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Quantitative Variation in Serine Transfer Ribonucleic Acid During Estrogen-Induced Phosphoprotein Synthesis in Rooster Liver*

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ABSTRACT: In roosters, hepatic synthesis of the yolk phosphoprotein, phosvitin, is induced by estrogen treatment. Phosvitin has an unusually high content of serine residues (over 50%), and an attempt was made to correlate phosvitin induction with changes in the benzoylated DEAE-cellulose chromatographic profile of *in vitro* acylated seryl transfer ribonucleic acid. Plasma phosvitin was used as an indicator of phosvitin induction, as newly synthesized phosvitin does not accumulate in the liver. Plasma phosvitin levels increased 150-fold after a single injection of estradiol-17β. Transfer ribonucleic acid and aminoacyl transfer ribonucleic acid ligases were prepared from livers of normal and estrogen-treated roosters. Unfractionated transfer ribonucleic acid isolated during the initial stimulatory period of phosvitin synthesis was acylated with serine to a significantly greater extent than was transfer ribonucleic acid from control animals, regardless of the source or concentration of ligase, concentration of transfer ribonucleic acid, duration of acylation incubation, or isotopic label. At least four seryl transfer ribonucleic acid peaks were observed in chromatograms of acylated transfer ribonucleic acid derived from control or estrogen-treated roosters. A marked relative increase in one major and in one minor peak was found during the rapid phase of phosvitin synthesis. This change in seryl transfer ribonucleic acid chromatographic pattern was independent of the isotopic label and the source of ligase. With decreasing levels of plasma phosvitin these changes in seryl transfer ribonucleic acid diminished and approached the control profile. Identical seryl transfer ribonucleic acid changes were again noted after a second dose of estradiol-17β. Similar chromatographic comparisons of aminoacyl transfer ribonucleic acids for glycine, alanine, valine, leucine, phenylalanine, tyrosine, threonine, methionine, arginine, histidine, lysine, and aspartic and glutamic acids before estrogen injection and during the initial rapid phase of phosvitin synthesis did not reveal major specific changes comparable to those observed in seryl transfer ribonucleic acid. Only small changes in minor species of glycyl, histidyl, and glutamyl transfer ribonucleic acid were observed. The correlation between phosvitin synthesis and specific seryl transfer ribonucleic acid levels, the relative lack of change in other aminoacyl transfer ribonucleic acids, and the predominance of serine in phosvitin suggest that the estrogen-induced seryl transfer ribonucleic acid alterations are related to phosvitin induction.

Multiple species of tRNA specific for a single amino acid (isoaccepting tRNAs) have been described in bacteria and higher organisms (Doctor *et al.*, 1961; Weiss and Kelmers, 1967; Caskey *et al.*, 1968; Yang and Novelli, 1968a). In some instances, isoaccepting tRNA species have been shown to recognize different synonym codons (Weisblum *et al.*, 1962; von Ehrenstein and Dais, 1963; Caskey *et al.*, 1968; Kano-Sueoka *et al.*, 1968; Sundharadas *et al.*, 1968). Changes in the chromatographic profiles of isoaccepting tRNAs have been observed in bacteria after phage infection (Kano-Sueoka and Sueoka, 1966) and during sporulation (Kaneko and Doi, 1966). In higher forms, qualitative and quantitative chromatographic differences have been found during wheat germination (Vold and Sypherd, 1968), and in mammalian cells between tissue types including tumor tissues (Axel *et al.*, 1967; Taylor *et al.*, 1967; Yang and Novelli, 1968a,b; Baliga

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et al., 1969) and after viral transformation (Subak-Sharpe *et al.*, 1966; Holland *et al.*, 1967). Much speculation has arisen regarding the possible role of variation in isoaccepting tRNA species as a mechanism of cellular regulation and differentiation (Novelli, 1967). We have utilized the well-known estrogen-induced hepatic synthesis of phosvitin in the domestic fowl to ascertain whether this physiologic response is reflected in alterations in isoaccepting tRNA species.

Phosvitin is an avian yolk protein synthesized in the liver of the laying hen and transported in the blood to the developing oöcyte (Heald and McLachlan, 1963). It is an unusual protein containing greater than 50% serine residues, almost all of which are phosphorylated (Heald and McLachlan, 1963; Allerton and Perlmann, 1965). Oligopeptides containing up to a maximum of six consecutive phosphoserine residues have been isolated from hydrolysates of phosvitin (Williams and Sanger, 1959). In male and immature birds, the hepatic synthesis of phosvitin can be induced by estrogen administration (Greengard *et al.*, 1964, 1965; Heald and McLachlan, 1965). This induction is characterized by a lag period of nearly 24 hr before an increase in plasma phosvitin can be measured (Gruber, 1967). However, the effect of estrogen on the liver is rapid and occurs prior to the increase in plasma phosvitin. Actinomycin D given simultaneously with the hormone blocks phosvitin synthesis. If the antibiotic is given 4 or 6 hr after estrogen, the inhibition is partial and at 10 hr inhibition cannot be detected. Puromycin inhibits only later, during the period of rapid phosvitin synthesis (Greengard *et al.*, 1964). The synthesis of phosvitin *in vivo* can be investigated by analysis of plasma samples, because even at times of rapid phosvitin synthesis no measurable amounts of phosvitin are present in rooster liver (Beuving, 1967).

This system was chosen for investigation because of the large induction of hepatic synthesis of a protein with high content of a single amino acid, and because the induction is abolished by agents which prevent RNA synthesis. In addition, since three to four species of tRNA corresponding to serine have been described in bacteria (Muench and Berg, 1966; Ishikura and Nishimura, 1968; Sundharadas *et al.*, 1968), fungi (Gillam *et al.*, 1967; Brown and Novelli, 1968), and mammalian hepatic and tumor tissue (Caskey *et al.*, 1968; Yang and Novelli, 1968a,b; Buck and Nass, 1969), it was expected that multiple tRNA species for serine would be found in avian liver, and that possibly the induction of phosvitin could be correlated with changes in one or more of these species.

Materials and Methods

White Leghorn roosters, weighing 1.8 to 2.0 kg, were injected intramuscularly with 10 mg/kg of estradiol 17β-benzoate (Nutritional Biochemicals Corp.) in sesame oil. Blood samples were collected from the wing vein into heparinized syringes at appropriate intervals. Proteins in plasma were precipitated with ten volumes of 10% (w/v) trichloroacetic acid. Lipid was removed from the precipitate by successive washings with chloroform–methanol (2:1), acetone, and ether. Alkali-labile phosphorus content was determined after barium hydroxide hydrolysis (Greengard *et al.*, 1964). This procedure is highly specific for phosvitin in plasma (Heald and McLachlan, 1963; Greengard *et al.*, 1965; Benowitz and Terepka, 1968).

Preparation of tRNA. Birds were sacrificed by injecting air into the wing vein. Livers were rapidly removed and chilled in ice-cold salt solution (Brunngraber, 1962). Cold, water-saturated phenol was added and the mixture was homogenized for 15 min at 4° in a Waring Blendor. Two additional phenol extractions were performed after which the tRNA was precipitated overnight at −20° by adding 0.1 volume of 2.0 M potassium acetate (pH 5.0) and 2.5 volumes of ethanol. The precipitate was dried and dissolved in 0.1 M Tris-buffer and the solution was adjusted to pH 10. Amino acids were stripped from the tRNA by incubation at 37° for 60 min. The pH was adjusted to 7.5 with acetic acid, and pancreatic DNase I (electrophoretically purified, RNase-free, Worthington Biochemical Corp.) (10 μg/ml) and $MgCl_2$ to a concentration of 0.005 M were added. After incubation for 15 min at 37°, a cold phenol extraction was performed and the tRNA was precipitated overnight. The precipitate was dried and dissolved in 0.3 M acetate buffer (pH 7.0), and the tRNA was purified by successive 2-propanol precipitations (Zubay, 1962). The final precipitate was dissolved in water, lyophilized, and stored under desiccation at −20°. The $A^{260/280}$ ratios were 1.85 to 1.95, and the yield from 100 g of liver averaged 80 mg of tRNA. tRNA thus prepared accepted amino acids in high yields; per cent acylation (mole of amino acid/mole of tRNA × 100) for the 14 amino acids used for chromatographic comparisons, ranged from 0.6% to 3.4%.

Preparation of Amino Acid tRNA Ligases. Pieces of liver were gently homogenized (Yang and Novelli, 1968a) in a Potter–Elvehjem Teflon–glass homogenizer. The homogenate was centrifuged at 30,000*g* for 20 min followed by 105,000*g* for 2 hr. The supernatant was passed through a 0.9 × 2.0 cm DEAE column (Whatman DE-32) equilibrated with a solution containing 0.01 M potassium phosphate (pH 7.5), 0.005 M KCl, 0.01 M fresh β-mercaptoethanol, and 10% glycerol (Muench and Berg, 1966). After a thorough wash with this solution, protein was eluted with a small volume of 0.01 M potassium phosphate (pH 6.5), containing 0.25 M KCl, and 0.01 M β-mercaptoethanol. The eluate, free of amino acids and tRNA, contained approximately 15 mg of protein/ml, and was stored in aliquots under liquid nitrogen. Protein in the preparation was determined by the biuret reaction (Layne, 1957) and tRNA by aminoacylation in the absence of added tRNA.

Acylation of tRNA. Acylation reaction mixtures (0.05 ml) contained the following components: 0.1 M Tris-HCl (pH 7.4), 0.01 M $MgCl_2$, 0.01 M KCl, 0.0004 M dithiothreitol, 0.005 M ATP (adjusted to pH 7.4 with KOH), 0.0001 M NaEDTA, 0.0001 M 19 nonradioactive amino acids, 0.00002 M radioactive amino acid, rooster liver tRNA, and 1.5 to 2.0 mg/ml of ligase protein. Isotopes were from New England Nuclear Corp., Nuclear-Chicago Corp., and Schwartz BioResearch; specific activities for ^3H varied between 500 and 28,200 mCi/mmole, and for ^{14}C between 50 and 495 mCi/mmole. Reaction mixtures were incubated at 37° for 30 min. For analysis, aminoacyl-tRNA was precipitated with an equal volume of cold 10% trichloroacetic acid, collected on glass fiber filters (Type A, Gelman Instr. Co.), dried, and counted in toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene in a Beckman liquid scintillation counter.

For preparation of aminoacyl-tRNA, 0.5 or 1.0 ml of acylation reaction mixtures containing 0.25–2.0 mg of rooster

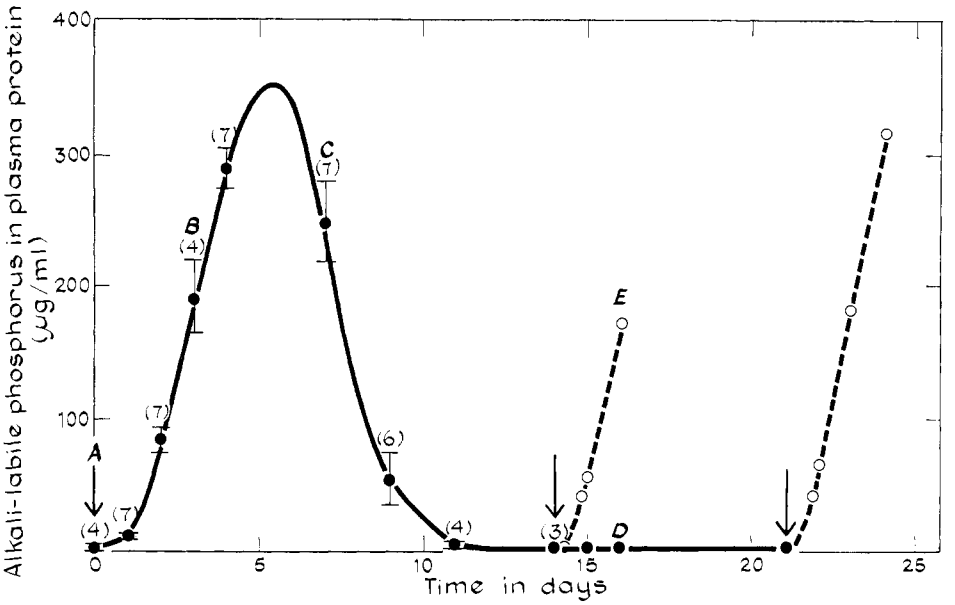


FIGURE 1: Plasma levels of phosphoprotein in roosters after estrogen treatment. Alkali-labile phosphorus in lipid-free plasma protein was measured following injection (arrow) of 10 mg/kg of estradiol 17β-benzoate. A second similar injection was given to two roosters at day 14 and to one rooster at day 21 (arrows). Points where tRNA and ligases were isolated for chromatographic comparisons are indicated as A, B, C, D, and E. Numbers of observations are in parentheses. Where indicated, values are means ± S.E.M.

liver tRNA were incubated at 37° for 30 min, chilled in ice, and passed through DEAE-cellulose columns according to Yang and Novelli (1968b). After washing extensively with a solution containing 0.25 M NaCl, 0.01 M $MgCl_2$, and 0.001 M EDTA (pH 4.5), aminoacyl-tRNA was eluted with 1.0 M NaCl, 0.01 M $MgCl_2$, and 0.001 M EDTA (pH 4.5). Fractions containing radioactive tRNA were pooled and diluted to a concentration of 0.45 M with respect to NaCl with a solution containing 0.01 M sodium acetate and 0.01 M $MgCl_2$ (pH 4.43). The preparations were stored at −20° until used for column chromatography.

Chromatographic Analysis of Aminoacyl-tRNA. Benzoylated DEAE-cellulose (BD-cellulose), 50–100 mesh, was either prepared according to Gillam *et al.* (1967), or purchased from Schwartz BioResearch. Columns, 0.9 × 20 cm, were washed with at least 300 ml of 2 M NaCl, followed by 200 ml of the initial buffer. The aminoacyl-tRNA was mixed with approximately 1.25 mg of stripped rooster liver tRNA and the mixture was applied to the column. The column was washed twice with 2-ml aliquots of initial buffer and eluted with 400 ml of a linear gradient of 0.45 to 1.0 M NaCl, containing 0.01 M $MgCl_2$ and 0.005 M sodium acetate (pH 4.43), except where indicated. Fractions (4 ml) were collected at a rate of 20 ml/hr at room temperature. Elution was then continued with about 50 ml of 15% (v/v) ethanol, containing 1.5 M NaCl and 0.005 M sodium acetate (pH 4.43). Each fraction was mixed with 0.25 mg of crude yeast RNA (Type XI, Sigma Chemical Co.), and precipitated by adding 50% trichloroacetic acid to a concentration of 10%. The precipitates were collected and counted as described above. For double labeling counting, appropriate corrections were made for ^3H and ^{14}C radioactivity appearing in the other channel. Calculations were made by a computer, which also plotted the chromatographic profiles.

Results

Plasma Phosphoprotein. Plasma phosphoprotein began to increase nearly 24 hr after estrogen administration and reached a maximum value approximately 150-fold greater than the control value on day 5 (Figure 1). Inorganic phosphate is 10.4% (by weight) of phosvitin (Allerton and Perlmann, 1965), allowing the calculation of phosvitin content from the values of alkali-labile, lipid-free phosphorus in plasma proteins. The control value for plasma phosvitin averaged 0.023 mg/ml and the maximum induced value was about 3.50 mg/ml. Plasma phosphoprotein fell after 6 days and reached the control level 11 days after estrogen administration. After a second estrogen injection at day 14, there was a much shorter lag period prior to the increase in plasma phosphoprotein (point E, Figure 1) (*cf.* Beuving, 1967). Three weeks after the initial estrogen administration this short lag period was still observed (Figure 1). Although there is a difference in the extent of the lag period after the initial and subsequent estrogen injections, the maximum rate of plasma phosphoprotein accretion is similar (*ca.* 1.15 mg of phosvitin/ml per day) after each dose of hormone.

Acylation of tRNA with Serine. Aminoacyl-tRNA ligase and tRNA from control (point A, Figure 1) and estrogen-treated (point B, Figure 1) roosters were compared (Figures 2 and 3). The synthesis of seryl-tRNA as a function of tRNA and enzyme origin and tRNA concentration is illustrated in Figure 2. The incorporation of serine into tRNA from estrogen-treated animals was greater than that from control animals with both enzymes. This difference is independent of enzyme concentration, or time of incubation (Figure 3). Seryl-tRNA synthesis was proportional to the concentration of tRNA, regardless of the source of enzyme or tRNA. Preparations of seryl-tRNA for chromatography were acylated

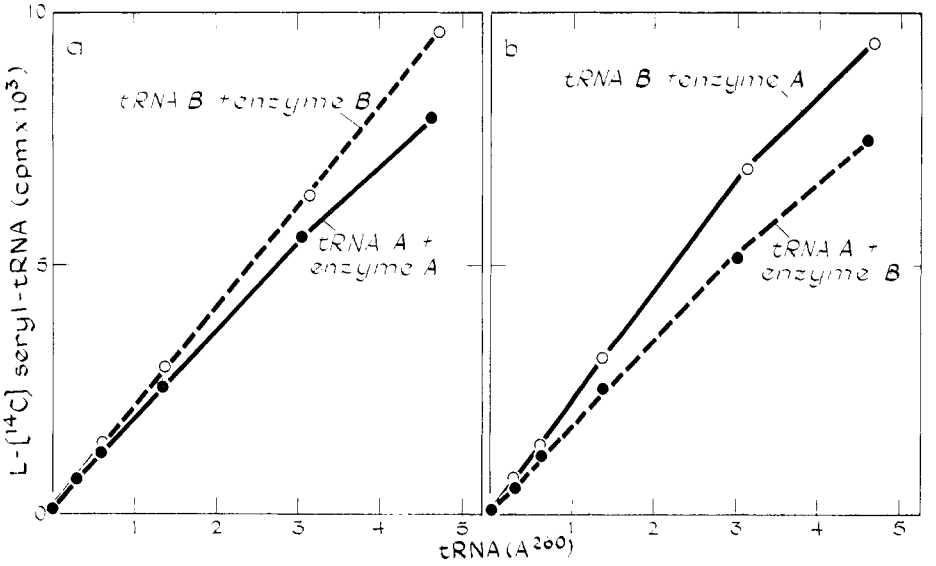


FIGURE 2: Acylation of tRNA from livers of control (point A, Figure 1) and estrogen-treated (point B, Figure 1) animals with L-[U-^{14}C]serine (specific activity 50 mCi/mmole) by ligases from control (A) and estrogen-treated (B) animals. The amounts of ligases used were 79 μg of protein (ligase A) and 73 μg of protein (ligase B). (a) Each tRNA was acylated by its respective ligase. (b) Ligases interchanged. For further experimental details, see Methods.

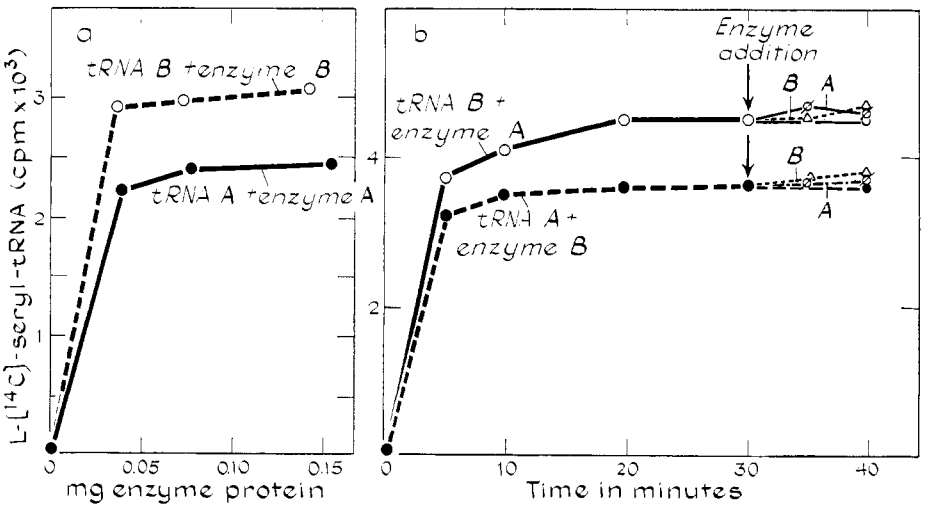


FIGURE 3: Comparison of acylation of hepatic tRNA derived from control (A) and estrogen-treated (B) animals by ligases from control (A) and estrogen-treated (B) animals. L-[^{14}C]Serine (as in Figure 2) was used in all acylations. (a) Influence of ligase concentration; 68 μg of tRNA A and 70 μg of tRNA B were used. (b) Time-course of aminoacylation and the effect of further ligase addition. The amounts of ligases and tRNA were as follows: 31 μg of protein, ligase A, 29 μg of protein, ligase B, 102 μg of tRNA A, and 104 μg of tRNA B. The arrow indicates point of further addition of same amounts of ligases.

in reaction mixtures in which the concentration of tRNA was limiting. The time course of acylation (Figure 3b) indicates that addition of either ligase preparation to completed reaction mixtures did not increase the extent of serine acylation. The per cent acylation of tRNA B was significantly ($p < 0.01, t = 3.08$) greater (3.0%) than that of tRNA A (2.3%).

Chromatography of Seryl-tRNA. The chromatographic profiles of [^{14}C]- and [^{3}H]seryl-tRNA prepared from control (point A, Figure 1) or estrogen-treated (point B, Figure 1) roosters, respectively, are compared in Figure 4. Peak I was chosen as a reference for plotting the chromatographic profiles. Four seryl-tRNA peaks were seen in both preparations; there was a large relative increase in two seryl-tRNA peaks (II and III) derived from estrogen-treated roosters. Identical results for seryl-tRNA were obtained when isotopic label and enzymes were interchanged (Figure 4b). To eliminate any possible variation due to different enzyme preparations during subsequent comparisons of control and estrogen-treated tRNA only the control enzyme was used except where indicated.

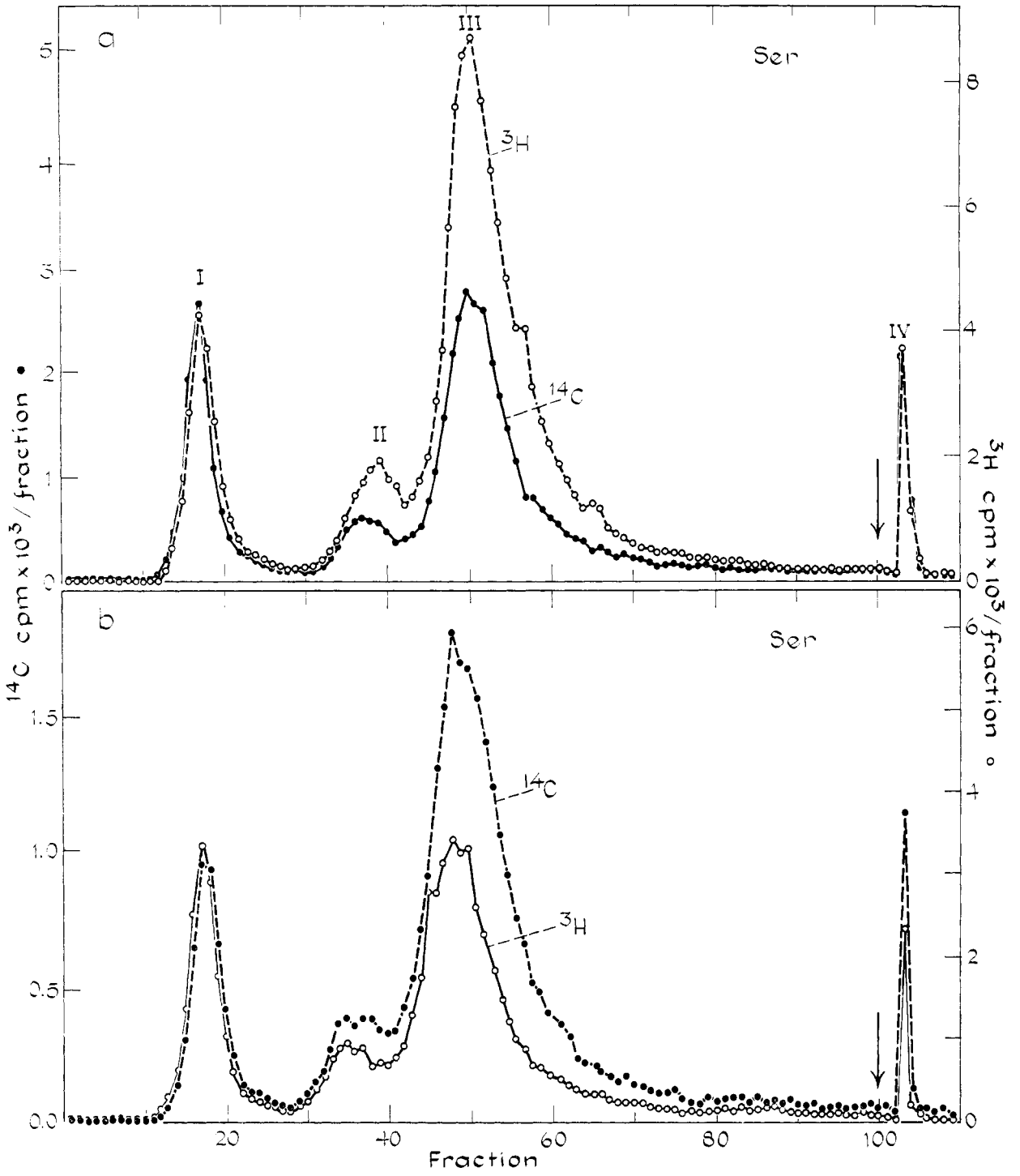


FIGURE 4: BD-cellulose chromatography of seryl-tRNA. Columns (0.9 × 20 cm) were eluted with a linear gradient (400 ml: 0.6–1.0 M, containing 0.01 M $MgCl_2$ and 0.005 M sodium acetate, pH 4.43). Elution was continued (arrow) with 15% ethanol, 1.5 M NaCl, and 0.005 M sodium acetate, pH 4.43. Fractions (4 ml) were collected at 20 ml/hr at room temperature and trichloroacetic acid precipitated. Carrier tRNA was added to each sample. (a) tRNA isolated from livers of control animals (point A, Figure 1) and acylated with L-[U-^{14}C]serine (specific activity 123 mCi/mmole) (●) cochromatographed with tRNA isolated from livers of estrogen-treated animals (point B, Figure 1) and acylated with L-[U-^{3}H]serine (specific activity 3730 mCi/mmole) (○). Each tRNA was acylated by its respective ligase. (b) As (a) above, but isotopes and ligases interchanged. [^{3}H]Seryl-tRNA (A) (○) acylated with ligase B cochromatographed with [^{14}C]seryl-tRNA (B) (●) acylated with ligase A. tRNA A was prepared from two and tRNA B from three livers.

In an attempt to correlate seryl-tRNA changes with alterations in phosvitin synthesis, [^{3}H]seryl-tRNA derived from tRNA isolated during the descending portion of the plasma phosphoprotein curve (point C, Figure 1), after its return to the control level (point D, Figure 1) and after a second estrogen injection (point E, Figure 1), were individually cochromatographed with [^{14}C]seryl-tRNA derived from untreated animals (point A, Figure 1). The chromatographic

TABLE I: Summary of Seryl-tRNA Chromatography.

Seryl-tRNA Preparation[a]		Radioactivity in Each Peak			
		Radioactivity in Peak I			
		Peak			
tRNA	Ligase	I	II	III	IV
A	A	1.00	0.39 ± 0.04[b]	2.25 ± 0.12[b]	0.29 ± 0.06[b]
A	B	1.00	0.43	2.49	0.17
B	B	1.00	0.67	4.68	0.25
B	A	1.00	0.63	4.69	0.28
C	C	1.00	0.46	2.85	0.24
D	A	1.00	0.31	1.73	0.18
E	A	1.00	0.47	3.51	0.36

[a] tRNA and ligase prepared from livers at designated intervals (designations as in Figure 1). [b] Values are means ± standard deviation (n = 5).

profiles observed with these preparations (Figure 5) indicate that the changes in levels of seryl-tRNA II and III accompany the alterations in plasma phosvitin concentration.

To compare the changes in seryl-tRNA fractions before and after the hormone injection, the counts under each peak were calculated and expressed relative to seryl-tRNA I, which was taken to be unity (Table I). The values for each tRNA preparation may be compared with the values (mean ± std dev) for the control tRNA. Seryl-tRNA III is markedly increased in preparations B and E, and less so in preparation C. Seryl-tRNA II is increased in preparation B, but the increases in preparations C and E are of borderline significance. The ratios for preparation D are uniformly low, but only peak III is less than two standard deviations below the mean of the control. The ratios for seryl-tRNA IV are within the variation of the control values.

The presence of serine in radioactive tRNA peaks from control and estrogen-treated animals was verified by ethanol precipitation of the tRNA and deacylation. The amino acids obtained were spotted on paper (Whatman 3MM) with authentic serine, and subjected both to chromatography (butanol–acetic acid–H_2O, 12:3:5) and electrophoresis at pH 1.9 (4% formic acid, 4.5 kV). Unstained strips were dried and counted in toluene–2,5-diphenyloxazole–1,4-bis[2-(5-phenyloxazolyl)]benzene. Radioactive serine (both ^3H and ^{14}C) derived from each chromatographic peak was observed in both systems.

Chromatography of Other Aminoacyl-tRNAs. Similar chromatographic comparisons of glycine, alanine, valine, leucine, phenylalanine, tyrosine, threonine, methionine, arginine, histidine, lysine, and aspartic and glutamic acid tRNAs before estrogen injection and during the initial rapid phase of phosvitin synthesis (point B, Figure 1) did not reveal major specific changes comparable to those observed in seryl-tRNA (Figure 6). Slight differences in minor species of glycyl-, histidyl-, and glutamyl-tRNA were observed. Glycyl- and histidyl-tRNA chromatography was repeated yielding the same results. The nonamide aminoacyl-tRNAs not tested

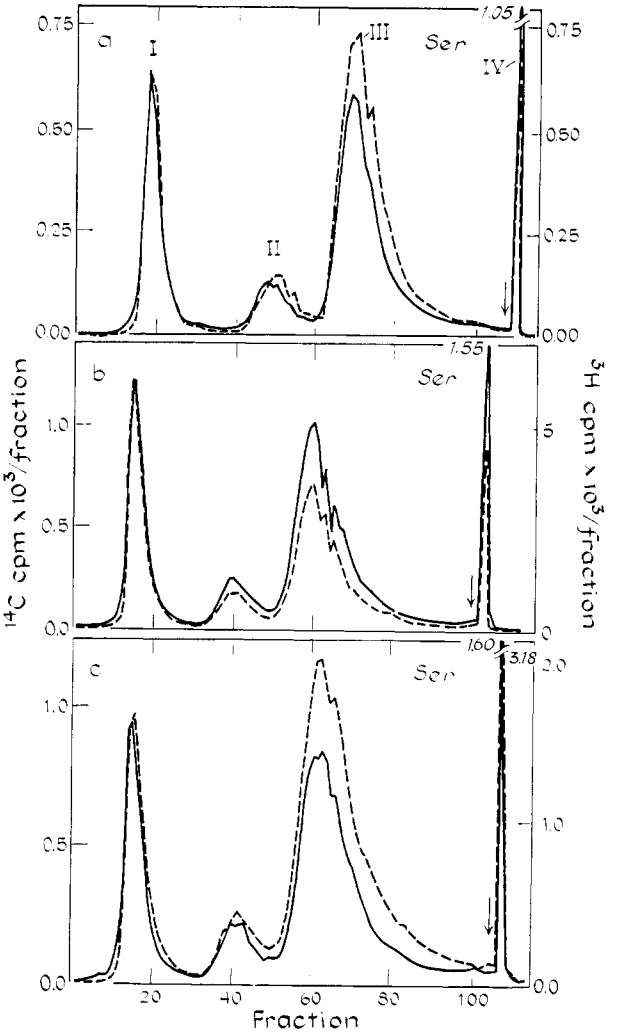
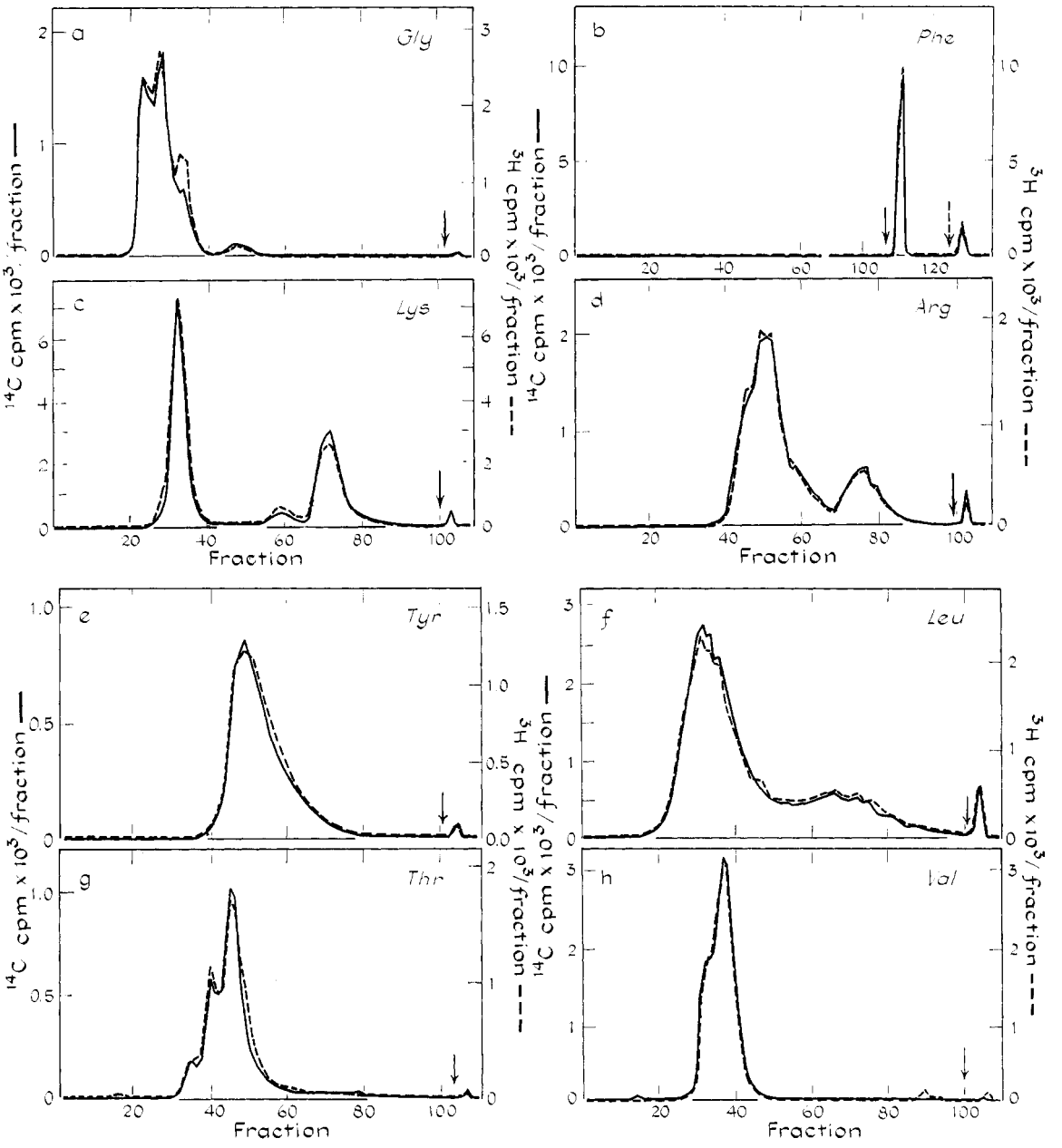


FIGURE 5: BD-cellulose chromatography of seryl-tRNAs derived from roosters at various times after estrogen administration. Chromatographic details are as in Figure 4. (a) tRNA from livers of control animals (point A, Figure 1) and acylated with L-[^{14}C]serine (solid line) cochromatographed with tRNA from livers of estrogen-treated animals (point C, Figure 1) and acylated with L-[^{3}H]serine (dotted line). Each tRNA was acylated by its respective ligase. (b) [^{14}C]Seryl-tRNA (A) (solid line) cochromatographed with [^{3}H]-seryl-tRNA (D) (dotted line). Control ligase A was used in both acylations. (c) [^{14}C]Seryl-tRNA (A) (solid line) cochromatographed with [^{3}H]seryl-tRNA (E) (dotted line). Control ligase A was used in both acylations.

(proline, tryptophan, cysteine, and isoleucine) account for less than 3% of the total residues in phosvitin (Allerton and Perlmann, 1965). The exact content of glutamine and asparagine in phosvitin is not known and these aminoacyl-tRNAs were not tested.

Discussion

The predominant alterations occurring in rooster liver as a consequence of estrogen administration are restricted to marked increases in two species of seryl-tRNA. One of these peaks is a major species of seryl-tRNA in rooster liver. Of the other aminoacyl-tRNAs studied, only minor species of glycyl-, histidyl-, and glutamyl-tRNA were altered. Estrogen



administration has complex effects on protein synthesis in avian liver. In addition to the induction of phosvitin synthesis, estrogen probably stimulates the syntheses of lipovitelline, an yolk lipoglycoprotein, and a less well-studied plasma β-lipoprotein fraction closely related to the egg yolk low-density lipoprotein (Schjeide *et al.*, 1963). Changes in lipid metabolism have also been described (Schjeide *et al.*, 1963; Hawkins and Heald, 1966). The synthesis of yolk proteins (vitellinogenesis) is quantitatively the dominant hepatic process resulting from estrogen administration. There is a concomitant decrease in the concentration of other plasma proteins of hepatic origin (Schjeide *et al.*, 1963). It is notable that estrogen does not markedly increase the rate of synthesis of total hepatic proteins (Heald and McLachlan, 1965) or nonphosvitin phosphoproteins (Greengard *et al.*, 1965).

Serine represents over 50% of the amino acid residues of phosvitin, and almost all of these are phosphorylated (Allerton and Perlmann, 1965). The greatest elevations of seryl-tRNA II and III were found at the point of most rapid accretion of plasma phosvitin. These seryl-tRNA species decreased toward normal values with the fall in plasma phosvitin. In data not presented here, injection of sesame oil alone did not cause an increase in plasma phosphoprotein or in the seryl-tRNA peaks. A second administration of estrogen was followed by an increase in plasma phosphoprotein and also in seryl-tRNA II and III. Since phosvitin does not accumulate in the liver, the correlation between plasma phosvitin and hepatic seryl-tRNA II and III levels, and the lack of specific changes in other tRNAs together with the predominance of serine in the protein, suggest that the estrogen-induced seryl-tRNA changes are related to the induction of phosvitin synthesis.

Lipovitellin and yolk low-density lipoprotein, probably synthesized by the liver during estrogen-stimulated vitellino-

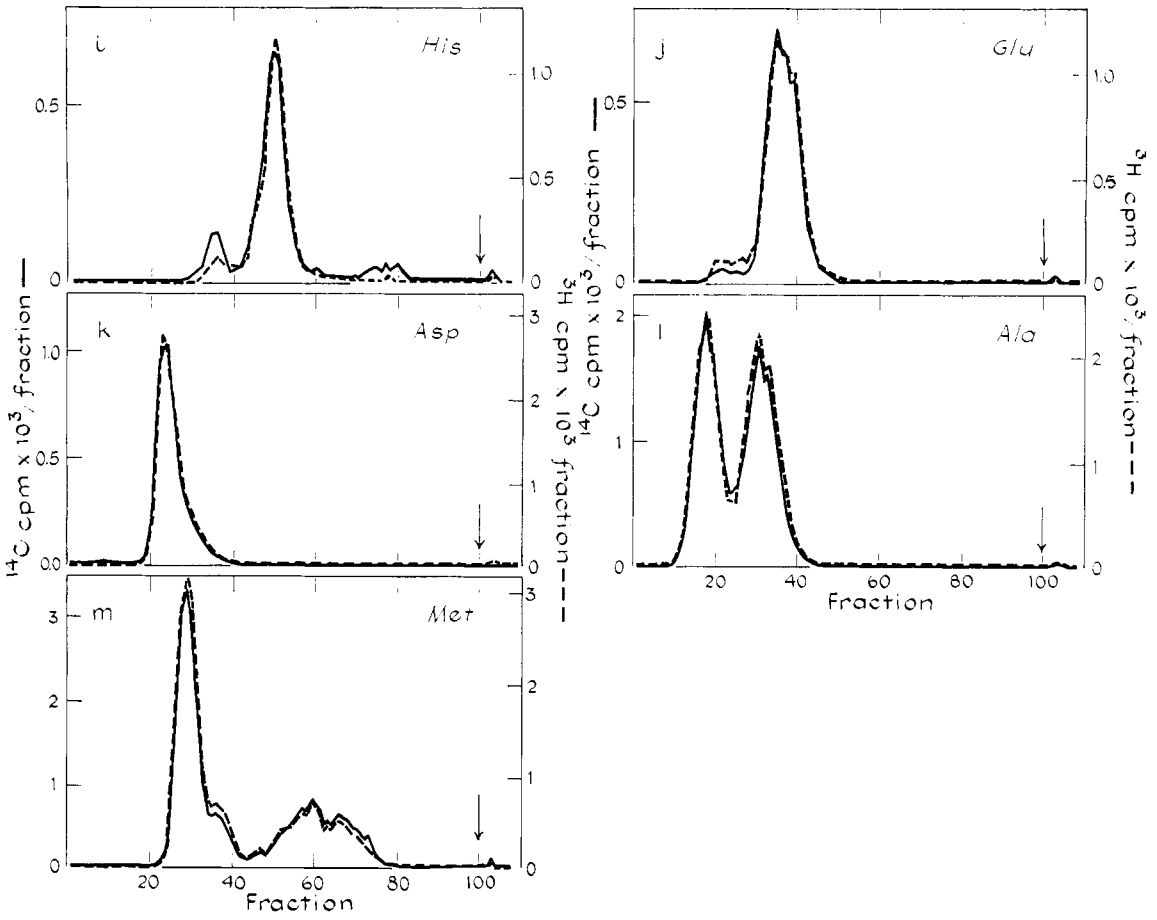


FIGURE 6: BD-cellulose chromatography of 13 other aminoacyl-tRNAs. Chromatographic details are as in Figure 4, except 0.45–1.0 M NaCl-buffer gradient in all aminoacyl-tRNAs other than tyrosyl- and leucyl-tRNAs (where 0.6–1.0 M gradient was used). The aminoacyl-tRNAs are indicated in the Figure. tRNA from livers of control (A) animals was acylated with L-[^{14}C]amino acid (solid line) and tRNA from livers of estrogen-treated (B) animals with L-[^{3}H]amino acid (dotted line). Control ligase (A) was used in all acylations. For phenylalanyl-tRNA, an additional ethanol solution (dotted arrow) was used (30% ethanol, 1.5 M NaCl, 0.005 M sodium acetate, pH 4.43). a–h of Figure 5 is on facing page.

genesis, contain a relatively low content of serine (10% or less) (Schjeide *et al.*, 1963; Steer *et al.*, 1968). No specific aminoacyl-tRNA changes were found that could be related to the induction of the synthesis of these proteins. Correlating amino acid composition of a protein with aminoacyl-tRNA levels may be an oversimplification. However, both qualitative and quantitative differences in aminoacyl-tRNAs have been described for mouse immunoglobulin-producing tumors that synthesize distinct proteins (Yang and Novelli, 1968a,b; Mushinski and Potter, 1969).

Most serine residues in phosvitin are phosphorylated, as is a small proportion of threonine residues (Allerton and Perlmann, 1965). However, no change in threonyl-tRNA chromatography or, in data not presented here, in threonine acylation was detected after hormone treatment.

An estimate of reproducibility of the chromatographic procedure is possible from Table I and the chromatographic profiles. The difference in seryl-tRNA elution characteristics in Figures 4 and 5 is due to differences in the preparation of BD-cellulose. The larger degree of variation seen in the ethanol-eluted fraction of seryl-tRNA (peak IV) may be due in part to variability in the small per cent of tRNA which is nonselectively retained by the resin and eluted by ethanol buffers (Gillam *et al.*, 1967).

The quantitative differences in seryl-tRNA preparations derived from control and estrogen-treated animals are not due to the source of ligase. Detailed comparisons of serine, glycine, and threonine acylations with ligases from both sources revealed no significant quantitative differences between ligase preparations. However, in data not shown here, small shifts in the chromatographic position of several aminoacyl-tRNA peaks were noted when the enzyme preparation derived from estrogen-treated animals was used.

The effect of estrogen on the liver of immature chicks is rapid and occurs prior to the increase in phosvitin synthesis. Actinomycin D given simultaneously with the hormone blocks phosphoprotein synthesis, but no inhibition is detectable when the antibiotic is given 10 hr after the hormone (Greengard *et al.*, 1964). Puromycin inhibits only later, during the period of rapid phosphoprotein synthesis. Within 2 hr after estrogen administration to immature animals, DNA–RNA hybridization reveals new hepatic RNA species qualitatively identical with hepatic RNA of laying hens (Hahn *et al.*, 1969). These findings are compatible with the hypothesis that the specific tRNA molecules which increase in response to estrogen administration are related to the induction of phosvitin synthesis.

Estrogens have also been shown to increase RNA synthesis

and induce ovalbumin synthesis in the immature chick oviduct (Dingman *et al.*, 1969). The major RNA change subsequent to estrogen treatment is a large increase in nuclear tRNA. This increase was seen in oviducts of normally maturing pullets, but was not observed in laying hens. DNA–RNA hybridization studies suggest that the RNA molecules induced by estrogen in the liver and in the oviduct are not homologous (Hahn *et al.*, 1969), consistent with the specific nature of the seryl-tRNA alterations observed during hepatic vitellinogenesis.

We do not know whether the relative increase in seryl-tRNA II and III is due to increased synthesis, decreased degradation, tRNA modification, or a combination of these processes. The effect is apparently not due to alterations in the aminoacyl-tRNA ligase. Since different seryl-tRNA peaks respond uniquely to the hormone, the process that increases the level of tRNA molecules is probably highly selective. Chromatographically distinct isoaccepting tRNA species often respond to different sets of codons, and thus are probably products of separate genes. Further studies are necessary to determine the codon–anticodon relationships and whether the elevated seryl-tRNA species are otherwise distinct from the nonelevated seryl-tRNA peaks. It is unknown whether any of these tRNAs are of mitochondrial origin. This possibility is not likely since mitochondrial tRNA accounts for only a small proportion of total liver tRNA. In addition, the ligase preparation was derived from mitochondria-free supernatant and, at least in rat liver, cytoplasmic seryl-tRNA ligase does not acylate mitochondrial tRNA (Buck and Nass, 1969).

The concentration of certain species of aminoacyl-tRNA (and the frequency of the corresponding mRNA codons) may regulate the rate of synthesis of certain proteins. Anderson (1969) has recently confirmed this concept in a synthetic polynucleotide-directed model system. However, there are other potential mechanisms by which changes in aminoacyl-tRNA can regulate protein synthesis. Although numerous variations in aminoacyl-tRNA (quantitative and qualitative) have been described in higher organisms, the biological significance of these alterations remains unclear. The present study is unique in that the synthesis of a specific protein has been correlated with an alteration in a discrete fraction of a specific aminoacyl-tRNA. The precise relationship between phosvitin synthesis and seryl-tRNA levels is currently under investigation.

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The Molecular Determinants of the A11 and A12 Allotypic Specificities in Rabbit Immunoglobulin*

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ABSTRACT: The finding that group a allotypes, segregating genetic markers determined by primary structure of the Fd region, are present on the distinctive heavy chains of several classes of rabbit immunoglobulin has posed a problem in understanding the nature of the genetic control of immunoglobulin synthesis. Recently a new system of allotypic markers, A11 and A12, detected by hemagglutination techniques has been identified on the γ heavy chain of rabbit immunoglobulin G. In the present paper it is shown that A11 correlated with methionine on the N-terminal side of the half-cystine participating in the inter-γ-chain bond, whereas A12 correlates with threonine at this position. The significance of the methionine–threonine interchange sharing the same chain with amino acid sequences determining the group a allotypes is considered in relationship to the genetic control of the biosynthesis of heavy and light chains of immunoglobulin.

Segregating genetic markers recognized by their differing intraspecies antigenic specificities were first reported by Oudin and named allotypes[1] (Oudin, 1956). These were shown to constitute two groups distinguished by their genetic behavior (Oudin, 1960). The group a determinants a1, a2, and a3 are present on the Fd portion of the heavy (H) chains and the group b determinants b4, b5, b6, and b9 (Dubiski and Muller, 1967) are present on the light (L) chain (Stemke, 1964). The determinants within each of groups a and b behave as alleles, and the two groups are not genetically linked to one another (Dubiski *et al.*, 1962).

Studies of peptides obtained by cyanogen bromide treatment indicated that the amino acid composition of a peptide present at the N-terminal end of the Fd fragment correlates with the group a allotype of the γ chain (Koshland, 1967; Porter, 1967; Koshland *et al.*, 1968; Prahl and Porter, 1968). Recently Wilkinson (1969a) has related these differences directly to amino acid sequence variations for the a1 and a3 specificities. A replacement of methionine for threonine in the hinge region of rabbit γ chain which appeared to correlate with allotype in pools of a3 and of a1 IgG has been reported (Porter, 1967; Prahl and Porter, 1968). Methionine at this position was found in 60–80% of the molecules of the a3 pool. Koshland (1967) was unable to confirm this replacement in purified antiphenylarsonic acid and antiphenyl β-lactoside antibodies from rabbits homozygous for the a3 determinant.

Recently Mandy and Todd (1968, 1969, 1970) have described another pair of allotypic specificities, A11 and A12. These specificities are detected by inhibition of hemagglutination. Their determinants are in the hinge region of the γ chain. Each of these specificities is associated with each of the group a allotypes so that all combinations are possible, *e.g.*, a1,A11, a1,A12, etc. Despite this variation, breeding studies have shown them to be linked to the group a specificities (Zullo *et al.*, 1968; Mandy and Todd, 1970).

We report here an investigation of the interchange of methionine and threonine in γ chains of IgG from rabbits homozygous for the group a allotype determinants as well as for A11 and A12. It will be shown that the presence of methionine correlates with the presence of the A11 and the presence of threonine correlates with A12, while the composition of the N-terminal peptide (Koshland, 1967; Prahl and Porter, 1968; Wilkinson, 1969b) remains characteristic of the group a allotype.

Materials and Methods

Serologic Methods. Typing for groups a and b was done by interfacial precipitation reactions (ring tests) using antiallotype sera prepared by injection of rabbits with rabbit antiovalbumin specific precipitate following the method of Oudin (1960). Typing for A11 and A12 was done by inhibition of hemagglutination as described by Mandy and Todd (1969, 1970).

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[1] The nomenclature employed for rabbit immunoglobulins, their chains, and fragments follows that recommended by a Committee of the World Health Organization (1964). The notation for allotypes is that of Dray *et al.* (1962) except that the initial A has been omitted for those specificities assigned to a definite genetic group, *e.g.*, a1 in lieu of Aa1.