Evidence for the Absence of the Terminal Adenine Nucleotide at the Amino Acid-Acceptor End of Transfer Ribonucleic Acid in Non-Lactating Bovine Mammary Gland and its Inhibitory Effect on the Aminoacylation of Rat Liver Transfer Ribonucleic Acid

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1. tRNA isolated from non-lactating bovine mammary gland competitively inhibits the formation of aminoacyl-tRNA in the rat liver system. 2. Non-lactating bovine mammary gland tRNA and twice-pyrophosphorolysed rat liver tRNA are unable to accept amino acids in a reaction catalysed by aminoacyl-tRNA synthetases from either rat liver or bovine mammary gland. Deacylated rat liver tRNA can however be aminoacylated in the presence of either enzyme. 3. Bovine mammary gland tRNA lacks the terminal adenine nucleotide at the 3′-terminus amino acid acceptor end, which can be replaced by incubation in the presence of rat liver nucleotide-incorporating enzyme, ATP and CTP. 4. The enzymically modified bovine tRNA (tRNApCpCpA) can bind labelled amino acids to form aminoacyl-tRNA, which can then transfer its labelled amino acids to growing polypeptide chains on ribosomes. 5. Molecules of rat liver tRNA or bovine mammary gland tRNA that lack the terminal adenine nucleotide or the terminal cytosine and adenine nucleotides inhibit the aminoacylation of normal rat liver tRNA to varying degrees. tRNA molecules lacking the terminal −pCpCpA nucleotide sequence exhibit the major inhibitory effect. 6. The enzyme fraction from bovine mammary gland corresponding to that containing the nucleotide-incorporating enzyme in rat liver is unable to catalyse the incorporation of cytosine and adenine nucleotides in pyrophosphorolysed rat liver tRNA and deacylated bovine tRNA. This fraction also markedly inhibits the action of the rat liver nucleotide-incorporating enzyme.

In recent years considerable attention has been paid to the enzymic mechanisms involved in protein synthesis. The initial steps involve the activation of amino acids, for which it has been shown that there is a specific enzyme (aminoacyl-tRNA synthetase) for each of the 20 amino acids found in proteins. This is followed by the addition of the activated amino acids to the 2′- or 3′-hydroxyl group of the terminal adenine nucleotide in tRNA (Hecht, Stephenson & Zamecnik, 1958; Zachau, Acs & Lipmann, 1958). The cellular fraction of the pH 5 enzymes contains all the constituents necessary for the activation of amino acids and the subsequent formation of aminoacyl-tRNA complexes. These constituents include, in particular, aminoacyl-tRNA synthetases (Hoagland, Keller & Zamecnik, 1956) and tRNA (Hoagland, Zamecnik & Stephenson, 1957). The amino acid acceptor molecules have been shown to contain a specific

nucleotide end-grouping consisting of two cytosine nucleotides and a terminal adenine nucleotide, since tRNA isolated from a 'preincubated pH 5 fraction' of rat liver is able to accept two cytidylate units per adenylate unit incorporated (Hecht, Zamecnik, Stephenson & Scott, 1958). Terminal addition of adenine nucleotides has been observed in several laboratories by Paterson & LePage (1957), Canellakis (1957) and Edmonds & Abrams (1957), who reported that ATP rather than ADP is the precursor in the reaction. These observations have been shown that tRNA has the terminal sequence −pCpCpA at the 3′-terminus amino acid acceptor end.

Much research has been carried out on the formation of aminoacyl-tRNA in both homologous and heterologous systems with aminoacyl-tRNA synthetases and tRNA species from the same source and from different sources (see review by Novelli,

1967). In addition it has been shown that homogenates of lactating mammary gland are able to catalyse ATP-dependent amino acid activation and transfer to tRNA (Frazer, Shimizu & Gutfreund, 1959; Bucovaz & Davis, 1961).

It has been shown however that the pH 5 enzyme fraction prepared from non-lactating bovine mammary gland is incapable of forming aminoacyl-tRNA complexes (Herrington & Hawtrey, 1969a). This enzyme fraction was also found to inhibit markedly the aminoacylation of tRNA in the rat liver system as well as the transfer of labelled amino acids from rat liver amino[^{14}C]acyl-tRNA to protein in rat liver polyribosomes. The inhibition observed was attributed to both ribonuclease activity and tRNA effects.

The present paper discusses the inhibitory effect of bovine mammary gland tRNA on the aminoacylation of rat liver tRNA with synthetase enzymes from both rat liver and non-lactating bovine mammary gland. Evidence is also presented to illustrate the existence of active and functional aminoacyl-tRNA synthetases in the bovine pH 5 enzyme fraction, which are probably incapable of functioning in the system *in vivo* owing to the presence of inhibitory tRNA.

This inhibitory effect may be due to the absence of the terminal adenine nucleotide at the amino acid-acceptor end of non-lactating bovine mammary gland tRNA. Once the terminal nucleotide has been incorporated, the RNA is able to accept amino acids and is capable of transferring these amino acids to a growing polypeptide chain on the ribosome.

MATERIALS AND METHODS

Chemicals. ATP (disodium salt), phosphoenolpyruvate (tricyclohexylammonium salt), crystalline GSH, and pyruvate kinase were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. CTP (tripotassium salt) and streptomycin sulphate were purchased from Calbiochem, Los Angeles, Calif., U.S.A., and GTP (trisodium salt) from Sigma Chemical Co., St Louis, Mo., U.S.A. Crystalline ribonuclease (grade 3) was from Seravac Laboratories (Pty.) Ltd., Cape Town, South Africa, and protamine sulphate was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Yeast ^{14}C-labelled protein hydrolysate (1500 μCi/mg) and ^{3}H-labelled ATP (tetralithium salt; 4.1 Ci/mmol) were obtained from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. All solutions were prepared before use and adjusted to pH 7.6 with 0.3 M-KOH.

Scintillation chemicals. 2,5-diphenyloxazole (PPO) and 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP) were purchased from Packard Instrument Co. Inc., La Grange, Ill., U.S.A. The scintillation fluid contained 0.5% PPO and 0.03% POPOP in chromatography-grade toluene.

Tissue. Mammary tissue from adult non-lactating cows was used for the preparation of tRNA and amino-acyl-tRNA synthetases. Livers from Wistar albino rats (150–250 g body wt.) were used for preparation of subcellular fractions. The rats were starved for 24 h before being killed by a blow on the head.

Preparation of subcellular fractions. Rat liver and bovine mammary gland homogenates were prepared in medium A, containing (final concns.): 0.25 M-sucrose, 5 mM-MgCl$_2$, 25 mM-KCl and 50 mM-tris–HCl buffer, pH 7.6, as described by Hawtrey, Schirren & Dijkstra (1963) and Herrington & Hawtrey (1969b) respectively.

(1) *Polyribosomes.* Rat liver polyribosomes were prepared by the method of Wettstein, Staehelin & Noll (1963) as modified for centrifuge tubes of the no. 30 rotor (Spinco model L 50 ultracentrifuge) by Hawtrey & Nourse (1966). The polyribosomes were suspended in medium A and left overnight at 0°C. Denatured material was removed by centrifugation at 1400 g for 10 min. The resultant polyribosome suspension was used immediately.

(2) *pH 5 enzyme fractions.* These were prepared from cell sap from rat liver and non-lactating bovine mammary gland as described by Hawtrey *et al.* (1963). The fractions were suspended in medium A, assayed for protein concentration and stored at −15°C until required.

(3) *Deacylated tRNA.* tRNA was isolated from the pH 5 enzyme fractions by the method of Herrington & Hawtrey (1969a) before being deacylated by incubation with 1.8 M-tris–HCl buffer, pH 8.0, at 37°C for 1 h (Sarin & Zamecnik, 1964). The deacylated tRNA was reprecipitated twice with ethanol and potassium acetate, dissolved in water and dialysed against water for 6–8 h.

(4) *Twice-pyrophosphorolysed rat liver tRNA.* This was prepared from rat liver pH 5 enzyme by the method of Daniel & Littauer (1963). The samples of RNA were stored at −15°C and thawed just before use.

(5) *Aminoacyl-tRNA synthetases.* These were prepared from the pH 5 enzyme fractions of rat liver and non-lactating bovine mammary gland by a modification of the procedure of Hele (1961). Most of the RNA was removed by addition of a freshly prepared solution of protamine sulphate (10 mg/ml) to the pH 5 fraction over a period of 5 min, in the proportion of 3 mg of protamine/100 mg of pH 5 enzyme protein. The precipitate was removed by centrifugation at 25000 g for 3 min with a Spinco no. 30 rotor (Beckman model L 50 ultracentrifuge). The crude enzyme fraction was obtained by adding 0.5 ml of cold 10 M-potassium acetate–20 mM-EDTA (sodium salt) and 2.8 g of (NH$_4$)$_2$SO$_4$/10 ml of supernatant. The resultant precipitate was dissolved in 20 ml of medium A and further purified by treatment with calcium phosphate gel (Keilin & Hartree, 1938) as described by Hele (1961).

The final fraction, obtained by precipitation with (NH$_4$)$_2$SO$_4$ at approx. 40% saturation, was dissolved in a minimum volume of medium A and dialysed extensively against medium A for 8–10 h. The 'RNA-low' fraction, which contained no RNA detectable by the method described by Herrington & Hawtrey (1969b), was stored overnight at −15°C and thawed just before use. Enzyme preparations made by this method were fairly unstable and lost over half their activity after two cycles of freezing and thawing.

Approximately 5 mg of synthetase-enzyme protein was obtained from 10 g of rat liver, whereas 100 g of mammary tissue was required to prepare the same amount of bovine

enzyme. The amount of adipose and connective tissue present in the gland has not, however, been taken into account.

(6) Nucleotide-incorporating enzyme. The enzyme was prepared as described by Daniel & Littauer (1963) except that the calcium phosphate-gel-adsorption step was omitted from the procedure. The protein fraction obtained at 100% $(NH_4)_2SO_4$ saturation was dissolved in a minimum volume of medium A and dialysed extensively against medium A for approx. 20h until free of sulphate. The enzyme preparation was stored at $-15°C$ and remained stable after three cycles of freezing and thawing.

Addition of cytosine nucleotides and a terminal adenine nucleotide to tRNA. Cytosine and adenine nucleotides were added to the terminal ends of twice-pyrophosphorolysed rat liver tRNA and normal deacylated bovine mammary gland tRNA as follows. The incubation medium contained rat liver nucleotide-incorporating enzyme (500 μg of protein), 1 mM-ATP, 0.5 mM-CTP, 5 mM-phosphoenolpyruvate, 50 μg of pyruvate kinase, 0.2 M-sucrose, 6 mM-MgCl₂, 16 mM-KCl, 32 mM-tris–HCl buffer, pH7.6, and 3 mg of either pyrophosphorolysed rat liver tRNA or deacylated bovine tRNA in a total volume of 10 ml. Incubations were at 37°C for 20 min. When only cytosine nucleotides were to be incorporated, ATP was omitted from the incubation medium. At the end of the incubation the samples were chilled in ice, mixed with an equal volume of 90% (w/v) phenol and shaken at room temperature for 60 min. The emulsion was broken by centrifugation at 1400g for 30 min and the RNA components were precipitated from the aqueous phase at $-15°C$ by the addition of 0.1 vol. of 20% (w/v) potassium acetate and 2.5 vol. of 96% (v/v) ethanol. The RNA was reprecipitated three times with ethanol and potassium acetate, dissolved in water and dialysed against water for 6–8h (eight changes).

Preparation of ¹⁴C-labelled aminoacyl-tRNA complexes. The tRNA of rat liver pH5 enzyme was labelled with yeast ¹⁴C-labelled protein hydrolysate by the method described by Hawtrey (1965). The preparation, which was stored at $-15°C$, was found to have a specific radioactivity of 124×10^3 c.p.m./mg of RNA.

Twice-pyrophosphorolysed rat liver tRNA and deacylated bovine mammary gland tRNA, to which had been added cytosine and terminal adenine nucleotides, were labelled with yeast ¹⁴C-labelled protein hydrolysate as follows. The incubation medium contained rat liver amino[¹⁴C]acyl-tRNA synthetases (800 μg of protein), 15 μmol of ATP, 8 μmol of GSH, yeast ¹⁴C-labelled protein hydrolysate (5 μCi), 0.2 M-sucrose, 7 mM-MgCl₂, 16 mM-KCl, 32 mM-tris–HCl buffer, pH7.6, and either 1 mg of rat liver RNApCpCpA* or bovine mammary gland RNApCpCpA in a total volume of 8 ml. Incubations were at 37°C for 20 min. The ¹⁴C-labelled aminoacyl-tRNA complexes were isolated by the phenol extraction method as described above. The rat liver and bovine mammary gland ¹⁴C-labelled aminoacyl-tRNA species were found to have specific radioactivities of 23.9×10^3 and 13.05×10^3 c.p.m./mg of RNA respectively.

Measurement of combination of labelled amino acids

with tRNA. This assay determined the yield of aminoacyl-tRNA formed when the enzyme, ATP, GSH and amino acids were present in excess and the amount of acceptor RNA was limiting. The incubation mixture contained 3 μmol of ATP, 1.6 μmol of GSH, deacylated rat liver tRNA (150 μg), yeast ¹⁴C-labelled protein hydrolysate (1 μCi), 0.2 M-sucrose, 35 mM-tris–HCl buffer, pH7.6, 17 mM-KCl, 7 mM-MgCl₂ and aminoacyl-tRNA synthetases (80–130 μg of protein) in a total volume of 0.9 ml. Unless otherwise stated, incubations were at 37°C for 20 min, in duplicate.

The reactions were stopped by the addition of an equal volume of cold 10% (w/v) trichloroacetic acid. After standing for 20 min at 4°C the precipitates were washed successively with cold 5% (w/v) trichloroacetic acid, ethanol–ether (1:1, v/v), and ether before being filtered and thoroughly washed on Millipore HA filters (pore size 0.45 μm). The filters were dried in air, placed in counting vials with 10 ml of scintillation fluid and counted in a liquid-scintillation spectrometer (Packard Tri-Carb model 2002).

Determinations of RNA and protein. Protein was determined by the biuret method of Gornall, Bardawill & David (1949) with bovine serum albumin as standard. The concentration of RNA was measured spectrophotometrically at 260 nm ($E_{1cm}^{1\%}$ 170) (Hawtrey, Nourse & King, 1966).

RESULTS

Effect of tRNA from different sources on the labelling of tRNA in rat liver pH5 *enzyme.* Deacylated tRNA isolated from the pH5 enzyme fraction of non-lactating bovine mammary gland strongly inhibits the incorporation of labelled amino acids into protein by rat liver polyribosomes (Herrington & Hawtrey, 1969a). Deacylated tRNA isolated from rat liver, however, has a stimulatory effect. Since it has been shown that the bovine pH5 fraction also prevents the aminoacylation of tRNA in rat liver pH5 enzyme, further studies were conducted to observe the effect of bovine mammary gland tRNA on the formation of rat liver amino-[¹⁴C]acyl-tRNA.

The results in Fig. 1 show that the bovine mammary gland tRNA strongly inhibits aminoacyl-tRNA formation in the rat liver system, the degree of inhibition increasing rapidly with the amount of tRNA added, up to a maximum of 76% inhibition at 800 μg. As would be expected, the addition of deacylated rat liver tRNA to the system has a stimulatory effect on aminoacylation owing to the increased amount of tRNA available to accept amino acids.

Three factors suggest that the RNA components isolated from the pH5 enzyme fractions of non-lactating bovine mammary gland and rat liver are tRNA: (1) they have sedimentation coefficient of approx. 4S, as shown by sucrose-density-gradient centrifugation; (2) treatment of the RNA components with an equal volume of 4 M-lithium

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* Abbreviations: tRNA–, tRNA lacking the terminal cytosine and adenine nucleotides; tRNApCpC, tRNA lacking the terminal adenine nucleotide.
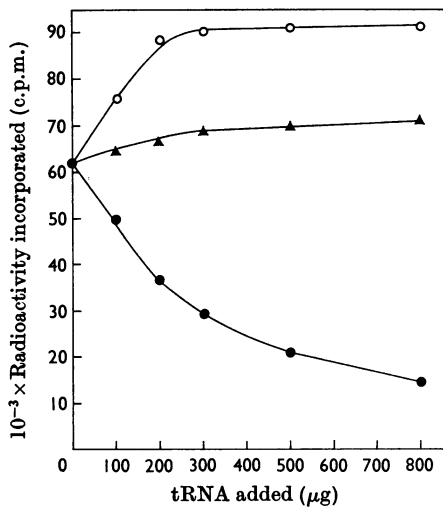
Fig. 1. Effect of deacylated tRNA from rat liver (O), bovine liver (▲) and non-lactating bovine mammary gland (●) on the formation of rat liver amino[^{14}C]acyl-tRNA. The incubation medium consisted of rat liver pH 5 enzyme (1 mg of protein), 2μmol of ATP, 1.6μmol of GSH, yeast ^{14}C-labelled protein hydrolysate (1μCi), 35mM-tris–HCl buffer, pH 7.6, 17mM-KCl, 7mM-MgCl$_2$ and increasing concentrations of the indicated tRNA in a total volume of 1 ml. All incubations were carried out in duplicate at 37°C for 15 min.
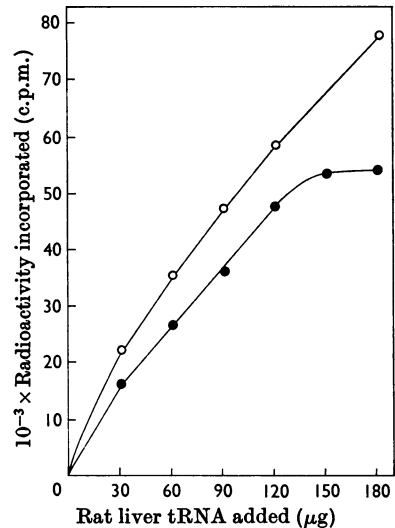

Fig. 2. Kinetics of aminoacyl-tRNA formation by rat liver aminoacyl-tRNA synthetases in the presence of 300μg of rat liver tRNA (□), 150μg of rat liver tRNA (O) and 150μg of non-lactating bovine mammary gland tRNA (●). See the Materials and Methods section for the conditions used for the assay of aminoacyl-tRNA formation.

chloride produces no precipitate, which would otherwise indicate the presence of RNA having a sedimentation coefficient greater than 4S; (3) both exhibit a hydrogen-bonded nature, as shown by their thermal-denaturation profiles.

Fig. 3. Formation of aminoacyl-tRNA as a function of the amount of acceptor rat liver tRNA in the absence (O) and presence (●) of 100μg of non-lactating bovine mammary gland tRNA. The incubation medium consisted of rat liver aminoacyl-tRNA synthetases (130μg of protein), deacylated tRNA, 3μmol of ATP, 1.6μmol of GSH, yeast ^{14}C-labelled protein hydrolysate (1μCi), 35mM-tris–HCl buffer, pH 7.6, 17mM-KCl, 7mM-MgCl$_2$, in a total volume of 0.9 ml. All incubations were carried out in duplicate at 37°C for 20 min.
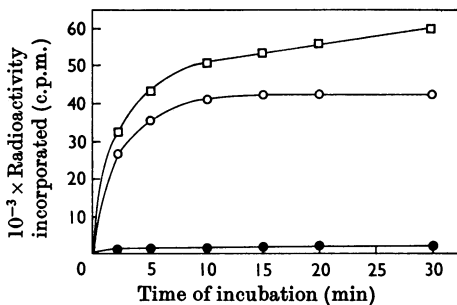
Kinetics of rat liver aminoacyl-tRNA synthesis. An examination of the kinetics of aminoacyl-tRNA formation shows that the reaction proceeds rapidly with time, reaching a limit at 15 min, when 150μg of deacylated rat liver tRNA was used as acceptor in the presence of rat liver aminoacyl-tRNA synthetases (130μg of protein) (Fig. 2). An increase in the concentration of rat liver tRNA produced a rate-stimulating effect, with an increase in V_{max}. Very little variation in behaviour was observed between one preparation of tRNA and another. In addition, Fig. 2 shows that when bovine mammary gland tRNA (150μg) was used as substrate in the presence of the rat liver synthetases, very little activation occurred, suggesting that this tRNA is incapable of accepting amino acids.

If in the first case the reaction was allowed to proceed to completion in the presence of varying amounts of rat liver acceptor RNA, the amount of aminoacyl-tRNA formed was a linear function of the amount of acceptor RNA added (Fig. 3). By introducing a fixed quantity of bovine tRNA (100μg) into the system, a decrease in the yield of aminoacyl-tRNA was observed owing to the inhibitory effect of this RNA on the aminoacylation of rat liver tRNA.
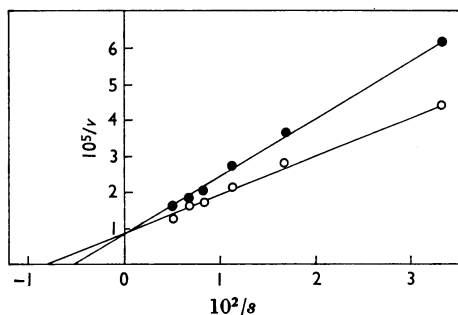
Fig. 4. Lineweaver–Burk reciprocal plots showing the effect of deacylated bovine tRNA on the values of K_m and V_{max}. determined for the aminoacylation of rat liver tRNA. The values for the reciprocals were obtained from the results shown in Fig. 3.
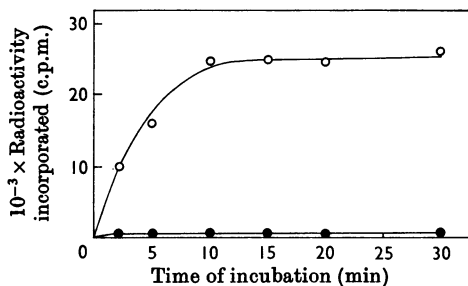

Fig. 5. Kinetics of aminoacyl-tRNA formation by non-lactating bovine mammary gland aminoacyl-tRNA synthetases in the presence of 150 μg of deacylated rat liver tRNA (○), and 150 μg of non-lactating bovine mammary gland tRNA. See the Materials and Methods section for the conditions used for the assay of aminoacyl-tRNA formation.

Application of the reciprocal-plot procedure of Lineweaver & Burk (1934) to the results shown in Fig. 3 showed that the inhibition exerted by bovine tRNA on the formation of rat liver aminoacyl-tRNA was competitive, with K_m approx. 1.2×10^{-9} M (Fig. 4). This value appears to be lower than that reported for synthetases from other sources (Allende et al. 1966; Baldwin & Berg, 1966; Hele & Barth, 1966).

Synthetase enzymes of non-lactating bovine mammary gland. A number of investigators have studied the formation of aminoacyl-tRNA in heterologous systems with aminoacyl-tRNA synthetases from different sources. The results in Fig. 5 show that non-lactating bovine mammary gland contains active synthetase enzymes that are capable of adding amino acids to the terminal adenosine group of rat liver tRNA but not to that of bovine tRNA.

Table 1. *Requirements for the aminoacylation of rat liver tRNA by non-lactating bovine mammary gland aminoacyl-tRNA synthetases*

The complete system consisted of bovine aminoacyl-tRNA synthetases (75 μg of protein), 150 μg of deacylated rat liver tRNA, 3 μmol of ATP, 1.6 μmol of GSH, yeast ^{14}C-labelled protein hydrolysate (1 μCi), 35 mM-tris–HCl buffer, pH 7.6, 17 mM-KCl, 7 mM-MgCl$_2$, in a total volume of 0.9 ml. All incubations were carried out in duplicate at 37°C for 20 min.

System	Aminoacyl-tRNA formation (c.p.m.)
Complete	37 344
Without ATP	255
Without GSH	4 975
Without ATP and GSH	575
Without MgCl$_2$	126
+Ribonuclease (10 μg)*	139

* The complete system was preincubated with ribonuclease (10 μg) for 5 min at 37°C.

These enzymes appear to be as active as those from rat liver on a protein-for-protein basis, the reaction being completed in 15 min. This shows that the method for the preparation of the synthetase enzymes removes almost all the tRNA, since contaminating bovine tRNA would decrease the yield of aminoacyl-tRNA formed.

Aminoacylation of tRNA was observed in the presence of ATP, GSH, Mg^{2+}, deacylated rat liver tRNA, ^{14}C-labelled amino acids and synthetase enzymes prepared from the pH 5 fraction of non-lactating bovine mammary gland (Table 1). In each case, omission of any one of these components results in a marked decrease in the amount of aminoacyl-tRNA formed. Preincubation of the system with ribonuclease prevented the aminoacylation of rat liver tRNA, owing to its degradation.

Incorporation of ribonucleotides into RNA. A number of investigators have demonstrated the incorporation of adenine and cytosine nucleotides into tRNA (Edmonds & Abrams, 1957; Hecht et al. 1958; Hecht, Stephenson & Zamecnik, 1959; Daniel & Littauer, 1963). It has been shown that ATP and CTP are the immediate precursors in this reaction. Table 2 shows the extent of incorporation of ^{3}H-labelled AMP into normal deacylated bovine tRNA and twice-pyrophosphorolysed rat liver tRNA. It is known that the addition of the cytosine nucleotide is independent of the presence of other nucleotides, whereas incorporation of the adenine nucleotide is enhanced by the addition of CTP. The results show that untreated bovine mammary gland tRNA and treated rat liver tRNA are able to accept approximately the same amount of

Table 2. *Addition of the terminal adenine nucleotide to tRNA under different conditions*

The incorporation of the labelled adenine nucleotide was determined during incubation at 37°C of rat liver nucleotide-incorporating enzyme (50 μg of protein), the indicated tRNA (150 μg), 5 μmol of phosphoenol-pyruvate, 50 μg of pyruvate kinase, 0.2 M-sucrose, 35 mM-tris–HCl buffer, pH 7.6, 17 mM-KCl and 7 mM-MgCl$_2$. Further additions of 0.2 μmol of CTP, 2 μmol of unlabelled ATP and 2 μmol of [^{3}H]ATP were added where indicated. Incubation times (in min) are shown in parentheses.

		Amount of nucleotide incorporated (c.p.m./mg of tRNA)	
Initial additions	Subsequent additions	Pyrophosphorolysed rat liver tRNA	Deacylated bovine tRNA
RNA+[^{3}H]ATP+CTP (10)	None	8 007	9 553
RNA+[^{3}H]ATP+CTP (20)	None	11 667	11 000
RNA+[^{3}H]ATP (10)	None	7 587	7 342
RNA+[^{3}H]ATP (10)	+CTP (10)	9 167	9 927
RNA+[^{12}C]ATP+CTP (10)	+[^{3}H]ATP (10)	1 227	940

Table 3. *End-group-nucleotide requirement of tRNA for aminoacylation by rat liver and bovine synthetase enzymes*

The incubation medium contained aminoacyl-tRNA synthetases, 3 μmol of ATP, 1.6 μmol of GSH, yeast ^{14}C-labelled protein hydrolysate (1 μCi), 7 mM-MgCl$_2$, 16 mM-KCl, 35 mM-tris–HCl buffer, pH 7.6, and 200 μg of the indicated RNA in a total volume of 1 ml. All incubations were carried out in duplicate at 37°C for 20 min.

	^{14}C-labelled amino acids incorporated into tRNA (c.p.m.)	
tRNA component added	Rat liver synthetases (150 μg of protein)	Bovine synthetases (80 μg of protein)
Deacylated rat liver tRNA	69 650	36 294
Twice-pyrophosphorolysed rat liver tRNA	97	837
Rat liver tRNApCpC	6 703	3 673
Rat liver tRNApCpCpA	43 737	23 460
Deacylated bovine tRNA	361	0
Bovine tRNApCpC	6 472	3 107
Bovine tRNApCpCpA	24 548	11 048

Table 4. *Inhibition of aminoacylation in the rat liver system by RNA components with different end-group nucleotides*

The incubation medium contained rat liver aminoacyl-tRNA synthetases (150 μg of protein), 3 μmol of ATP, 1.6 μmol of GSH, yeast ^{14}C-labelled protein hydrolysate (1 μCi), deacylated rat liver tRNA (200 μg), 0.2 M-sucrose, 7 mM-MgCl$_2$, 17 mM-KCl, 35 mM-tris–HCl buffer, pH 7.6, and 200 μg of the indicated tRNA in a total volume of 1 ml. All incubations were carried out in duplicate at 37°C for 20 min.

tRNA component added	Radioactivity incorporated (c.p.m.)	Stimulation (%)
Deacylated rat liver tRNA	69 650	—
Twice-pyrophosphorolysed rat liver tRNA	38 974	−44.0
Rat liver tRNApCpC	59 084	−15.2
Rat liver tRNApCpCpA	85 786	+23.2
Deacylated bovine tRNA	61 884	−11.2
Bovine tRNApCpC	58 079	−16.6
Bovine tRNApCpCpA	79 961	+14.8

labelled AMP. The extent of labelling appears to be dependent on time, since after 20 min of incubation the incorporation of AMP was increased. Omission of CTP from the incubation medium still resulted in attachment of ^{3}H-labelled AMP at the 3'-terminus amino acid-acceptor end in both RNA species, but not to the same extent as when CTP was present.

Addition of amino acids to isolated tRNA components with incomplete and complete end grouping. The results in Figs. 2 and 5 conclusively show that tRNA isolated from the pH 5 enzyme fraction of non-lactating bovine mammary gland is unable to

accept amino acids in a reaction catalysed either by its own synthetase enzymes or by those from rat liver. Deacylated rat liver tRNA, on the other hand, is readily able to accept amino acids. However, when the terminal cytosine and adenine nucleotides are removed from rat liver tRNA by pyrophosphorolysis the molecule is no longer able to form the aminoacyl-tRNA complex (Table 3).

Incubation of bovine mammary gland tRNA and pyrophosphorolysed rat liver tRNA in the presence of ATP, CTP and the rat liver nucleotide-incorporating enzyme results in the addition of the terminal nucleotide group (–pCpCpA). As a result of this addition both RNA components were capable of accepting amino acids and synthesizing amino-acyl-tRNA when the reaction was catalysed by

aminoacyl-tRNA synthetases from either rat liver or bovine mammary gland.

Effect exerted by modified tRNA components on the aminoacylation of rat liver tRNA. Table 4 shows that deacylated bovine mammary gland tRNA inhibits the aminoacylation of rat liver tRNA by approx. 11.2%. Removal of the terminal –pCpCpA nucleotides from rat liver tRNA by pyrophosphorolysis produces an RNA molecule that is unable to accept amino acids and also inhibits synthesis of rat liver aminoacyl-tRNA, the inhibitory effect being greater than that exerted by normal deacylated bovine tRNA. However, when cytosine and adenine nucleotides are enzymically introduced on the end of both these RNA inhibitors, not only are they capable of accepting amino acids but also have a marked stimulatory effect on the yield of aminoacyl-tRNA formed in the presence of rat liver tRNA and synthetase enzymes.

The results in Table 3 show that pyrophosphorolysed rat liver tRNA and deacylated bovine tRNA that have been enzymically modified to contain theoretically only terminal cytosine nucleotides (tRNApCpC) can accept amino acids. These molecules are however able to inhibit the aminoacylation of rat liver tRNA to the same extent as that shown by normal deacylated bovine mammary gland tRNA. It is possible that the system used for the incorporation of cytosine nucleotides is not entirely free of ATP and as a result a few RNA molecules would be formed that contain a terminal –pCpCpA nucleotide sequence.

Additional evidence that bovine mammary gland tRNA that has been modified by the addition of terminal cytosine and adenine nucleotides can function as normal tRNA is given in Table 5. The modified tRNA can transfer labelled amino acids attached to the 2'- or 3'-hydroxyl group of the terminal adenosine group to growing polypeptide chains on the ribosomes. The efficiency of transfer is equal to that shown by rat liver aminoacyl-tRNA,

being of the order of 25%. It is however possible that under the experimental conditions used for the isolation of the tRNApCpCpA–amino acid, some amino acids may well be lost from the RNA.

Nucleotide-incorporating enzyme. The nucleotide-incorporating enzyme prepared from the pH5 fraction of rat liver by the method of Daniel & Littauer (1963) has been shown to add cytosine and adenine nucleotides to the 3'-terminus amino acid acceptor end of deacylated bovine mammary gland tRNA and twice-pyrophosphorolysed rat liver tRNA (Tables 2, 3, 5 and 6). The resultant RNA components (tRNApCpCpA) are able to accept amino acids in a reaction catalysed by aminoacyl-tRNA synthetases from either rat liver or bovine mammary gland.

If however the enzyme fraction corresponding to the nucleotide-incorporating enzyme of rat liver is obtained from non-lactating bovine mammary gland and used in an attempt to introduce cytosine and adenosine nucleotides on to bovine mammary gland tRNA, the results reported in Table 6 are obtained. It appears that this enzyme is totally incapable of adding the terminal –pCpCpA nucleotide sequence, since the resultant rat liver and bovine mammary gland tRNA components are unable to accept amino acids. Whether this enzyme modifies the pyrophosphorolysed rat liver or bovine tRNA in any way or merely does not catalyse the incorporation reaction is not known.

By mixing equal protein concentrations of rat liver nucleotide-incorporating enzyme and the corresponding bovine enzyme fraction, one would expect the cytosine and adenine nucleotides to be incorporated terminally into pyrophosphorolysed rat liver tRNA and deacylated bovine tRNA. The results presented in Table 6 show that the bovine mammary gland enzyme fraction is able to block completely the action of the rat liver enzyme, since the resulting tRNA components are still unable to accept amino acids.

Table 5. *Transfer of labelled amino acids from different amino[14C]acyl-tRNA components into protein by rat liver polyribosomes*

The incubation medium contained rat liver polyribosomes (0.4 mg of protein), rat liver pH5 enzyme (1 mg of protein), 0.5 μmol of GTP, 1.6 μmol of GSH, 5 μmol of phosphoenolpyruvate, 50 μg of pyruvate kinase, 0.2 M-sucrose, 6 mM-MgCl$_2$, 17 mM-KCl, 35 mM-tris–HCl buffer, pH 7.6, and the indicated amino[14C]acyl-tRNA components in a total volume of 1.7 ml. All incubations were carried out in duplicate at 37°C for 15 min. For details of preparation of amino[14C]acyl-tRNA, see the Materials and Methods section.

Amino[14C]acyl-tRNA component added	Radioactivity added to the incorporation medium (c.p.m.)	Radioactivity incorporated into protein (c.p.m.)	Percentage of total radioactivity incorporated into protein
Rat liver amino[14C]acyl-tRNA	30420	7879	25.9
Rat liver tRNApCpCpA–amino acid	9264	3038	32.8
Bovine tRNApCpCpA–amino acid	5060	1290	25.5

Table 6. *Effect of nucleotide-incorporating enzymes from rat liver and bovine mammary gland on the addition of the terminal adenosine nucleotide to different tRNA components as judged by amino acid acceptance*

The amino acid-incorporating medium contained rat liver aminoacyl-tRNA synthetases (100 μg of protein), 3 μmol of ATP, 1.6 μmol of GSH, yeast ^{14}C-labelled protein hydrolysate (1 μCi), 0.2 M-sucrose, 8 mM-MgCl$_2$, 16 mM-KCl, 32 mM-tris–HCl buffer, pH 7.6, and 200 μg of the indicated tRNA in a total volume of 1.3 ml. All incubations were carried out in duplicate at 37°C for 20 min. For preparation of RNA components see the Materials and Methods section. Postulated nucleotide end-sequences of the resultant tRNA components are shown in brackets.

Conditions for first pretreatment of different tRNA components	Conditions for second treatment of tRNA components after first pretreatment	^{14}C-labelled amino acids incorporated into tRNA (c.p.m.)
Rat liver tRNA, deacylated (tRNApCpCpA)	None (tRNApCpCpA)	80088
Bovine mammary gland tRNA, deacylated (tRNA–?)	None (tRNA–?)	309
Rat liver tRNA, twice pyrophosphorolysed (tRNA–)	None (tRNA–)	18
Rat liver tRNA, twice pyrophosphorolysed (tRNA–)	Rat liver nucleotide-incorporating enzyme, ATP, CTP (tRNApCpCpA)	82652
Bovine mammary gland tRNA deacylated (tRNA–?)	Rat liver nucleotide-incorporating enzyme, ATP, CTP (tRNApCpCpA)	49215
Rat liver tRNA, twice pyrophosphorolysed (tRNA–)	Bovine mammary gland nucleotide-incorporating enzyme, ATP, CTP (tRNA–?)	3344
Bovine mammary gland tRNA, deacylated (tRNA–?)	Bovine mammary gland nucleotide-incorporating enzyme, ATP, CTP (tRNA–?)	0
Rat liver tRNA, twice pyrophosphorolysed (tRNA–)	Rat liver and bovine mammary gland nucleotide-incorporating enzymes, ATP, CTP (tRNA–?)	2108
Bovine mammary gland tRNA, deacylated (tRNA–?)	Rat liver and bovine mammary gland nucleotide-incorporating enzymes, ATP, CTP (tRNA–?)	0

DISCUSSION

The fact that tRNA is able to inhibit the incorporation of amino acids in a protein-synthesizing system is known. Aaronson, Korner & Munro (1966) have shown that the stimulation of [14C]-leucine incorporation into protein in an *Escherichia coli* S-30 system, brought about by liver microsomal RNA or poly U as template, was inhibited by rat liver tRNA and to a lesser extent by *E. coli* tRNA. In addition, Herrington & Hawtrey (1969a) have shown that tRNA isolated from the pH 5 enzyme fraction of non-lactating bovine mammary gland inhibits total protein synthesis.

The results presented show that the bovine mammary gland tRNA will also inhibit the formation of aminoacyl-tRNA when rat liver pH 5 enzyme is used as the source of acceptor RNA and aminoacyl-tRNA synthetases. A significant feature however is that tRNA isolated from the pH 5 enzyme fraction of bovine liver has no inhibitory effect but, like the rat liver tRNA, will actually stimulate the formation of the activated complex. It appears that tRNA isolated from a 'functional' organ in the cow is capable of being aminoacylated, whereas tRNA from an essentially 'non-functional' organ is not capable of being aminoacylated.

The reciprocal plot of Lineweaver & Burk (1934)

clearly shows that the bovine tRNA acts as a competitive inhibitor of the aminoacylation of rat liver tRNA (Fig. 4). Hence, although the bovine tRNA cannot be aminoacylated it nevertheless partially prevents the aminoacylation of rat liver tRNA by competing for the active sites on the synthetase enzymes.

Detailed studies have been made on the formation of aminoacyl-tRNA in heterologous systems by using aminoacyl-tRNA synthetases from one source and tRNA from a different source (Berg, Bergmann, Ofengand & Dieckmann, 1961; Benzer & Weisblum, 1961; Yamane & Sueoka, 1963; Lagerkvist & Waldenstrom, 1964). They concluded that the extent of combination of amino acids with tRNA is dependent on the source of the enzyme, the tRNA and the particular amino acid used.

The results presented show that rat liver tRNA can be aminoacylated by synthetase enzymes from either rat liver or non-lactating bovine mammary gland, the efficiency of the bovine enzyme being almost equal to that of rat liver. Similar effects have been observed in which Makman & Cantoni (1966), studying purified seryl-tRNA synthetase from yeast, reported that rat liver tRNA had about the same serine-acceptor capacity irrespective of whether the yeast or rat liver enzyme was used.

Enzymes from either rat liver or bovine mammary

gland are, however, unable to aminoacylate bovine tRNA. Hence, although the bovine mammary gland pH5 enzyme cannot synthesize aminoacyl-tRNA, it nevertheless contains an activating-enzyme system that is functional but whose effect is masked owing to the presence of the tRNA. Once separated from the tRNA the enzymes are capable of performing their required functions.

The RNA isolated from the pH5 enzyme fraction of rat liver generally contains the cytosine and adenine nucleotide end group and it is at least partially saturated with amino acids. The nucleotides can be removed from the RNA by incubating the pH5 enzyme in the presence of PP_i and absence of CTP and ATP, resulting in pyrophosphorolysed tRNA (Daniel & Littauer, 1963). The resultant tRNA, like deacylated bovine mammary gland tRNA, is unable to accept amino acids and markedly inhibits the synthesis of rat liver aminoacyl-tRNA. Demonstration of the steps involving the catalytic incorporation of CMP and AMP into pyrophosphorolysed tRNA by the rat liver nucleotide-incorporating enzyme to form the terminal trinucleotide sequence (tRNApCpCpA) may be represented by the reactions suggested by Hecht *et al.* (1959).

$$tRNA + 2\,CTP \;\rightleftharpoons\; tRNApCpC + 2\,PP_i \qquad (1)$$

$$tRNApCpC + ATP \;\rightleftharpoons\; tRNApCpCpA + PP_i \qquad (2)$$

From our results it appears that pyrophosphorolysed rat liver tRNA is able to accept ^3H-labelled AMP whether CTP was included in the incorporation mixture or not. The evidence presented shows that normal bovine mammary gland tRNA accepts the same amount of AMP as pyrophosphorolysed rat liver tRNA, in a reaction in which the presence of CTP enhances the incorporation. This suggests that the tRNA isolated from the pH5 enzyme fraction of non-lactating bovine mammary gland lacks the terminal trinucleotide sequence, –pCpCpA. From the results however one can only conclude that the terminal adenine nucleotide of bovine tRNA is definitely missing. The terminal structure may, therefore, be proposed as being tRNApCpC or tRNA–. This observation explains the inability of bovine tRNA to accept amino acids owing to the absence of the 2'- or 3'-hydroxyl groups of the terminal adenine nucleotide (Zachau *et al.* 1958). This may be substantiated by the fact that bovine tRNA competitively inhibits the aminoacylation of rat liver tRNA but once the terminal –pCpCpA trinucleotide grouping has been incorporated it will in fact stimulate the synthesis of rat liver aminoacyl-tRNA. In addition, Priess, Berg, Ofengand, Bergmann & Dieckmann (1959) have shown that periodate oxidation of the vicinal hydroxyl groups

of the terminal adenosine group in tRNA destroys its capacity to accept an amino acid, and that such a molecule competitively inhibits the aminoacylation of untreated tRNA. Like bovine mammary gland tRNA, this treated RNA exhibits inhibition that is competitive with normal tRNA (Torres-Gallardo & Kern, 1965). These facts coupled with the results presented provide conclusive evidence for the absence of a terminal adenine nucleotide in bovine mammary gland tRNA.

From the results presented in Table 3 one may postulate that bovine tRNA lacks the complete terminal trinucleotide sequence and not just the terminal adenine nucleotide. Both pyrophosphorolysed rat liver tRNA and deacylated bovine tRNA to which have been incorporated terminal cytosine nucleotides (tRNApCpC) are able to accept amino acids with approx. one-tenth the affinity of intact tRNA, an observation that has also been reported for yeast by Makman & Cantoni (1966).

The results presented in Table 6 clearly show that the nucleotide-incorporating enzyme fraction from bovine non-lactating mammary gland (that enzyme fraction corresponding to the rat liver nucleotide-incorporating enzyme), which is itself inactive, nevertheless strongly inhibits the action of the corresponding enzyme fraction from rat liver. This is noteworthy in that it appears that other enzymic factors are playing a role in the addition of nucleotides to the amino acid-acceptor terminal of tRNA.

The results presented in this paper suggest that protein synthesis in the non-lactating bovine mammary gland is under control at the level of tRNA. More specifically, the control is situated at the amino acid-acceptor terminal of tRNA, in that a large percentage of bovine tRNA molecules lack a terminal adenosine unit and therefore cannot accept amino acids. In this connexion it is noteworthy that Cannon (1965) has reported work on the relationship between protein synthesis and tRNA end-group instability in *E. coli*. The concept of the metabolic significance of terminal adenosine-group instability in tRNA (Cannon, 1965) certainly applies to non-lactating bovine mammary gland tRNA.

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