Acknowledgments

The authors express their gratitude to E. R. Shelton for assistance, and to Dr. H. Weissbach for a gift of EF-Tu used in some of the initial experiments.

References

Ames, B. N. (1966), *Methods Enzymol. 8*, 115.
Brot, N., Yamasaki, E., Redfield, B., and Weissbach, H. (1970), *Biochem. Biophys. Res. Commun. 40*, 698.
Fink, L. M., Goto, T., Frankel, F., and Weinstein, I. B. (1968), *Biochem. Biophys. Res. Commun. 32*, 963.
Fraenkel-Conrat, H., Singer, B., and Tsugita, A. (1961), *Virology 14*, 54.
Gillam, I., Blew, D., Warrington, R. C., Tigerstrom, M., and Tener, G. M. (1968), *Biochemistry 7*, 3459.
Gordon, J. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1574.
Klyde, B. J., and Bernfield, M. R. (1973), *Biochemistry 12*, 3757.
Leder, P., Singer, M. F., and Brimacombe, R. L. C. (1965), *Biochemistry 4*, 1561.
Loftfield, R. B. (1972), *Progr. Nucl. Acid Res. 12*, 87.
Mäenpää, P. H., and Bernfield, M. R. (1969), *Biochemistry 8*, 4926.
Miller, D. L., and Weissbach, H. (1970), *Arch. Biochem. Biophys. 141*, 26.
Müller, P., Wehrli, W., and Staehelin, M. (1971), *Biochemistry 10*, 1885.
Okita, G. T., Kabura, J. J., Richardson, F., and LeRoy, G. V. (1957), *Nucleonics 15*, 111.
Pearson, R. L., Hancher, C. W., Weiss, J. F., Holladay, D. W., and Kelmers, A. D. (1973), *Biochim. Biophys. Acta 294*, 236.
Petrissant, G., Boisnard, M., and Puissant, C. (1971), *Biochimie 53*, 1105.
Ravel, J. M., Shorey, R. L., and Shive, W. (1968), *Biochem. Biophys. Res. Commun. 32*, 9.
Roy, K. L., and Söll, D. (1970), *J. Biol. Chem. 245*, 1394.
Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.
Schofield, P., and Zamecnik, P. C. (1968), *Biochim. Biophys. Acta 155*, 410.
Skoultchi, A., Ono, Y., Moon, H. M., and Lengyel, P. (1968), *Proc. Nat. Acad. Sci. U. S. 60*, 675.
Weissbach, H., Miller, D. L., and Hachmann, J. (1970), *Arch. Biochem. Biophys. 137*, 262.
Weissbach, H., Redfield, B., and Brot, N. (1971), *Arch. Biochem. Biophys. 145*, 676.
Wimmer, E., Maxwell, I. H., and Tener, G. M. (1968), *Biochemistry 7*, 2623.
Work, T. S., and Work, E. (1970), Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 1, New York, N. Y., American Elsevier, p 170.

Rate of Serine Transfer Ribonucleic Acid Synthesis During Estrogen-Induced Phosphoprotein Synthesis in Chick Liver†

Barry J. Klyde and Merton R. Bernfield*

ABSTRACT: The estrogen-induced hepatic synthesis of the yolk phosphoprotein, phosvitin, in roosters is accompanied by a 25% increase in the serine acceptance of hepatic tRNA which is limited to two serine isoacceptors and not dependent on AA-tRNA synthetase (P. H. Mäenpää and M. R. Bernfield, *Biochemistry 8*, 4926 (1969). In chicks, estrogen is now shown to cause a similar increase in the concentration of hepatic Ser-tRNA as well as changes in the acylation of other tRNA species during phosvitin induction. To determine whether the change in Ser-tRNA level is due to a specific increase in synthesis, the rate of tRNA^{Ser} synthesis relative to the rate of total tRNA synthesis in hormone-treated chicks was compared to that in control chicks. The relative synthetic rate was determined by a double-label method in which nonspecific effects of the hormone were avoided. The incorporation of [^{3}H]- and [^{14}C]orotic acid, over a brief period *in vivo* or in liver slices, into purified tRNA^{Ser} from estrogen-treated and control birds was compared to the incorporation into unfractionated tRNA from these birds. No appreciable change in the relative rate of tRNA^{Ser} synthesis was found during a period when the level of Ser-tRNA continued to increase. Kinetic analysis of the data suggests that mechanisms other than increased synthesis are responsible for the increased serine acceptance of hepatic tRNA during estrogen-induced phosvitin synthesis. Several possibilities are discussed, including a decrease in degradation rate or an estrogen-evoked specific tRNA modification.

Estrogen-induced increases in different classes of RNA have often been assumed to be the result of increased gene transcription. Evidence that such increases in RNA reflect augmented synthetic rate include the estrogen-evoked increased incorporation of precursors into rRNA and tRNA of rat uteri (Billing *et al.*, 1969) and into all rooster liver RNA fractions (Coolsma and Gruber, 1968). Inhibition studies with actinomycin D (Greengard *et al.*, 1964) and DNA–RNA hybridization experiments (Hahn *et al.*, 1969) have suggested that the estrogen-induced phosphoprotein synthesis in rooster

† From the Division of Developmental Biology, Department of Pediatrics, Stanford University School of Medicine, Stanford, California 94305. *Received April 17, 1973.* This work supported by Grants GM-15086 and HD-02147 from the National Institutes of Health. B. J. K. is a trainee of the Medical Scientist Training Program under National Institutes of Health Grant GM-1922.

liver is mediated by selective increases in RNA synthesis. However, Tomkins and Martin (1970) have cautioned that such experiments are not concrete evidence for a hormone-induced increase in the rate of specific gene transcription, but may reflect an alteration in precursor pool size, transport, degradation, or nonspecific synthesis of RNA.

Administration of estrogen to roosters induces the hepatic synthesis of phosvitin, a phosphoprotein containing more than 50% serine residues (Heald and McLachlan, 1965; Allerton and Perlmann, 1965) and causes more than a 25% increase in the serine-accepting activity of hepatic tRNA (Mäenpää and Bernfield, 1969). This increase is limited to two of the four serine isoacceptors and is independent of the source or concentration of AA-tRNA synthetase, concentration of tRNA, duration of acylation incubation or isotope in serine. Similar estrogen-induced changes in serine acceptance are seen when hepatic tRNA from laying hens and immature birds is compared (Beck *et al.* 1970). However Busby and Hele (1970) found increased tRNA acceptance of additional amino acids after estrogen treatment.

The estrogen-induced increase in Ser-tRNA presents an opportunity to determine whether a hormone-evoked quantitative change in a direct transcriptional product is caused by (a) increased synthesis, (b) decreased degradation, (c) activation, alteration or release of preexisting molecules, or (d) some combination of these processes. In the work reported in this paper we have investigated whether the rate of $tRNA^{Ser}$ synthesis relative to the rate of total tRNA synthesis is altered during estrogen-induced phosvitin synthesis in chicks. The data do not reveal an appreciable change in the relative rate of $tRNA^{Ser}$ synthesis, and kinetic analysis suggests that alternative mechanisms are responsible for the increase in Ser-tRNA concentration.

Materials and Methods

Radioisotopes. Labeled amino acids were obtained from the usual commercial sources. [5-^{14}C]Orotic acid (61 Ci/mol) and [5-^{3}H]orotic acid (18,000 and 23,000 Ci per mol) were obtained from Amersham-Searle.

Animals. Eight-day-old white Leghorn male chicks were used for *in vivo* incorporation of label rather than adult birds because less radioactive RNA precursor was needed and more animals could be used, thus reducing the effect of individual variations. Liver slices were prepared from 8-day-old male chicks for *in vitro* labeling. Chicks were injected intramuscularly with 40 mg/kg of 17β-estradiol benzoate (Nutritional Biochemicals Corp.) in sesame oil; control birds were injected with sesame oil only. Blood samples obtained after decapitation of the chicks were analyzed for phosvitin as described previously (Mäenpää and Bernfield, 1969).

tRNA Labeling. For *in vivo* labeling, two chicks were injected with hormone, and two with sesame oil. After 41 hr, each hormone-treated chick received 2.5 mCi of [^{3}H]orotic acid intraperitoneally in several injection sites and each control chick received 0.50 mCi of [^{14}C]orotic acid in the same way. Livers were removed and tRNA was isolated 3 hr after precursor administration.

For *in vitro* labeling, liver slices about 0.5 mm thick were prepared from groups of four to seven chicks injected with either hormone or sesame oil 40 to 41 hr previously. Slices from estrogen-treated and control birds were incubated separately under conditions described by Heald and McLachlan (1965), except that penicillin (800 units/ml) and streptomycin (200 μg/ml) were added. Medium for the slices from hormone-treated birds contained 10 μCi/ml of [^{14}C]-orotic acid and medium for control slices contained 50 μC/ml of [^{3}H]orotic acid with 50 ml of medium for each group. After 3-hr incubation, the slices were chilled on ice and tRNA was isolated.

The 3-hr-labeling period is short relative to the half-life of tRNA, 80 hr (Quincey and Wilson, 1969) to 5 days (Hanoune and Agarwal, 1970) in rat liver, and long relative to the half-life of pre-tRNA, 5–10 min in HeLa cells (Bernhardt and Darnell, 1969), thus minimizing the effects of tRNA degradation and pre-tRNA maturation.

Preparation of tRNA. Labeled tissues from control and hormone-treated birds were pooled, and mixed double-labeled tRNA was isolated as described (Klyde and Bernfield, 1973). Pooling of livers reduces the contribution of isolation artifacts, such as differences in activity of nucleases or CCA pyrophosphorylase. Unlabeled tRNA was prepared from 5 hormone-treated chicks and 5 control chicks 44 hr after hormone administration, and from 13 hormone-treated and 14 control chicks 65 hr after estrogen administration.

Acylation of tRNA. Mixed double-labeled tRNA, consisting of tRNA from control birds labeled with one isotope and tRNA from estrogen-treated birds labeled with another isotope, was acylated with [^{3}H]serine as described (Klyde and Bernfield, 1973). The amino acid acceptance of unlabeled tRNA was determined as previously described (Mäenpää and Bernfield, 1969). Synthetase from control birds was used for all acylations because of the previous demonstration that the source of synthetase does not influence the extent of amino acid acceptance (Mäenpää and Bernfield, 1969).

Purification of $tRNA^{Ser}$ and Determination of Purity. The method of purification and determination of purity of $tRNA^{Ser}$ from double-labeled tRNA acylated with [^{3}H]serine is described in the accompanying paper (Klyde and Bernfield, 1973).

Comparison of Rates of $tRNA^{Ser}$ Synthesis. The ratio of isotopes (isotope in estrogen-treated birds per isotope in control birds) in purified $tRNA^{Ser}$ was divided by that in unfractionated tRNA. Comparison of the isotope ratios eliminates the effect of differences in precursor dose or uptake and obviates the need to correct for variation in pool size, assuming that all tRNA species are synthesized from the same precursor pool. This method does not allow an estimate of absolute synthetic rate, but instead is used to evaluate the hormone-induced change in synthetic rate of $tRNA^{Ser}$. The expression used was

$$\left(\frac{\text{E dpm}}{\text{C dpm}}\right)_{\text{in } tRNA^{Ser}} \Big/ \left(\frac{\text{E dpm}}{\text{C dpm}}\right)_{\text{in total tRNA}} \qquad (1)$$

where E refers to tRNA from estrogen-treated chicks and C to tRNA from control chicks. This ratio is equivalent to

$$\left(\frac{tRNA^{Ser}\ \text{dpm}}{\text{total tRNA dpm}}\right)_{E} \Big/ \left(\frac{tRNA^{Ser}\ \text{dpm}}{\text{total tRNA dpm}}\right)_{C} \qquad (2)$$

which describes the estrogen-induced change in synthetic rate of $tRNA^{Ser}$. In this way the synthetic rate of $tRNA^{Ser}$ relative to total tRNA in hormone-treated birds can be compared with that in control birds. Use of the ratio of $tRNA^{Ser}$ radioactivity to total tRNA radioactivity for both hormone-treated and control chicks cancels out the contribution of a possible hormone-induced general increase in tRNA synthetic rate, and permits comparison of the estrogen-influenced $tRNA^{Ser}$ synthetic rate with that of controls.

An estrogen-induced change in the extent of modification of pyrimidine bases at the 5 position, such as methylation and pseudo uracil formation, would alter the amount of label in bases derived from [5-^{3}H]orotic acid. This is a potential source of error, since a hormone-induced increase in tRNA modification would cause loss of ^{3}H label and result in underestimation of synthetic rate. This source of error was bypassed by: (a) using [5-^{14}C]orotic acid for estrogen-treated chicks, and [5-^{3}H]-orotic acid for control chicks in the two experiments utilizing *in vitro* labeling of tRNA. A hormone-induced change in extent of tRNA modification would thus not alter tRNA labeling since ^{14}C would not be expected to be lost. Differences in extent of modification between tRNA species in the control chicks receiving [^{3}H]orotic acid are irrelevant, since labeling in control tRNA was the base line with which labeling in hormone-influenced tRNA was compared; (b) reversing labels in the *in vivo* incorporation experiment, so that hormone-treated chicks received [5-^{3}H]orotic acid and control chicks [5-^{14}C]orotic acid. In this way it could be determined if the *in vivo* and *in vitro* experiments were comparable, and if an isotope effect was present.

Isotope ratios in tRNA were determined on aliquots containing 300–700 pmol of purified [^{3}H]Ser-tRNA, as a ternary complex with EF-Tu–GTP, and on aliquots of unfractionated tRNA from the starting material used to purify the [^{3}H]Ser-tRNA. Unlabeled serine (20 μmol) and 0.5 mg of carrier RNA (yeast RNA type XI, Sigma) were added to each sample, and the solutions were adjusted to pH 13 with 1 N KOH and incubated for 40 min at 0°. These deacylation conditions were designed to quantitatively deacylate the [^{3}H]-serine without causing any loss of trichloroacetic acid precipitable label in tRNA, and were shown to completely deacylate Ser-tRNA and to remove 99.8% of the label from [^{3}H]Val-tRNA, the AA-tRNA most resistant to alkali deacylation (Sarin and Zamecnik, 1964). The deacylated tRNA was precipitated (10% trichloroacetic acid, 20 min at 0°), collected by centrifugation (15 min, 30,000*g*) and dissolved in 3 ml of ice-cold 0.1 N KOH containing 6.7 mM unlabeled serine. Following precipitation, collection and solubilization as before, the tRNA was precipitated with trichloroacetic acid onto glass-fiber filters. The filters were dried, placed in scintillation vials, and incubated for 3–4 hr at 50° with 150 μl of water and 1 ml of 0.6 N NCS (Amersham Searle) to solubilize the tRNA and eliminate any distortion of the isotope ratio caused by self-absorption of ^{3}H β particles. After the vials had cooled, 10 ml of toluene-based scintillation fluid was added to each. Samples were counted in a Beckman LS-233 scintillation counter, with less than 0.1% ^{3}H counted in the ^{14}C window, for 50 min or until 2.5×10^5 counts were recorded. Efficiencies obtained were 39% for ^{3}H and 62% for ^{14}C.

Results

Estrogen Responsiveness of Chicks. PLASMA PHOSPHOPROTEIN. Chicks were found to require four times the estrogen dose previously used in roosters (Mäenpää and Bernfield, 1969) to produce a plasma phosphoprotein response comparable in duration to that produced in adult birds. The induction of phosphoprotein in chicks was qualitatively similar to that seen in roosters, but the magnitude of the response was substantially less (Figure 1).

EXTENT OF ACYLATION. Aminoacylation of hepatic tRNA from control and estrogen-treated chicks (44 hr after hormone administration) was performed with 19 amino acids under conditions where acylation was linearly proportional to tRNA concentration. Amino acid acceptance was determined relative to the total amount of tRNA, so that differences in acceptance reflect changes in concentration. Significant differences in extent of acylation were found for five amino acids (Table I). Estrogen treatment brought about an increase in tRNA acceptance of arginine (11%), leucine (27%), and serine (17%), and a decrease in acceptance of histidine (−4.5%) and tyrosine (−13%). tRNA prepared from chicks injected 65 hr previously with estrogen accepted significantly more leucine (32%) and serine (16%) than tRNA prepared from control chicks. Thus, chicks resemble roosters and hens in displaying increased serine acceptance by hepatic tRNA during estrogen-induced hepatic synthesis of phosvitin.

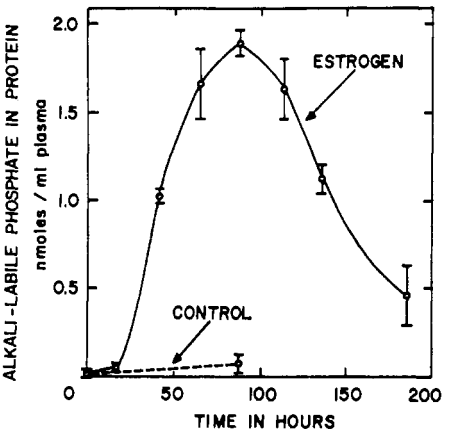

FIGURE 1: Plasma phosphoprotein levels in chicks after estrogen treatment. Alkali-labile phosphate in lipid-free plasma protein was measured at various times after injection of 40 mg/kg of 17β-estradiol benzoate into 8-day-old male chicks. Control birds received an equal volume of sesame oil, the hormone solvent. Values are means ±SEM for six observations.

TABLE I: Amino Acid Acceptance of Hepatic tRNA from Estrogen-Treated and Control Chicks.[a]

Amino Acid	Acylation of Control tRNA μmole of AA/ μmole of tRNA $\times$ 100	% Change in Acylation 44 hr after Estrogen[b]	*P* Value
Arg	6.8	+11	<0.05
Leu	3.7 (3.1)[c]	+27 (+32)	<0.01
Ser	3.1 (3.1)	+17 (+16)	<0.001
His	4.7	−4.5	<0.02
Tyr	0.51	−13	<0.02

[a] Acceptance of 14 other amino acids was not significantly different for tRNA from estrogen-treated and control chicks: Ala, Asp, Cys, Gln, Glu, Gly, Ile, Lys, Met, Phe, Pro, Thr, Trp, Val. [b] Per cent difference determined from slopes of curves of amino acid acceptance *vs.* tRNA concentration. Slopes were calculated by the method of least squares and compared by regression analysis, and differences between slopes tested for significance with Student's "t" test. There were 10 degrees of freedom (df) ($N + N' - 4$) in all cases but Arg, Cys, and Tyr (8 df) and Met (2 df). [c] Values in parentheses obtained for tRNA from chicks injected 65 hr previously with estrogen. *P* values were identical with those obtained after 44 hr.

TABLE II: Estrogen Responsiveness of Chicks.

Parameter	Time after Administration of Estrogen or Sesame Oil[a]	
	44 hr	65 hr
	% Increase over Control	
Liver weight	58	72
tRNA per liver	32	103
Ser-tRNA per total tRNA[b]	17	16

[a] 5 and 13 control, and 5 and 14 estrogen-treated birds at 44 and 65 hr, respectively. [b] From Table I.

LIVER WEIGHT AND APPEARANCE. Hormone-treated chicks used to prepare unlabeled tRNA showed a 58% increase in liver weight relative to control chicks 44 hr after estrogen administration, and a 72% increase 65 hr after hormone administration (Table II). Livers of estrogen-treated birds varied from tan to yellow in color, while livers of control birds were dark brown or red-brown. Livers from the three groups of hormone-treated chicks used in the precursor incorporation experiments showed the expected color change and weighed 46–81% more than livers of control chicks at 40–41 hr after estrogen treatment. In comparison, liver weight of hormone-treated roosters is more than twice that of control roosters 64 hr after estrogen administration (B. J. Klyde, unpublished result).

TOTAL tRNA. Hormone-treated chicks used to prepare unlabeled tRNA showed a 32% increase in amount of tRNA per liver compared to control chicks 44 hr after estrogen administration, and more than twice as much tRNA per liver at 65 hr (Table II).

These assessments of estrogen responsiveness (plasma phosphoprotein, tRNA acylation, alteration in liver weight, appearance, and tRNA content) suggest that estrogen-treated chicks show similar, but not as extensive, changes as hormone-treated roosters. The data also indicate that the chicks used for evaluation of the synthetic rate of $tRNA^{Ser}$ responded to estrogen to the same extent as the birds which demonstrated a 17% increase in serine acceptance of hepatic tRNA.

Comparison of Synthetic Rates of $tRNA^{Ser}$. Mixed double-labeled hepatic tRNA was prepared from (a) estrogen-treated and control chicks injected 44 hr previously with hormone or hormone vehicle and 3 hr previously with labeled orotic acid (*in vivo* incorporation), and from (b) liver slices of control and estrogen-treated chicks injected 40–41 hr previously with hormone or vehicle and then incubated for 3 hr with precursors *in vitro*. The mixed, double-labeled tRNA preparations were acylated with [^{3}H]serine, the seryl-tRNA–EF-Tu–GTP complex was purified and the isotope ratios of the purified $tRNA^{Ser}$ were compared with that of unfractionated tRNA from the same preparation (Table III).

Assessment of the relative synthetic rate of $tRNA^{Ser}$ is dependent upon the purity of the $tRNA^{Ser}$ preparation. Assuming that the tRNA contaminating the purified $tRNA^{Ser}$ has the same isotope ratio as the unfractionated tRNA, the relative synthetic rate of absolutely pure $tRNA^{Ser}$ (assumed to be 1600 pmol/A_{260}) can be derived from the isotope ratios and the experimentally determined purity (Table III). In the calculations, the isotope ratio in $tRNA^{Ser}$ was divided by that in unfractionated tRNA, yielding the proportionate change in synthetic rate of $tRNA^{Ser}$ due to estrogen, as outlined in Materials and Methods. The hormone-influenced synthetic rate of $tRNA^{Ser}$ compared to that in control chicks (represented as 1.000) for the three precursor incorporation experiments was 1.041 ± 0.046 (mean ± SD), indicating that the synthetic rate of $tRNA^{Ser}$ relative to total tRNA in estrogen-treated chicks is not substantially different from that in control chicks.

The purity of the isolated $tRNA^{Ser}$ in expt 1 was 1717 pmol/A_{260}, or 107% if 1600 pmol/A_{260} is assumed to be 100% pure. If it is assumed that the error in purity determination is ±10%, it can be calculated that the resultant error in determination of the relative rate of $tRNA^{Ser}$ synthesis (Table III) is of the order of ±0.01.

Discussion

In the work presented in this report, hepatic tRNA from estrogen-stimulated chicks displayed a 17% increase in serine acceptance at a time when the rate of $tRNA^{Ser}$ synthesis relative to total tRNA in hormone-treated chicks was determined to be not appreciably different from that in control chicks. A mechanism other than increased synthesis is thus probably responsible for producing the increase in Ser-tRNA concentration in estrogen-treated chicks.

The rate of synthesis of a molecular species derived from a single precursor is generally zero order, and the rate of degradation is usually first order. The assumed kinetic changes which could produce the observed increase in $tRNA^{Ser}$ are (1) a switch from a basal, zero-order rate of synthesis to a different, increased zero-order rate; (2) a switch from a basal, first-order rate of degradation to a different, reduced first-order rate; (3) change in the rate constants for both synthesis and degradation; and (4) change in neither rate constant, with the increase in $tRNA^{Ser}$ caused by alterations in maturation, tRNA modification, or some other process not acting through changes in the rates of synthesis or degradation.

Degradation of tRNA in mammalian liver has been shown to follow first-order kinetics (Quincey and Wilson, 1969; several workers cited by Hanoune and Agarwal, 1970), and it is likely that the same is true for avian tRNA in this experiment. In this work the concentration of hepatic $tRNA^{Ser}$ in hormone-treated chicks showed the same increase relative to controls at both 44 and 65 hr after hormone administration. The constancy of the increased concentration of $tRNA^{Ser}$ during a time period when total tRNA per liver is increasing (Table II) indicates that synthesis follows zero order kinetics, if it is assumed that the first-order rate constant for degradation does not change during the same time period.

The experiments reported in this paper were designed to detect a hormone-induced change in the rate of synthesis of $tRNA^{Ser}$. A hormone-evoked change in the rate of $tRNA^{Ser}$ degradation, with no effect on synthesis, would not be detected directly and would result in only an unaltered rate of synthesis in the present work. Similarly, if the $tRNA^{Ser}$ increase was caused by a process such as tRNA modification which did not involve changes in either the rate of synthesis or degradation, it would also be detected in these experiments as only an unaltered rate of synthesis. However, if both degradation and synthesis were stimulated by estrogen, this would have been detected as an increase in the rate of $tRNA^{Ser}$ synthesis. No appreciable increase in the rate of $tRNA^{Ser}$ synthesis was seen, indicating that either the rate of $tRNA^{Ser}$ degradation is increased by estrogen, or that the hormone does not cause a change in the rate constant for synthesis or

TABLE III: Synthesis Rate of tRNA^{Ser} in Hormone-Treated Chicks Relative to That in Control Chicks.

Expt	tRNA	tRNA Radioactivity dpm ^{3}H	tRNA Radioactivity dpm ^{14}C	Isotope Ratio[b]	Rel Ratio[c]	tRNA^{Ser} Purity (pmole/A_{260})	Rel Synthetic Rate of Pure tRNA^{Ser}[d]
(1) Chick, *in vivo*	tRNA^{Ser}	6,808	795.0	8.564	1.026	1717	1.026
E = ^{3}H[a]	Unfractionated	31,280	3747	8.348			
(2) Chick, liver slices	tRNA^{Ser}	3,567	568.9	0.1595	1.060	923	1.104
E = ^{14}C	Unfractionated	8,223	1237	0.1504			
(3) Chick, liver slices	tRNA^{Ser}	5,510	1077	0.1955	0.9952	1270	0.9938
E = ^{14}C	Unfractionated	12,260	2408	0.1964			

[a] E = isotope in tRNA from hormone-treated chicks. [b] Disintegrations per minute in tRNA from estrogen-treated birds divided by disintegrations per minute in tRNA from control birds. [c] Isotope ratio of tRNA^{Ser} divided by isotope ratio of unfractionated tRNA. [d] Pure tRNA assumed to be 1600 pmol/A_{260} unit. Computed for expt 2, for example, using the formula $(923/1600)(x) + [(1600 - 923)/1600] = 1.060$, where x = relative synthetic rate of the pure tRNA^{Ser}.

degradation but instead alters a process such as tRNA modification or maturation.

What relative synthetic rate for tRNA^{Ser} would be required to produce the observed 17% increase in serine acceptance, assuming that synthetic rate was the only parameter altered? The new relative synthetic rate of tRNA^{Ser} after estrogen administration can be approximated by using the equations of Schimke and Doyle (1970), which describe the approach of an enzyme level to a new steady state following changes in rates of synthesis and degradation. From Schimke and Doyle (1970), K_s and K_d are the steady state rate constants of synthesis and degradation, and in the present study are the rate constants for tRNA in the control chicks. K_s' and K_d' are the constants achieved after hormone stimulation and correspond to the constants in the estrogen-treated chicks. Assume that the rate constants of tRNA^{Ser} synthesis in hormone-treated and control chicks, K_s' and K_s, have already been corrected by division with the rate constants of total tRNA synthesis in hormone-treated and control chicks, to eliminate any hormone effect on the synthetic rate of all species and permit evaluation of the specific hormone effect on tRNA^{Ser}.

If tRNA$_0^{Ser}$ is the basal concentration of tRNA^{Ser} and tRNA$_t^{Ser}$ is the hormonally induced concentration at time t, and assuming for purposes of calculation that the degradation rate is unchanged ($K_d' = K_d$), then the relative increase in tRNA^{Ser} at time t is described by

$$\left(\frac{\text{tRNA}_t^{Ser}}{\text{tRNA}_0^{Ser}}\right) = \left(\frac{K_s'}{K_d'\text{tRNA}_0^{Ser}}\right) - \left(\frac{K_s'}{K_d'\text{tRNA}_0^{Ser}} - 1\right)e^{-K_d't} \quad (3)$$

substituting $K_d' = (K_s/\text{tRNA}_0^{Ser})$ in eq 3 yields

$$\left(\frac{\text{tRNA}_t^{Ser}}{\text{tRNA}_0^{Ser}}\right) = \left(\frac{K_s'}{K_s}\right) - \left(\frac{K_s'}{K_s} - 1\right)e^{-(K_s/\text{tRNA}_0^{Ser})t} \quad (4)$$

The expression K_s'/K_s describes the proportionate increase in tRNA^{Ser} synthetic rate or the tRNA^{Ser} synthetic rate in hormone-treated chicks relative to that in control chicks, and can be calculated using data from rat liver on the turnover of tRNA (11,000 molecules min^{-1} cell^{-1}) and the number of tRNA molecules per cell (7.5×10^7) from Quincey and Wilson (1969). Then, $K_s = A \times 11{,}000$ molecules of tRNA min^{-1}, and tRNA$_0^{Ser} = A \times 7.5 \times 10^7$, where A is the proportion of the total tRNA that is tRNA^{Ser}. It can be seen that A cancels out in the equation, and need not be considered.

If it is assumed that an elevated tRNA^{Ser} synthetic rate prevails from the time of hormone administration until about 40 hr, when a 17% increase in serine acceptance was measured, then it can be calculated that $K_s'/K_s = 1.57$. If it is assumed that there is a 20-hr lag, similar to the lag seen for the phosphoprotein response (Figure 1), before the tRNA^{Ser} synthetic rate is increased, then the 17% increase in Ser-tRNA seen at about 40 hr would have to be achieved after only 20 hr at the increased synthetic rate. K_s'/K_s can then be calculated to be 2.06. From these approximate calculations it can be seen that the increase in synthetic rate would have to be between 57 and 106% to reasonably account for the 17% increase in Ser-tRNA.

Failure to observe an increased rate of tRNA^{Ser} synthesis at about 40 hr after estrogen treatment, at a time when tRNA^{Ser} concentration is increased by 17%, might be explained by postulating a brief increase in tRNA^{Ser} synthetic rate which has subsided and is therefore undetectable by 40 hr. However, the data of Table II show that the concentration of tRNA^{Ser} remains elevated even at 65 hr, despite a continuing increase in the total amount of tRNA per liver, indicating that the mechanisms responsible are still operative beyond the time when tRNA^{Ser} synthetic rate was studied.

The quantitative increase in seryl-tRNA during phosvitin synthesis is seen regardless of acylation conditions, enzyme source, and age or sex of bird (Mäenpää and Bernfield, 1969; Beck *et al.*, 1970). The selective change in two of four isoacceptors has been demonstrated by chromatography (Mäenpää and Bernfield, 1969; Beck *et al.*, 1970) and confirmed by ribosomal binding (Mäenpää, 1972), procedures dependent on prior serine acylation. The method of tRNA^{Ser} purification in this study is similarly dependent on prior serine acylation. Although it seems clear that the concentration of physiologically available Ser-tRNA is increased during phosvitin synthesis, failure to find a corresponding increase in tRNA synthesis might mean that the concentration of tRNA^{Ser} is unchanged. To examine the possibility that serine acceptance may not accurately reflect tRNA^{Ser} concentration, the level of

$tRNA^{Ser}$ must be measured by a procedure not dependent upon serine acylation.

Determination of the rate of $tRNA^{Ser}$ synthesis following purification with EF-Tu assumes that all $tRNA^{Ser}$ species are complexed. If some $tRNA^{Ser}$ species were not complexed, the determined synthetic rate would be inaccurate. Complex formation does occur with all $tRNA^{Ser}$ species because the chromatographic profile of purified Ser-tRNA on BD-cellulose resembles that of unpurified Ser-tRNA (Klyde and Bernfield, 1973). Recovery, in the purified complex, of more than 75% of the Ser-tRNA in unfractionated tRNA indicates that it is unlikely that a major $tRNA^{Ser}$ species is unreactive with EF-Tu (Klyde and Bernfield, 1973).

The increase in Ser-tRNA may be due to alteration in precursor processing, transport, or storage, or in tRNA modification, degradation or other steps. Precursors of tRNA, pre-tRNA, have a half-life of 5–15 min in mammalian cells (Bernhardt and Darnell, 1969; Choe and Taylor, 1972), appear in the cytoplasm within 3–5 min after manufacture and apparently do not accumulate in the nucleus (Burdon, 1971). Change in the rate of transport of specific pre-tRNA species from the nucleus is therefore unlikely to be a regulatory step which is altered by estrogen. Precursor storage is probably not involved because pre-tRNA does not seem to be associated with cytoplasmic organelles or bound to protein (Burdon and Clason, 1969), although it is difficult to completely exclude the possibility of intracellular compartmentalization of pre-tRNA or mature tRNA. Eukaryote pre-tRNA does not possess the modifications present in mature tRNA (Burdon *et al.*, 1967). Methylation or other modifications may regulate tRNA maturation; methionine starvation of HeLa cells slows the conversion of pre-tRNA to tRNA, but pre-tRNA does not accumulate and the methyl accepting capacity of tRNA increases (Bernhardt and Darnell, 1969).

Estrogen administration increases tRNA methylase activity in chick oviduct (Hacker, 1969) and in rat liver (Sheid *et al.*, 1970). In rooster liver, the activity of certain specific tRNA methylases is increased by estrogen treatment and others are decreased, although total tRNA methylase activity is decreased (Mays and Borek, 1971). These assessments of methylase activity were performed with a heterologous substrate, *E. coli* tRNA, and therefore do not necessarily indicate that tRNA methylated *in vivo* possesses more methyl groups as a result of estrogen treatment. Methylation of previously undermethylated tRNA has been shown to increase its amino acid accepting activity (Shugart *et al.*, 1968). Degradation of rRNA is thought to be prevented by methyl groups which are introduced during posttranscriptional maturation (Vaughan *et al.*, 1967), but unlike methyl-deficient rRNA precursors, methyl-deficient tRNA is apparently quite stable (Burdon, 1971). Methylation of tRNA has been shown to be altered by estrogen administration and possesses the specificity required for a process affecting only a few of the many tRNA species. It is conceivable that an estrogen-evoked specific methylation of certain $tRNA^{Ser}$ species might cause this population of $tRNA^{Ser}$ to be more efficiently acylated, or less susceptible to degradation.

An additional possible explanation is that as a result of a direct interaction of Ser-tRNA with estrogen, similar to the binding of estradiol to yeast Ser-tRNA observed by Chin and Kidson (1971), a greater proportion of Ser-tRNA is extracted during tRNA isolation. Such binding might also impede degradation. However, estradiol binds more firmly to yeast Phe-tRNA and no increase in Phe-tRNA is seen during phosvitin induction, and rooster liver Ser-tRNA does not bind estradiol (P. Mäenpää, personal communication).

Changes in the levels of specific AA-tRNAs have been found during developmental and oncologic processes and have been correlated with the synthesis of specific proteins. Garel *et al.* (1970) found that coincident with the increased synthesis of silkworm fibroin, a protein containing a large amount of just a few amino acids, the acylation of tRNA species accepting those amino acids increased. The increased collagen synthesis in rat granulation tissue is accompanied by an increase in proline accepting activity (Lanks and Weinstein, 1970). In the work presented in this paper, demonstration that there is no increase in the synthesis of specific tRNA species at a time when these species are increasing in amount suggests that mechanisms other than synthesis may be important in regulating specific tRNA levels.

References

Allerton, S. E., and Perlmann, G. E. (1965), *J. Biol. Chem. 240*, 3892.

Beck, G., Hentzen, D., and Ebel, J. (1970). *Biochim. Biophys. Acta 213*, 55.

Bernhardt, D., and Darnell, J. E. (1969), *J. Mol. Biol. 42*, 43.

Billing, R. J., Barbirolli, B., and Smellie, R. M. S. (1969), *Biochim. Biophys. Acta 190*, 60.

Burdon, R. H. (1971), *Progr. Nucl. Acid Res. Mol. Biol. 11*, 33.

Burdon, R. H., and Clason, A. E. (1969), *J. Mol. Biol. 39*, 113.

Burdon, R. H., Martin, B. T., and Lal, B. M. (1967). *J. Mol. Biol. 28*, 357.

Busby, W. F., and Hele, P. (1970), *Biochim. Biophys. Acta 224*, 413.

Chin, R., and Kidson, C. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 2448.

Choe, B. K., and Taylor, M. W. (1972), *Biochim. Biophys. Acta 272*, 275.

Coolsma, J. W. T., and Gruber, M. (1968), *Biochim. Biophys. Acta 169*, 306.

Garel, J. P., Mandel, P., Chavancy, G., and Daillie, J. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 7*, 327.

Greengard, O., Gordon, M., Smith, M. A., and Acs, G. (1964), *J. Biol. Chem. 239*, 2079.

Hacker, B. (1969), *Biochim. Biophys. Acta 186*, 214.

Hahn, W. E., Schjeide, O. A., and Gorbman, A. (1969), *Proc. Nat. Acad. Sci. U. S. 62*, 112.

Hanoune, J., and Agarwal, M. K. (1970), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 11*, 78.

Heald, P. J., and McLachlan, P. M. (1965), *Biochem. J. 87*, 571.

Klyde, B. J., and Bernfield, M. R. (1973), *Biochemistry 12*, 3752.

Lanks, K. W., and Weinstein, I. B. (1970), *Biochem. Biophys. Res. Commun. 40*, 708.

Mäenpää, P. H. (1972), *Biochem. Biophys. Res. Commun. 47*, 971.

Mäenpää, P. H., and Bernfield, M. R. (1969), *Biochemistry 8*, 4926.

Mays, L. L., and Borek, E. (1971), *Biochemistry 10*, 4612.

Quincey, R. V., and Wilson, S. H. (1969), *Proc. Nat. Acad. Sci. U. S. 64*, 981.

Sarin, P. S., and Zamecnik, P. C. (1964), *Biochim. Biophys. Acta 91*, 653.

Schimke, R. T., and Doyle, D. (1970), *Annu. Rev. Biochem. 39*, 929.

Sheid, B., Bilik, E., and Biempica, L. (1970), *Arch. Biochem. Biophys. 140*, 437.

Shugart, L., Chastain, B. H., Novelli, G. D., and Stulberg, M. P. (1968), *Biochem. Biophys. Res. Commun. 31*, 404.

Tomkins, G. M., and Martin, D. W. (1970), *Annu. Rev. Genet. 4*, 91.

Vaughan, M. H., Soeiro, R., Warner, J. R., and Darnell, J. E. (1967), *Proc. Nat. Acad. Sci. U. S. 58*, 1527.

Amino Acid Sequence of a κ Bence Jones Protein from a Case of Primary Amyloidosis†

Frank W. Putnam,* E. J. Whitley, Jr.,‡ C. Paul,§ and J. N. Davidson¶

ABSTRACT: Amino acid sequence analysis has been done on a κ Bence Jones protein (Tew) from a case of primary amyloidosis with the objective of determining the sequence of the variable region. Twenty-two tryptic peptides accounting for 182 residues were isolated and were completely or partially sequenced. Chymotryptic digestion yielded 32 peptides which supplied many overlaps. Sequenator analysis was performed for the first 42 residues of the amino terminus. From the combined data the sequence of the variable region (residues 1–108) was deduced. The composition, partial sequence data, and alignment of the peptides of the constant region (residues 109–214) correspond exactly to the sequence established for human κ light chains of the same allotype. The V region includes five extra residues (30a–30e) and is characteristic of the κII subgroup. A computer analysis of the sequence of the Tew Bence Jones protein in comparison with other human κ light chains was undertaken to establish quantitative criteria for subgroup classification. In terms of minimum nucleotide mutations the Tew protein differs from other subgroup κII proteins by an average of only 0.2 base/amino acid residue position, whereas other human κ chains differ from Tew by an average of 0.4–0.6 base/position. The Bence Jones protein and the tissue amyloid protein from this patient appear to be identical in primary structure as indicated by identity in the amino-terminal sequence for 27 residues and similarity in peptide maps, amino acid composition, and other properties.

Since our report of the first complete amino acid sequence of a human κ Bence Jones protein (Putnam *et al.*, 1966), almost 5000 residue positions have been reported for other human light chains; yet, no two human light chains have yet proved to be identical in sequence. Each of the two types of light chains, κ and λ, has been shown to be divided almost precisely in half into a variable (V) region comprising the NH_2-terminal half and a constant (C) region comprising the COOH-terminal half (Putnam, 1969). The C region containing residues 109–214 is identical in sequence for all human κ light chains except for an inherited variation at position 191, where either valine or leucine may be present according to the allotype. In contrast, the V regions of any two human κ chains differ by a minimum of about ten and a maximum of about 60 residues. Human λ light chains conform to a similar principle of structure, as do the light chains of most other species. Nonetheless, some human κ chains are more related in amino acid sequence in their V regions than are others. Such structurally related light chains are said to belong to the same subgroup. Three such subgroups have been proposed for human kappa light chains, *i.e.*, κI, κII, and κIII (Putnam, 1969; Milstein, 1969; Hilschmann, 1969).[1] However, the subgroups have been defined arbitrarily and are based largely on short NH_2-terminal sequences rather than on complete sequences of the V region. Since the existence of subgroups is a fundamental postulate of several theories of antibody diversity, a more exacting analysis is needed of the subgroup classification of a series of light chains that have been completely or nearly completely sequenced. This article presents the proof for the amino acid sequence of a human Bence Jones protein (Tew) which belongs to the κII subgroup, and discusses the sequence relationship of this light chain to other κ light chains. This Bence Jones protein was isolated from the urine of a patient who died of primary amyloidosis (Case 14 of Osserman *et al.*, 1964), and the sequence of the Bence Jones protein and of the amyloid protein isolated from his tissues is identical as far as the latter could be determined (Terry *et al.*, 1973).

Experimental Procedure

Bence Jones protein Tew was precipitated from the urine of the patient by adjustment to 60% ammonium sulfate and was purified by use of a DEAE-Sephadex A-50 column (Bernier

† From the Department of Zoology, Indiana University, Bloomington, Indiana 47401. *Received April 24, 1973.* This work was supported in part by grants from the National Institutes of Health (CA 08497), the American Cancer Society (NP-10), and the Damon Runyon Fund for Cancer Research (DRG 1134). The manual sequence data are documented in the M.S. thesis of E. J. W. (1969).

‡ Present address: Department of Biochemistry, University of Minnesota, Saint Paul, Minn. 55101.

§ Present address: Laboratoire de Chimie générale, Université Libre de Bruxelles, Belgium.

¶ Present address: Biology Department, Harvard University, Cambridge, Mass. 02138. National Institutes of Health trainee supported by GM-00036.

[1] For nomenclature of human κ chain subgroups see the Discussion and also statements in *Bull. W. H. O.* [(1969), *41*, 975] and in *Biochemistry* [(1972), *11*, 3311].