in dry pyridine, a new fluorescent compound with an R_F of 0.83 in butanol–acetic acid–water (5:2:3) was detected; the spectrum of this compound, shown in Figure 4, is identical with the spectrum of acetylcytidine at pH 7 (von Montagu *et al.*, 1968). Like acetylcytidine, it is extremely labile to base, hydrolyzing rapidly in 0.34 M ammonia to give cytidine.

Keith and Ebel (1968) studied the acylation of tRNA with acetic anhydride, and found that, of the four major nucleoside residues in tRNA, only cytidine was acetylated, forming N^6-acetylcytidine. The rapid loss of a portion of the radioactivity from [^{14}C]phenoxyacetylated rat liver tRNA on mild base hydrolysis could be explained by the acylation of a small number of cytidine residues (Table VI). These residues would be hydrolyzed under the conditions used for enzymic digestion of the tRNA, and may, in fact, account for the small amount of [^{14}C]phenoxyacetate recovered from the digests.

TABLE VI: Ammonolysis of Acylated tRNA.[a]

Amino Acid Assayed	Treatment	Act. Eluted from BD-cellulose with Soln D (% of Total)	Total Acceptor Act. Eluted with Solns B and D (pmoles/A_{260} Unit)
Asparagine	Acylation	94	43
	Acylation + ammonolysis	88	33
Glycine	Acylation	15	16
	Acylation + ammonolysis	2	15
Isoleucine	Acylation	78	222
	Acylation + ammonolysis	72	220
Lysine	Acylation	21	24
	Acylation + ammonolysis	8	15
Proline	Acylation	5	8
	Acylation + ammonolysis	6	7
Serine	Acylation	36	67
	Acylation + ammonolysis	22	70
Threonine	Acylation	80	39
	Acylation + ammonolysis	74	35
Tyrosine	Acylation	92	11
	Acylation + ammonolysis	87	10

[a] tRNA from rat liver was treated with phenoxyacetoxysuccinimide. The tRNA was precipitated with ethanol, washed, and dissolved in water. An aliquot was treated with 0.3 M ammonia for 1 hr at 37°, precipitated with ethanol, and chromatographed on BD-cellulose.

The results obtained after hydrolysis of phenoxyacetylated rat liver tRNA would suggest that those tRNA species that undergo a small amount of acylation form acylated complexes that are readily hydrolyzed by weak base.

The function of the reactive base in those species of rat liver tRNA that undergo extensive acylation has not been elucidated in this work. The characterization of the structure of the radioactive nucleoside isolated from *E. coli* tRNA is being actively pursued. This nucleoside is not present in yeast, tRNA, since all the [^{14}C]phenoxyacetate incorporated is removed by hydrolysis in dilute ammonia. This would also explain why the degree of acylation of yeast tRNA is much lower than that of *E. coli* and rat liver tRNA under the same conditions.

References

Akashi, S., Murachi, T., Ishihara, H., and Goto, H. (1965), *J. Biochem. (Tokyo) 58*, 162.

Brunngraber, E. F. (1962), *Biochem. Biophys. Res. Commun. 8*, 1.

Carbon, J. A., Hung, L., and Jones, D. S. (1965), *Proc. Nat. Acad. Sci. U. S. 53*, 979.
Caskey, C. T., Beaudet, A., and Nirenberg, M. (1968), *J. Mol. Biol. 37*, 99.
Chladek, S., Zemlicka, J., and Gut, V. (1969), *Biochem. Biophys. Res. Commun. 35*, 306.
de Groot, N., Lapidot, Y., Panet, A., and Wolman, Y. (1966), *Biochem. Biophys. Res. Commun. 25*, 17.
Fink, L. M., Goto, T., Frankel, F., and Weinstein, I. B. (1968), *Biochem. Biophys. Res. Commun. 32*, 963.
Fiske, C. W., and Subbarow, Y. (1925), *J. Biol. Chem. 66*, 375.
Fox, J. J., Van Praag, D., Wempen, I., Doerr, I. L., Cheong, L., Knool, J. E., Eidinoff, M. L., Bendich, A., and Brown, B. G. (1959), *J. Amer. Chem. Soc. 81*, 178.
Gillam, I., Blew, D., Warrington, R. C., von Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.
Gillam, I., Millward, S., Blew, D., von Tigerstrom, M., Wimmer, E., and Tener, G. M. (1967), *Biochemistry 6*, 3043.
Griffiths, E. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 10*, 225.
Hanes, C. S., and Isherwood, F. A. (1949), *Nature (London) 164*, 1107.
Hayes, N. V., and Branch, C. E. K. (1943), *J. Amer. Chem. Soc. 65*, 1555.
Kagawa, T., Fukinbara, T., and Sumiki, Y. (1963), *Agr. Biol. Chem. 27*, 598.
Keith, G., and Ebel, J.-P. (1968), *C. R. Acad. Sci. 266*, 1066.
Knorre, D. G., Pustoshilova, N. M., Teplova, N. M., Shamovskii, G. G. (1965), *Biokhimiya 30*, 1218.
Lipsett, M. N. (1966), *Cold Spring Harbor Symp. Quant. Biol. 31*, 449.
Magasanik, B., Vischer, E., Donigen, R., Elson, E., and Chargaff, E. (1950), *J. Biol. Chem. 186*, 37.
Mandell, J. D., and Hershey, A. D. (1960), *Anal. Biochem. 1*, 66.
Maxwell, I. H., Wimmer, E., and Tener, G. M. (1968), *Biochemistry 7*, 2629.
Nishizuka, Y., and Lipmann, F. (1966), *Proc. Nat. Acad. Sci. U. S. 55*, 212.
Roy, K. L., and Söll, D. (1968), *Biochim. Biophys. Acta 161*, 572.
Schofield, P., Hoffman, B. M., and Rich, A. (1970), *Biochemistry 9*, 2525.
Sedat, J. W., Kelley, R. B., and Sinsheimer, R. L. (1967), *J. Mol. Biol. 26*, 537.
Stern, R., Zutra, L. E., and Littauer, U. Z. (1969), *Biochemistry 8*, 313.
Stuart, A., and Khorana, H. G. (1964), *J. Biol. Chem. 239*, 3885.
Sueoka, N., and Yamana, T. (1967), *Methods Enzymol. 12*, 658.
Uziel, M., Koh, C., and Cohn, W. E. (1968), *Anal. Biochem. 25*, 77.
von Montagu, M., Molemans, F., and Stock, J. (1968), *Bull Soc. Chim. Belg. 77*, 171.
Wyatt, G. R. (1951), *Biochem. J. 48*, 584.
Zubay, G. (1962), *J. Mol. Biol. 4*, 347.

C1q Protein of Human Complement†

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ABSTRACT: C1q is a subcomponent of the first component of complement which exhibits affinity for various immunoglobulins. It is an unusual protein in that, as a normal plasma constituent, its chemical composition bears resemblance to that of collagen. A method of isolation of C1q from human serum has been described which affords preparation of the protein in a high degree of ultracentrifugal and immunochemical homogeneity. Purification was 370-fold and the yield ranged between 3 and 12% of the amount of C1q in serum. The molecular weight of C1q was 393,000 or 410,000, depending on the method of determination. Evidence for dissociation into noncovalently linked subunits was obtained by electrophoresis in polyacrylamide gels containing 0.1% sodium dodecyl sulfate. C1q contains 181 residues of glycine, 17 residues of hydroxylysine, and 51 residues of hydroxyproline per 1000 amino acid residues. The total carbohydrate content of the protein is 9.8% by weight, 6.45% by weight consisting of approximately equimolar amounts of galactose and glucose. Evidence was obtained suggesting that a glucose–galactose moiety is linked to hydroxylysine.

The subcomponent C1q[1] represents one of three different proteins occurring in plasma as a calcium-dependent complex which constitutes the first component of complement (Lepow *et al.*, 1963). C1q is able to distinguish between various immunoglobulins in that it enters into protein–protein interaction with γG and γM, but not with γA, γD, and γE. One molecule of C1q carries five to six binding sites for γG. Interaction of C1q with immune complexes results in activation of the complement system. These antibody-like properties prompted a chemical investigation of the protein. An initial chemical exploration showed the presence of hydroxylysine

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[1] The nomenclature of complement used is that recommended by the World Health Organization (*Bull. WHO* (1968), *39*, 935). The components of complement (C) are designated numerically, C1–C9. The subcomponents of C1 are called C1q, C1r, and C1s. Intermediate reaction products of complement-dependent cytolysis consisting of cells (E), antibody to cell surface antigens (A), and complement components are symbolized by notations which indicate the components required for their formation: EAC1; EAC1,4; EAC1,4,2, etc.