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# The characterization of phosphoseryl tRNA from lactating bovine mammary gland

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## ABSTRACT

BD-cellulose and RPC-5 chromatography of tRNA isolated from lactating bovine mammary gland showed the presence of four seryl-tRNA isoacceptors. The species, $tRNA_{IV}^{Ser}$, with the strongest affinity for BD-cellulose (required ethanol in the elution buffer) could be phosphorylated in the presence of serine, [$\gamma$-$^{32}$P]-ATP, seryl-tRNA synthetase and phosphotransferase activity from the same tissue. O-Phosphoserine was identified as the $^{32}$P-labelled product after mild alkaline hydrolysis of this aminoacylated tRNA. Pancreatic ribonuclease treatment of the aminoacylated tRNA yielded a labelled product which was identified as phosphoseryladenosine. These results indicated there is a specific phosphoseryl tRNA species in lactating bovine mammary gland. It appears that the formation of phosphoseryl-tRNA proceeds by enzymic phosphorylation of seryl-tRNA.

## INTRODUCTION

A role for protein kinases in the enzymic phosphorylation of phosvitin (1) and casein (2) has been clearly demonstrated. These studies however, have not shown whether all the phosphate residues of these two phosphoproteins arise as a consequence of protein kinase activity or whether some phosphate residues are incorporated by other mechanisms (2). Protein kinases that can phosphorylate dephosphorylated bovine casein have been isolated from lactating rat mammary gland (3,4). However, in these studies the actual sites of phosphorylation were not investigated and as a result no indication was given as to whether these kinases phosphorylated only those sites which were originally found to be phosphorylated. Kinase activities have been isolated from lactating bovine mammary gland (5), however, these activities failed to phosphorylate several sites which are found to be phosphorylated *in vivo*.

Specifically, no protein kinase has been detected which can phosphorylate all the sites in the phosphate "cluster" regions of $\alpha_s$- and β-caseins (6). The unique arrangements of phosphoseryl residues in the "cluster" regions of $\alpha_s$- and β-caseins (7,8) and the failure to find a specific protein kinase suggests that perhaps a number of phosphate residues must be present in nascent casein polypeptides to serve as recognition sites during specific phosphorylation by the appropriate kinases. The incorporation of the phosphate required for recognition by the kinases could be ensured by introduction of phosphoserine into the nascent polypeptide during protein synthesis by a specific tRNA.

Phosphoseryl-tRNA has been found in tissue active in phosvitin synthesis (9,10) as well as in rat liver. Whilst participation in cell-free polypeptide synthesis has been demonstrated the incorporation of phosphate as phosphoserine has not been shown. It thus became of interest to determine if a phosphoseryl tRNA is present in lactating bovine mammary gland. The characterization of this tRNA species is reported herein.

## MATERIALS AND METHODS

*General.* Uniformly labelled L-[$^{14}$C]-serine (159mCi/mmole) and L-[3-$^{3}$H]-serine (5.3 Ci/mmole) were obtained from the Radiochemical Centre, Amersham; [γ-$^{32}$P]-ATP was prepared according to the method of Glynn and Chappell (11). BD-cellulose (12) was prepared by the method of Gillam *et al.* (13), RPC-5 was a product of Miles Laboratories and DEAE-cellulose was the Whatman DE-22 or DE-32 types. All other substrates and reagents were of analytical or A grade.

*Tissue.* Lactating bovine mammary glands were obtained from either the State Abattoirs, Sydney, or the Hawkesbury Agricultural College, Richmond, N.S.W. Glands were washed in distilled water to remove excessive milk then frozen in liquid nitrogen before transportation to the laboratory. The time taken from slaughter of the animal to freezing of the gland was usually 10 to 20 minutes.

*Preparation of crude tRNA.* Approximately 250g frozen tissue was homogenized in 1ℓ of 0.1M Tris-HCl, pH 8.0 which contained 10 mM EDTA and 1 mM 2-mercaptoethanol. Frozen tissue was broken into small pieces using a hammer then homogenized in approximately 250 ml of buffer in a heavy duty blendor. Homogenization was completed in a high speed blendor after addition of the remaining amount of buffer. The homogenate was cleared of connective tissue and large debris by centrifugation at 10,000g for 10 minutes. After three phenol extractions the crude RNA was isolated by ethanol precipitation. The RNA was resuspended in 250 ml of the original buffer, solid NaCl added to a final concentration of 1 M and the mixture stirred at 4°C for 1 hr. Any precipitate was removed by centrifugation and the crude tRNA isolated from the supernatant by ethanol precipitation.

The crude tRNA was further purified by DEAE-cellulose chromatography. The tRNA was applied to the column in a pH 7.5 buffer containing 0.3 M NaCl and eluted with a buffer containing 0.65 M NaCl. After ethanol precipitation the tRNA was dried using acetone and stored as a powder at -20°C.

*Aminoacyl-tRNA synthetases.* Although aminoacyl-tRNA synthetase activity could be isolated from lactating bovine mammary gland the activity obtained was usually very labile and enzyme storage was a problem. A more reliable source of synthetase was fresh unfrozen bovine liver; no differences between the specificities of the liver enzyme and the mammary gland enzyme were observed. For the present studies a crude synthetase fraction was suitable. Generally 50 g of tissue was homogenized in 250 ml of 0.05 M HEPES, pH 8.0 which contained 0.01 M $MgCl_2$, 1 mM 2-mercaptoethanol and 0.35 M sucrose. Homogenization was performed for 60 seconds in a high speed blendor. The homogenate was centrifuged at 10,000 g for 10 minutes and the supernatant re-centrifuged at 105,000 g for 60 minutes. The high speed supernatant fraction was stored as aliquots at -20°C. Enzyme prepared in this manner maintained seryl-tRNA synthetase activity for 5 to 6 weeks.

*Phosphotransferase activity.* Enzyme activity that transferred the γ-phosphate of [γ-$^{32}$P] ATP to seryl-

$tRNA_{IV}$ (phosphotransferase activity) was prepared from lactating bovine mammary gland. Frozen mammary gland was homogenized using the same buffer and conditions used in the synthetase preparation. The upper half of the high speed supernatant was collected and stored in aliquots at -20°C. To further fractionate phosphotransferase activity of this high speed supernatant chromatography on DEAE-cellulose (DE-32) was performed. The column (2.0 x 15 cm) was equilibrated, at 4°C, with a 0.01M Tris-HCl, pH 7.5, buffer containing 0.01M $MgCl_2$, 1mM 2-mercaptoethanol and 0.05 M KCl. Approximately 200 $E_{280}$ units was applied and, after washing with one bed volume of buffer containing 0.05M KCl protein was eluted using a linear gradient of 0.05M to 0.6M KCl in the above buffer. Fractions were assayed for seryl-tRNA synthetase activity as well as for phosphotransferase activity.

*Aminoacylation.* Serine acceptance assays were performed in reaction mixtures which contained: 12.5 µmoles HEPES (pH 8.0), 0.5 µmole $MgCl_2$, 0.1 µmole ATP and 314 pmoles $^{14}$C-serine (1 µl $^{14}$C-serine as supplied). The tRNA (0.5 to 1.0 $E_{260}$ unit) and synthetase were added separately. 120 µg protein resulted in an acceptance activity of 38 pmoles $^{14}$C-serine/$E_{260}$ unit of unfractionated tRNA. Total reaction volume was 70 µl and, after incubation at 37°C for 30 minutes, 30 µl samples were spotted onto Whatman 3MM filter paper discs. The amount of radioactivity precipitated by cold TCA treatment was then determined.

For chromatographic purpose the above reaction was increased 10 to 20 fold using either $^{14}$C-serine (16 nmoles) or $^{3}$H-serine (190 pmoles). The aminoacyl-tRNA was isolated by the method of Yang and Novelli (14) using small DE-22 columns.

*Phosphate acceptance.* Each 100 µl reaction mixture contained 50 µmoles HEPES (pH 8.0), 1.0 µmole $MgCl_2$, 0.15 µmole [$\gamma$-$^{32}$P] ATP (approximately 2 µ Ci) and 10 nmoles L-serine. To this reaction mixture was added tRNA (0.5 to 1.0 $E_{260}$ unit), synthetase (150 µg protein) and phosphotransferase (80 µg protein). These amounts of protein resulted in a phosphate acceptance of 14 pmoles $^{32}$P-phosphate/$E_{260}$ unit of un-

fractionated tRNA. The phosphotransferase activity used in all phosphate transfer reactions was the first peak of activity which eluted from DEAE-cellulose, see Figure 5. After incubation at 37°C for 30 minutes each reaction was applied to a small DE-22 column (1 ml bed volume) which was first washed with acetate buffer, pH 4.5, containing 0.35 M NaCl then the aminoacyl-tRNA eluted using 1 M NaCl buffer wash.

*Dual radioisotope determinations.* In experiments where either $^{14}$C-serine and $^{3}$H-serine or $^{14}$C-serine and $^{32}$P-phosphate were used the amount of radioactivity of each nuclide was determined using a Packard Model 2650 programmable scintillation spectrometer.

*High voltage electrophoresis of aminoacyl-tRNA degradation products.* Radioactively labelled ($^{14}$C/$^{32}$P) seryl-tRNA collected from DE-22, was digested with pancreatic ribonuclease A (10 µl of 10 mg/ml solution). Following incubation for 30 minutes at room temperature the reaction mixture was divided into three parts. One part was made 0.1M with respect to Tris-HCl, pH 9.5. Another was digested with acid phosphomonoesterase (10 µl of 10 mg/ml) and both were incubated for 30 minutes at room temperature. The third part was kept at 4°C. Following the 30 minute incubation period each of the reactions was halved and brought to either pH 1.9 or pH 6.4 by addition of the appropriate electrophoresis buffer.

High voltage electrophoresis was performed on Whatman 3 MM paper at either pH 1.9 (6.25% formic acid) or pH 6.4 (pyridine : acetic acid : water; 10 : 4 : 90) at 2,500 V for 45 minutes. Standards were localized by viewing under a U.V. lamp or staining with ninhydrin. Strips containing the samples were cut into 1.0 cm pieces and the radioactivity in each piece determined.

A sample of $^{14}$C-labelled seryladenosine was prepared for use as an electrophoresis standard by treatment of $^{14}$C-labelled seryl-tRNA from bovine liver with ribonuclease A. The seryladenosine was isolated by preparative high voltage electrophoresis at pH 1.9 on Whatman 3 MM.

The product was characterized by electrophoresis (pH 1.9 and pH 6.4) before and after alkaline hydrolysis and by comparison of its U.V. spectral properties to those of adenosine.

*BD-cellulose chromatography.* All BD-cellulose chromatography employed acetate buffer (0.05 M sodium acetate, pH 4.5 containing 15 mM $MgCl_2$ and 2 mM mercaptoethanol) using varying concentrations of NaCl. Flow rates were maintained at 60 ml/hr. For preparative work up to 3,000 $E_{260}$ units crude tRNA in 0.35 M Na Cl-acetate buffer was applied to a BD-cellulose column (2 x 40 cm) previously equilibrated in the same buffer. Elution was effected with a 1.5 M NaCl-acetate buffer wash followed by a 2.0 M NaCl, 15% ethanol-acetate buffer wash. The $tRNA^{Ser}$ which eluted in the 1.5 M NaCl wash will be referred to as $tRNA^{Ser}_{I-III}$ and that which eluted in the 2.0 M NaCl, 15% ethanol wash as $tRNA^{Ser}_{IV}$.

The procedures employed for tRNA chromatography on BD-cellulose by gradient elution are presented under respective figure legends.

*RPC-5 Chromatography.* Chromatography of aminoacyl-tRNA on a RPC-5 column (0.8 x 21 cm) was performed following the procedure of Kelmers and Heatherly, (15).

## RESULTS

*Column chromatography.* BD-cellulose elution profiles of $tRNA^{Ser}$ from various sources show a fraction of serine acceptance activity in the ethanol eluant (16,17). It has not been shown whether this fraction represents a minor $tRNA^{Ser}$ species or whether it is the result of non-specific binding of the bulk of $tRNA^{Ser}$ which is eluted from the BD-cellulose with the hydrophobic, high ionic strength buffer (18).

The elution characteristics of $tRNA^{Ser}_{I}$ from rat liver on BD-cellulose depend on the $Mg^{2+}$ concentration (17). That is, with no $Mg^{2+}$ present in the buffers, addition of ethanol is required for elution. For this reason 15 mM $MgCl_2$ was used in all buffers for BD-cellulose chromatography.

Because of the unique properties of the $tRNA^{Ser}_{IV}$ that are reported here we felt it essential to show that it is indeed a discrete tRNA species and not an artifact of chromatographic procedures. The tRNA eluted from BD-cellulose with a NaCl buffer was aminoacylated with $^{14}$C-serine whereas the tRNA remaining bound to BD-cellulose after the salt wash but eluting with the NaCl-ethanol buffer was amino-acylated with $^{3}$H-serine. These two fractions were then co-chromatographed on BD-cellulose (Figure 1). All three $^{14}$C-seryl-tRNAs eluted in the linear salt gradient and only $^{3}$H-seryl-tRNA eluted in the NaCl-ethanol gradient. It is thus apparent that $tRNA^{Ser}_{IV}$ is a chromatographically discrete species. This result was confirmed by co-chromatography of

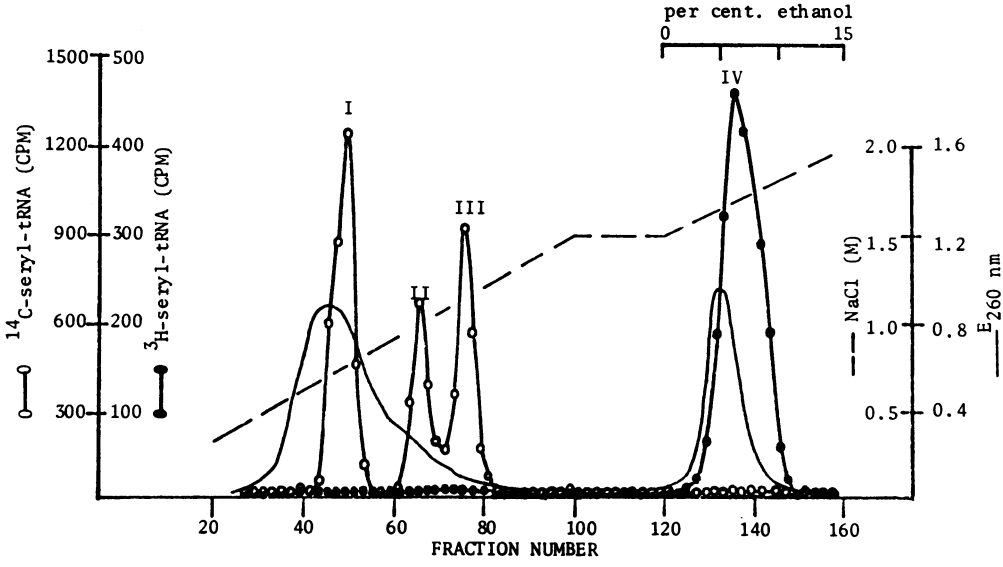


*Figure 1. Co-chromatography of tRNA fractions on BD-cellulose.* $^{14}$C-seryl-$tRNA_{I-III}$ (25 $E_{260}$ units; 40,000 cpm) and $^{3}$H-seryl-$tRNA_{IV}$ (20 $E_{260}$ units; 52,000 cpm) were pooled and 30 $E_{260}$ units unfractionated tRNA added as carrier. The sample (in 0.35 M NaCl-acetate buffer) was applied to a column (1.3 x 23 cm) on BD-cellulose and chromatography performed first using a linear gradient of NaCl (0.35 M to 1.5 M, 60 ml each) then a linear gradient of NaCl-ethanol (1.5 M NaCl to 2.0 M NaCl, 15% ethanol, 30 ml each) after an intermediary 1.5 M NaCl wash. Fractions of 1.5 ml were collected and radioactivity for each nuclide was determined by counting 0.1 ml samples of fractions.

similar samples on RPC-5 (Figure 2). Four $tRNA^{Ser}$ species were also obtained and $^{3}$H-seryl-tRNA ($tRNA^{Ser}_{IV}$) eluted as a single peak after elution of three $^{14}$C-seryl-tRNAs.

Chromatography of non-aminoacylated tRNA on BD-cellulose (Figure 3) demonstrated that the elution characteristics of the $tRNA^{Ser}$ species were not dependent on prior aminoacylation. Figure 3B shows the $^{14}$C-serine acceptance profile in which three species eluted in the NaCl gradient and one eluted in the NaCl-ethanol gradient in similar positions to the acylated species shown in Figure 1. When column fractions were assayed for phosphate acceptance utilizing [$\gamma$-$^{32}$P]-ATP in reaction mixtures, $tRNA^{Ser}_{IV}$ uniquely accepted the phosphate label (Figure 3C).

*Analysis of* $tRNA^{Ser}_{IV}$. To determine whether the $^{32}$P-phosphate incorporated by $tRNA^{Ser}_{IV}$ was covalently linked to

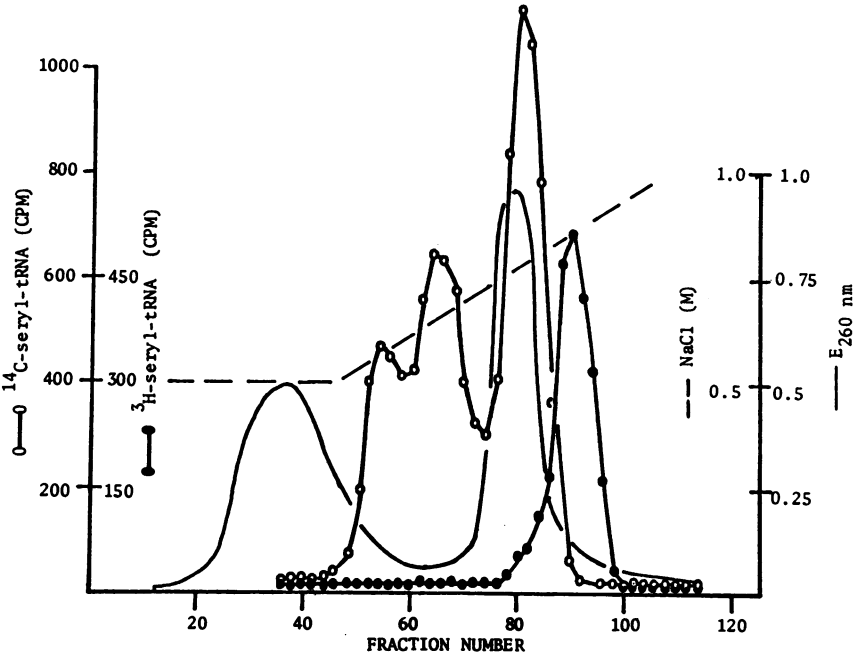


*Figure 2. Co-chromatography of tRNA fractions on RPC-5.* $^{14}$C-seryl-$tRNA_{I-III}$ (50 $E_{260}$ units; 95,000 cpm) and $^{3}$H-seryl-$tRNA_{IV}$ (10 $E_{260}$ units; 40,000 cpm) were pooled and applied to a column (0.8 x 21 cm) of RPC-5. Chromatography was performed using a linear gradient of NaCl (0.5 M to 1.0 M, 50 ml each). Fractions were collected and treated as for Figure 1.

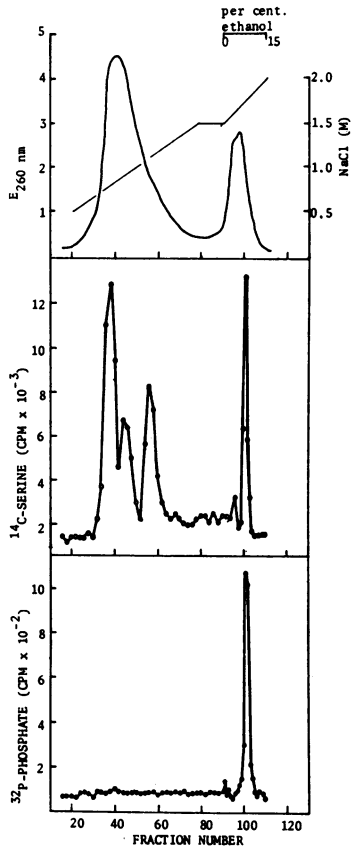


*Figure 3. Chromatography of unfractionated tRNA on BD-cellulose.* Approximately 2,000 $E_{260}$ units crude tRNA was applied to a column (2 x 40 cm) of BD-cellulose and chromatography performed first using a linear gradient of NaCl (0.45 M to 1.5 M, 300 ml each) then a linear gradient of NaCl - ethanol (1.5 M NaCl to 2.0 M NaCl, 15% ethanol, 200 ml each) after an intermediary 1.5 M NaCl wash. Fractions of 10 ml were collected and 0.5 ml samples were ethanol precipitated and assayed for serine and phosphate acceptance.

the serine moiety of seryl-tRNA, degradation of the aminoacylation product was performed followed by analysis of the labelled products by high voltage electrophoresis. The $tRNA^{Ser}_{IV}$, doubly labelled with $^{14}$C-serine and $^{32}$P-phosphate, was isolated using DEAE-cellulose then treated with ribonuclease A. Figure 4A,1 shows the separation of the labelled products on high voltage electrophoresis at pH 1.9. From the electrophoretic mobility and the labelling pattern the following assignments were made: 1, phosphoserine; II, phosphoseryladenosine; III, serine: IV, seryladenosine. When the ribonuclease reaction mixture was subjected to mild alkaline conditions and the products electrophoresed at pH 1.9 (Figure 4A,2) phosphoserine (I) and serine (III) were the major products. Presumably hydrolysis of phospho-

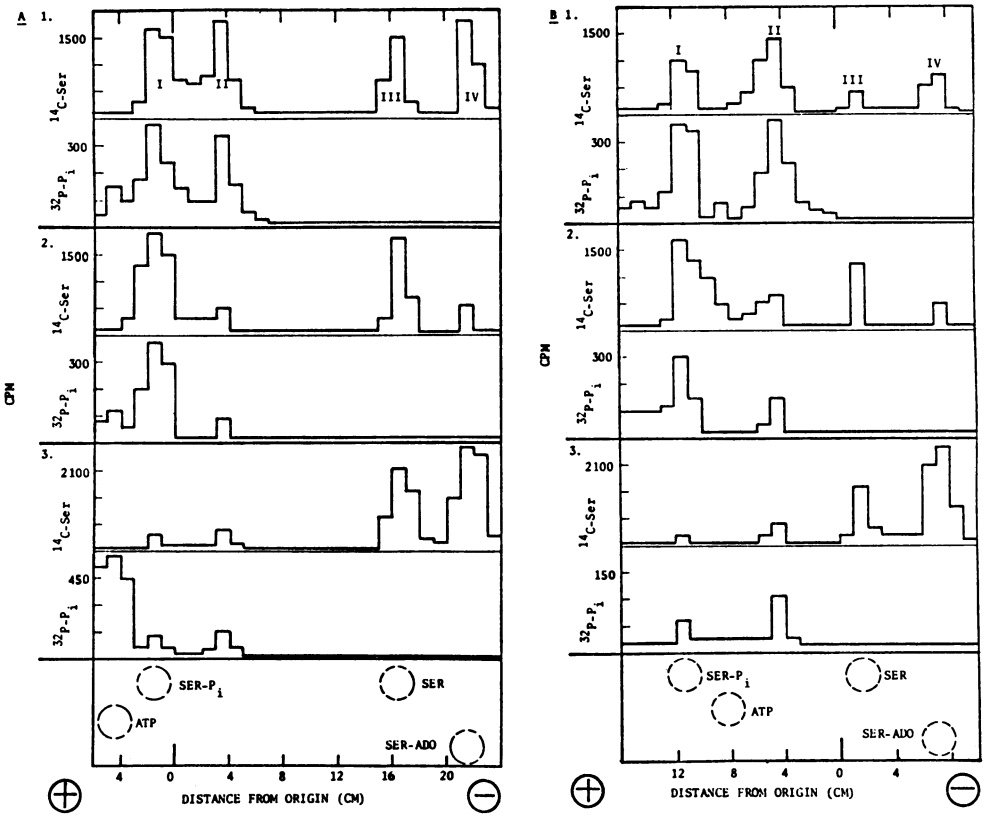


*Figure 4. High voltage electrophoresis.* $tRNA^{Ser}_{IV}$ (3 $E_{260}$ units) was aminoacylated using $^{14}$C-serine and [$\gamma$-$^{32}$P]ATP. Aminoacyl-tRNA was separated from reaction mixture components using DEAE-cellulose and treated as described in Materials and Methods. Samples were electrophoresed at pH 1.9 (Figure 4B). The electrophoresis profiles represent the result of treatment of ($^{14}$C/$^{32}$P) seryl-$tRNA_{IV}$ with 1. pancreatic ribonuclease A 2. ribonuclease then 0.1 M Tris-HCl, pH 9.5 3. ribonuclease then acid phosphatase. I, phosphoserine; II, phosphoseryl-adenosine; III, serine; IV, seryladenosine.

seryladenosine (II) and seryladenosine (IV) had occurred. Treatment of the ribonuclease reaction mixture with acid phosphomonoesterase showed depletion of the $^{32}$P-labelled components (I and II) yielding serine (III) and seryladenosine (IV) (Figure 4A,3). Thus the results of mild alkaline hydrolysis and acid phosphatase treatment of the ribonuclease A products lent support to the structural assignments. Electrophoresis of the above degradation products at pH 6.4

gave similar results. The component assigned as phosphoseryl-adenosine (II) was the major labelled product of ribonuclease treatment (Figure 4B, 1). This product was hydrolyzed to phosphoserine (I) by mild alkaline treatment (Figure 4B, 2) and dephosphorylated by acid phosphomonoesterase treatment to a product with identical electrophoretic mobility as seryl-adenosine (IV) (Figure 4B, 3).

Thus from all the supportive evidence given above it was concluded that phosphoserine is esterified to the terminal adenosine of $tRNA^{Ser}_{IV}$.

From gel filtration (Biogel AcA 54) analysis and sucrose density gradient centrifugation studies (results not shown) it was determined that $tRNA^{Ser}_{IV}$ has a molecular weight approximating 4S RNA. Actually this tRNA appeared on the leading edge of the bulk of the tRNAs in both systems indi-

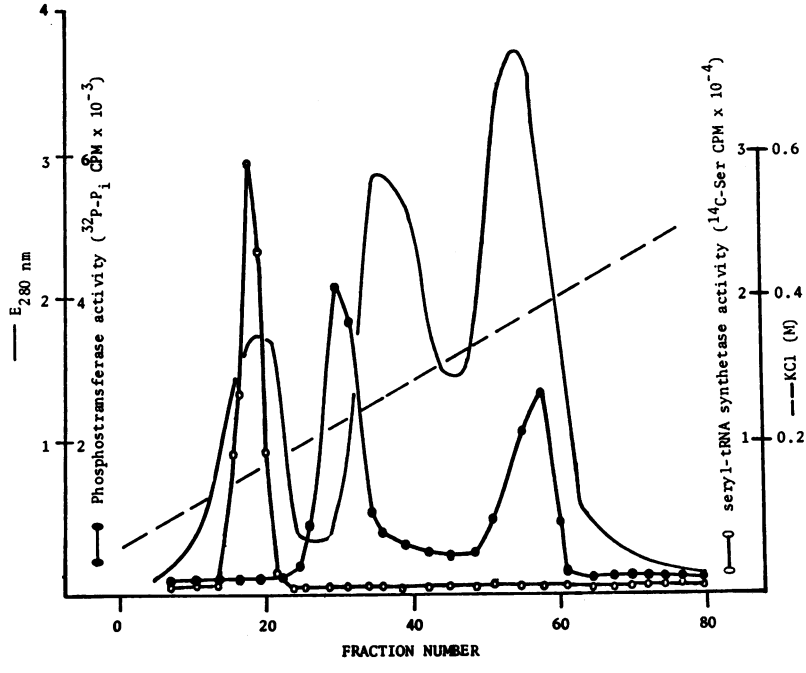


*Figure 5. Chromatography of mammary gland high speed supernatant on DEAE-cellulose.* Approximately 200 $E_{280}$ units of the supernatant obtained by centrifugation of mammary gland post-mitochondrial supernatant at 105,000 g were chromatographed on DE-32 as described. Aliquots (0.02 ml) of fractions were assayed for phosphotransferase activity and seryl-tRNA synthetase activity.

cating that probably this tRNA has the extra loop similar to other seryl-tRNA species (17).

*Fractionation of mammary gland supernatant.* Fractionation of a high speed supernatant from bovine mammary gland on DE-32 (Figure 5) gave two major fractions of phosphotransferase activity and one peak of seryl-tRNA synthetase activity. The phosphotransferase clearly separated from the synthetase activity. The significance of the two peaks of phosphotransferase is unclear.

## DISCUSSION

These studies demonstrate the presence of a specific phosphoseryl-tRNA in lactating bovine mammary gland. Phosphoseryl-tRNA appears to form by the transfer of the γ-phosphate of ATP to a specific seryl-tRNA. The appearance of seryl-tRNA and phosphoseryl-tRNA in a tRNA fraction showing a single peak of serine acceptance in two chromatographic systems may preclude the formation of phosphoseryl-tRNA by esterification of $tRNA^{Ser}_{IV}$ with phosphoserine. Although reaction conditions for phosphorylation were sub-optimal it cannot be excluded that incomplete formation of phosphoseryl-tRNA was due to heterogeneity in the tRNA fraction.

The validity of phosphoseryl-tRNA formation in lactating bovine mammary gland was further supported by the demonstration of a specific enzyme (phosphotransferase) whose activity was found to be dependent on the addition of $tRNA^{Ser}_{IV}$, seryl-tRNA synthetase and serine.

The function of phosphoseryl-tRNA is yet undetermined. The present studies were instigated as a result of a lack of understanding of the mechanism of phosphorylation of casein. However, the possibility of the role of phosphoseryl-tRNA being other than involvement in protein synthesis cannot be excluded. Examples of tRNA utilization other than for protein synthesis have been documented (19,20,21,22). The finding of hydroxypyruvyl-tRNA in *E. coli* and *M. xanthus* (23) has led to the suggestion that phosphoseryl-tRNA may play a role in the biosynthesis of serine (10). Reports demonstrating the pathways of serine biosynthesis (24,25) do not indicate a role for tRNA in these processes and the

formation of serine from phosphoserine, the last step in the 3-phosphoglycerate to serine pathway, is essentially irreversible (24). If phosphoseryl-tRNA was involved in this pathway it may allow the reversal of the phosphoserine to serine reaction. A further consideration is that owing to the high concentrations of serine required during casein (26) and phospholipid (27) syntheses in lactating bovine mammary gland then the role of phosphoseryl-tRNA may be in the maintenance of these levels.

Present studies are directed at determining the function of phosphoseryl-tRNA in lactating bovine mammary gland. Preliminary experiments using a homologous cell-free protein synthesis system indicate that both serine and phosphate can be incorporated from phosphoseryl-$tRNA_{IV}$ into hot TCA precipitable material. Whether phosphoserine is introduced intact has not been established and these investigations are continuing.

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12. Abbreviations: BD-cellulose, benzoylated DEAE-cellulose; RPC, reversed phase chromatography; phosphoserine (SER-P), O-phosphoryl-L-serine

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