Thyroid Ribonucleic Acid–Iodopeptides. Purification and Properties of a Thyroxine-Containing Complex from Porcine Thyroid†

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ABSTRACT: Complexes which contain RNA and iodoproteinaceous components are present in close association with the tRNA of mammalian thyroids. The complexes present in the tRNA fraction of porcine thyroid were fractionated from all or the bulk of the tRNA by chromatography on benzoylated DEAE-cellulose. The fraction containing the RNA–iodoproteinaceous complexes was shown to contain at least three components of very similar composition and properties. Preliminary analysis of the total fraction showed it contains orcinol-reactive material (presumably ribose), phosphorus, iodine, proteinaceous elements, and carbohydrate in addition to ribose. Amino acid analysis revealed that the proteinaceous portion of the complex contains significant quantities of eleven amino acids; fewer basic than acidic residues are present and the common aromatic amino acids are lacking. Thyroxine was the only iodinated component present in, and identified from, proteolytic digests of the complex. Based on total iodine content and an average molecular weight of 20,000 the complex could contain as much as 1 mol of thyroxine/2 mol of complex. Fractionation of tRNA from the complexes permitted experiments to examine if the complexes behave as tRNA under aminoacylation conditions and if the presence of the complexes affects the aminoacylation of tRNA. Two subfractions of the complex, tested at concentrations approximating those of tRNA, showed significant incorporation of seven of fifteen amino acids into cold trichloroacetic acid insoluble–hot trichloroacetic acid soluble form. As in the case of aminoacylation of tRNA, incorporation was abolished by pancreatic ribonuclease, was inhibited by inorganic pyrophosphate and was dependent on magnesium ion and ATP. The aminoacylation of thyroid tRNA, free of complex, was compared with that of tRNA plus complex added back at normal tRNA:complex ratios. Aminoacylation, of 11 of the 15 amino acids tested, was inhibited more than 10% in the presence of the complexes; the aminoacylation of aspartic acid was inhibited 47%. Little effect of the complex was observed on the aminoacylation of leucine, threonine, and histidine. In contrast to its inhibitory effects, the aminoacylation of isoleucine was stimulated in the presence of the complexes. The degree of stimulation varied from 48% to as much as 400% depending on the time of storage of the synthetase fraction and the concentration of the complex. The presence of the hormone-containing complexes in the thyroid, their close association with tRNA, and the varied effects on aminoacylation of tRNA, suggest, among other possibilities, that the complexes could play a role in control of protein biosynthesis at the translational level.

Ribonucleic acid–iodoproteinaceous complexes have been reported to be associated with the tRNA fraction of bovine, ovine, and porcine thyroid (Kull *et al.*, 1965; Kull, 1967; Kull and Soodak, 1971). The complexes were first observed in tRNA fractions prepared from calf thyroid glands using the procedure of Brunngraber (1962) and were shown to contain amino acids, iodine, and RNA components. Degradative experiments provided evidence that the complex was maintained by covalent bonds. Diiodotyrosine was identified from hydrolysates and the hormone thyroxine was tentatively identified. The complex was degraded by pancreatic ribonuclease and snake venom diesterase to components having iodine and amino acids and a higher molecular weight nuclease-resistant RNA-containing core (Kull and Soodak, 1971).

Experiments concerning the biological role of the calf thyroid RNA–iodoproteinaceous complex were complicated by the tenacity with which the complex remained associated with tRNA. Neither chromatography, electrophoresis, nor protein denaturants affected its association with tRNA.

We are studying the complexes present in the tRNA fraction of porcine thyroid and have achieved their separation from tRNA by the use of benzoylated DEAE-cellulose (BD-cellulose).[1] Thyroxine has been isolated from the highly purified complex which also contains carbohydrate other than the ribose of the RNA portion.

At concentrations comparable to tRNA the purified complex appears to incorporate 7 of the 15 amino acids tested into covalent linkage labile to hot trichloroacetic acid. Incorporation is dependent on magnesium ion, ATP and the presence of RNA.

At much lower concentrations the complex affects the aminoacylation of 12 of the 15 amino acids tested. Effects vary from pronounced inhibition to significant stimulation in the case of the aminoacylation of isoleucine.

Experimental Section

Materials. Pig thyroid glands were obtained frozen from

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[1] Abbreviations used are as listed in *Biochemistry 5*, 1445 (1966), with the following exceptions: BD-cellulose, benzoylated-DEAE-cellulose; "Y," the fluorescent base adjacent to the 3'-OH terminus of the anticodon region of the $tRNA^{Phe}$ from several species. The structure of this nucleoside has been identified by Nakanishi *et al.* (1970).

Pel-Freeze Biologicals, Inc. (Rogers, Ark.), and were stored at −75° until fractionated.

Whatman DE-23 was precycled and equilibrated as recommended by the manufacturers. BD-cellulose purchased from Gallard-Schlesinger Chemical Mfg. Corp. (Carle Place, N. Y.) was washed exhaustively with 40% ethanol–10% benzyl alcohol–1.0 M LiCl, 95% ethanol, water, and then 2.0 M NaCl. It was stored as suggested by Gillam and Tener (1971). G-25, G-50 Sephadex, DEAE A-50 Sephadex, and LH-20 were purchased from Pharmacia Fine Chemicals Inc. (Piscataway, N. Y.). L-[^{14}C]Amino acids were purchased from Schwarz BioResearch (Orangeburg, N. Y.) or New England Nuclear Corp. (Boston, Mass.). Pancreatic ribonuclease A (RAF) was purchased from Worthington Biochemical Corp. (Freehold, N. J.) and Pronase, B grade, from Calbiochem (Los Angeles, Calif.). All other reagents were obtained commercially and used without further purification.

Isolation of Thyroxine from Complex II. Total complex II was incubated with Pronase as previously described (Kull and Soodak, 1971). After incubation for 24 hr the mixture was dialyzed against 250 ml of water at room temperature for 8 hr. The dialyzable material was dried by rotary evaporation at 37° and then dissolved in 1.0 ml of ethyl acetate–methanol–2 N NH_4OH (400:100:40, v/v). The fraction was applied to a LH-20 column and iodinated amino acids were separated using the procedure of Williams *et al.* (1969). Fractions, detected by iodine assay (R. A. Gadski and F. J. Kull, unpublished data), which corresponded in position of elution to thyroxine standards, were concentrated by rotary evaporation and the presence of thyroxine in the fraction established by paper chromatography relative to standards (ascending, Whatman No. 3MM) using butanol–acetic acid–water (78:5:17, v/v). Iodine-containing spots were detected by the method of Gmelin and Virtanen (1959).

Aminoacyl-tRNA Synthetase. Frozen pig thyroids were sliced and homogenized in two volumes of a buffer composed of 0.05 M Tris-HCl (pH 7.5), 0.01 M 2-mercaptoethanol, and 10% glycerol (all work done at 4°). Following filtration through cheesecloth presoaked in buffer, the preparation was centrifuged at 12,000*g* for 20 min. The supernatant was removed, again filtered through cheesecloth, centrifuged at 105,000*g* for 1.5 hr, and the supernatant was applied to a DEAE-cellulose column preequilibrated in the homogenizing buffer. The column was washed until the A_{280} of the eluate was below 0.2 A_{280}/ml and the synthetase fraction was eluted with 0.05 M Tris-HCl (pH 7.5), 10% glycerol, 0.01 M 2-mercaptoethanol, and 0.3 M KCl. The 5-ml fractions were then assayed for aminoacyl-tRNA synthetase activity using thyroid tRNA and each of the amino acids used in this work. Fractions with maximal activity served as sources of aminoacyl-tRNA synthetases and were stored at −20°.

The partially purified pig thyroid leucyl- and tyrosyl-tRNA synthetases used for the experiments described in Table IV were gifts of Mr. W. Curatolo and Mr. J. Goodman, respectively.

Aminoacylation Reactions. Aminoacylation reactions were carried out in a final volume of 0.15 ml. Except where noted otherwise, final concentrations of components in all incubation mixtures were: 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 2.5 mM ATP, and each contained 1 μCi/ml of L-[^{14}C]Amino acid. The tRNA and complex II concentrations varied from experiment to experiment in the range of 2.1–13.5 A_{260}/ml. Either 25 or 50 μl of synthetase fractions was added to initiate reactions incubated at 25°. Radioactivity was measured by a Packard Tri-Carb liquid scintillation spectrometer Model 3375 following the procedure of Bollum (1959). (Details are given in figure and table legends.)

The presence of complex II was qualitatively monitored throughout this investigation by absorbance at 320 nm. Iodinated phenolic compounds are known to have significant absorbance at this wavelength (Gemmill, 1955; Edelhoch, 1962). Kull and Soodak (1971) pointed out that the iodine content and 320-nm absorption appeared to be proportionately related in the calf thyroid complex which they studied.

Purification of the RNA–Iodoproteinaceous Complex. Porcine thyroid glands were sliced while frozen and ground in a commercial meat grinder. Two volumes of a buffer composed of 0.2 M NaCl, 0.05 M Tris-HCl (pH 7.5), and 0.005 M EDTA were added per g of ground thyroid and the mixture was homogenized in a Waring Commercial blender by six, 10-sec bursts. The homogenate was filtered through a double layer of buffer-soaked cheesecloth and centrifuged at 12,000*g* for 20 min.

The postmitochondrial supernatant was decanted through cheesecloth and adjusted to 0.5% sodium dodecyl sulfate; one-third volume of cold, neutralized 87% phenol was added and the mixture was homogenized in the Waring blender for 20 min. Following centrifugation at 10,000*g* for 30 min the aqueous phase was removed and the pH was adjusted to 5.0–5.5, while stirring, by dropwise addition of acetic acid. After addition of three volumes of ethanol the total mixture was made 0.01 M barium acetate and refrigerated overnight. The precipitate that formed was removed by centrifugation and to ensure quantitative recovery the supernatant was filtered. The combined precipitates were suspended in 0.1 M Na_2SO_4 by homogenization using a hand homogenizer and $BaSO_4$ was removed by centrifugation. The supernatant was adjusted to 0.01 M Tris-HCl (pH 7.5) and 0.5% sodium dodecyl sulfate and the fraction was mixed vigorously with one-half volume of neutralized 87% phenol for 20 min followed by centrifugation for 30 min at 10,000*g*. The aqueous phase was carefully removed and the phenol treatment was repeated until neither interfacial material nor precipitate was observed. The final aqueous phase was removed, diluted in half, and applied directly to a DEAE-cellulose column previously equilibrated with 0.01 M Tris-HCl at pH 7.5. The column was washed with 0.15 M NaCl, 0.01 M Tris-HCl, and 0.001 M EDTA until the effluent absorbance was negligible. Washing with the same buffer plus 0.4 M NaCl removes a low molecular weight complex (complex I)[2] and elution with 1.0 M NaCl in the same buffer removed tRNA and complex II.

The 1.0 M NaCl eluate was adjusted to 0.2 M sodium acetate (pH 5.5), and three volumes of ethanol was added. The resulting precipitate was collected by centrifugation and dissolved in 0.3 M sodium acetate (pH 7.0). The fraction was further purified according to the procedure of Zubay (1962) using isopropyl alcohol fractional precipitation.

Deacylation of the fraction was carried out either following the procedure of Nathans and Lipmann (1961) or that of Kelmers *et al.* (1965). On occasion the deacylation procedure was repeated. The results we will describe were unaffected either by the method of deacylation or by its repetition. Tris-HCl or glycine buffer was removed by dialysis first against water and then against 0.01 M sodium acetate (pH 5.5) and 0.001 M $MgCl_2$.

The fraction was next applied to a BD-cellulose column

[2] F. J. Kull and R. A. Gadski, 1973, submitted for publication.

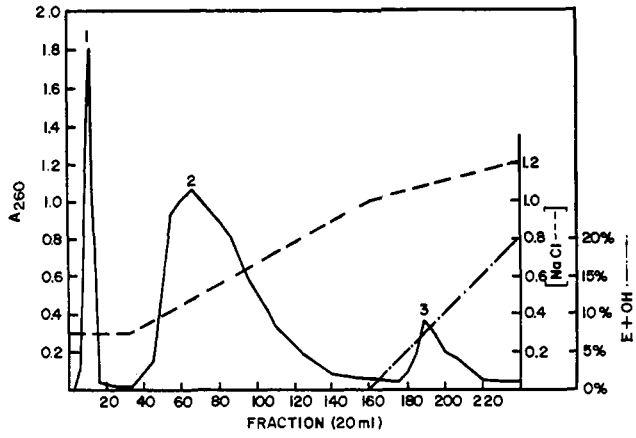

FIGURE 1: Chromatography of porcine thyroid tRNA on BD-cellulose. The tRNA–complex II fraction was prepared and deacylated as described in the Experimental Section and applied to the BD-cellulose column (1.2 × 15 cm). The column and tRNA fraction were both in 0.01 M sodium acetate (pH 5.5) and 0.001 M $MgCl_2$. After addition of the tRNA–complex II fraction the column was washed with 500 ml of the same buffer. The column was then eluted as shown in the figure; first by buffer plus 0.3 M NaCl, then by a linear NaCl gradient (0.3–1.0 M), and finally by simultaneous linear gradients of 1.0–1.2 M NaCl and 0–20% ethanol.

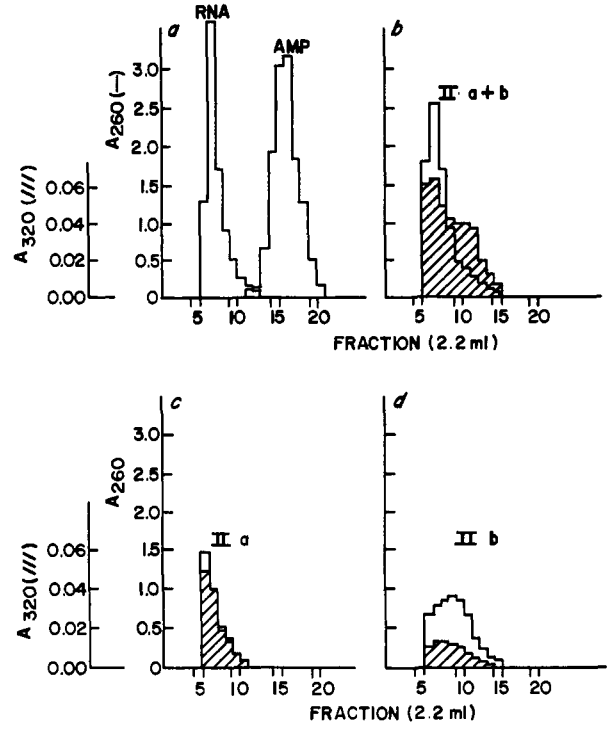

FIGURE 2: Chromatography and fractionation of complex IIa + b on Sephadex G-50. The complex II fraction obtained from BD-cellulose by 1.5 M NaCl, 25% ethanol, 0.01 M sodium acetate (pH 5.5), and 0.001 M $MgCl_2$ elution, was chromatographed on a Sephadex G-50 column (1 × 42 cm). Chromatography was carreid out in 0.01 M Tris-HCl (pH 7.5) and 0.2 M NaCl. tRNA and 2′(3′)AMP were used to determine the void and retained volumes, respectively, of the column (a). Following chromatography of fraction IIa + b (b), fractions 6–9 from b were combined, concentrated, and rechromatographed on the same column (c). Fractions 10–20 from b were also combined, concentrated, and rechromatographed (d).

that was prepared and equilibrated according to the procedure of Gillam *et al.* (1967). Chromatography was carried out as described in Figure 1.

Results

Purification of the RNA–Iodoproteinaceous Complex. The procedure described for the purification of complex II allows the fractionation of relatively large amounts of thyroid tissue in as short a period of time as possible and provides rapid and thorough deproteinization of the RNA without loss of either complex II, or the low molecular weight complex I which will be described elsewhere.[2] The procedure also provides for homogenization of thyroid glands while still frozen and eliminates difficult homogenization usually encountered with conventional tissue grinding techniques (caused by the high content of fascia in thyroid). Homogenization is carried out in 0.2 M NaCl buffer to inhibit possible nucleases (Krawiec and Eisenstadt, 1970). By the use of only one-third volume of phenol for the original phenol treatment, occasional and unpredictable problems in emulsion breaking that were encountered at greater phenol concentrations were avoided. Ethanolic precipitation in the presence of barium ions is necessary to quantitatively precipitate complex I and has the added advantage of decreasing the number of subsequent phenol treatments. The latter observation appears to be a result of the insolubility of protein–barium complexes in sodium sulfate and/or increased denaturation of protein by ethanol in the presence of barium ions. Usually a single phenol treatment is sufficient to remove all vestiges of phenol–sodium dodecyl sulfate precipitable protein after barium–ethanol precipitation. However, at least two such treatments were usually performed to ensure as complete as possible removal of contaminating protein at this early stage of purification.

Chromatography of the tRNA–complex fraction of BD-cellulose as shown in Figure 1 resulted in the separation of all or the bulk of tRNA from the complex. The elution pattern shows two minor (peaks 1 and 3) and one major peak of A_{260}-absorbing material. Peak 2 contains the majority of the fifteen species of tRNA for which we tested, whereas peaks 1 and 3 contained insignificant amounts of active tRNA and were not present in all preparations. Material eluting at positions corresponding to peaks 1 and 3 have been noted by other workers (Henes *et al.*, 1969) and are believed to be degraded rRNA and/or inactive tRNA. *N*-Phenoxyacetylaminoacyl-tRNA complexes as well as the tRNA esters of aromatic amino acids and tRNAs containing "Y base" have been found to elute from BD-cellulose in positions which correspond to peak 3 (Gillam *et al.*, 1967; Thiebe and Zachau, 1968; Fink *et al.*, 1971).

Under the conditions shown in Figure 1 no RNA–iodoproteinaceous complex was eluted from the column as judged by the absence of absorbance at 320 nm.

Following the conditions described in Figure 1, 25–30% of the total A_{320}-absorbing material applied to the column (complex IIa and IIb) could be eluted by washing the column with 25% ethanol, 1.5 M NaCl, 0.01 M sodium acetate (pH 5.5), and 0.001 M $MgCl_2$. Chromatography of this material on DEAE-Sephadex A-50 resulted in no further fractionation. However, on Sephadex G-50 a trailing shoulder was observed (Figure 2b). Rechromatography, of fractions pooled from the first column, on the G-50 column showed the fraction to be composed of at least two subfractions; a major fraction, IIa (Figure 2c), and a minor fraction, IIb (Figure 2d), which corresponded to the trailing shoulder.

TABLE I: Chemical Composition of the Total Complex II Fraction.

Analysis	$\mu g/A_{260}$ Yeast RNA	$\mu g/A_{260}$ Complex II	Standard	Reference
		Part A		
Total phosphorus	3.27	1.47	Yeast RNA, KH_2PO_4	Fisk and Subbarow (1925)
RNA (as 9.4% phosphorus)	35.70	15.70	Yeast RNA	
RNA (orcinol assay)	35.70	17.00	Yeast RNA	Schneider (1957)
Total carbohydrate	15.70	12.80	Yeast RNA	Dubois *et al.* (1956)
DNA	0	0	Porcine thyroid DNA	Dische (1930)
Protein	0	37.00	Bovine serum albumin	Miller (1959)
Iodine	0	0.70	Diiodotyrosine and KI	Bennotti and Bennotti (1963)

Component	% Composition	Daltons/Mol Wt[a]	Component (Residues/Mol)[b]
	Part B		
RNA	26.80	5,350	18.0
Carbohydrate (−ribose)	11.60	2,330	13.0
Protein	60.50	12,100	111.0
Iodine	1.14	230	1.8

[a] The average molecular weight of the complex II fraction was estimated to be 20,000 by Sephadex chromatography. [b] Average residue weights used in these calculations were: nucleotide, 300; monosaccharide, 180; amino acid, 110; iodine, 127.

TABLE II: Amino Acid Analysis of Total Complex II Fraction.[a]

Amino Acid	nmol of Amino Acid/A_{260}
Lysine	18.8
Aspartic acid	11.8
Threonine	34.4
Serine	10.2
Glutamic acid	15.0
Proline	12.6
Glycine[b]	15.5
Alanine	67.0
Methionine	34.7
Isoleucine	3.1
Leucine	9.3

[a] Amino acid analyses were performed using a Beckman Amino Acid Analyzer, Model 120B on samples hydrolyzed in 6 N HCl at 105° for 24 hr in sealed, evacuated tubes. Tryptophan, glutamine, and asparagine were not determined. Other amino acids not listed were either absent or present at less than 0.1-nmol quantities. [b] This value is possibly not quantitative because of glycine being produced during acid hydrolysis of purines.

The elution of the remainder of complex II (IIc) from the BD-cellulose required the use of a solvent composed of 40% ethanol, 10% benzyl alcohol, and 1.0 M LiCl.

Simultaneous and quantitative elution of all three complexes from BD-cellulose could be achieved by the ethanol–benzyl alcohol–LiCl solvent. Other solvents which we tested which were less hydrophobic and/or of less ionic strength failed to completely remove the 320-nm-absorbing material. The ethanol–benzyl alcohol–LiCl solvent rapidly dehydrates BD-cellulose and markedly decreases flow rates. We have found that better and more rapid results are obtained if the packing is removed from the column after the final NaCl–ethanol gradient and the complex is eluted from the BD-cellulose by stirring with the ethanol–benzyl alcohol–LiCl solvent followed by centrifugation.

After elution from BD-cellulose the complex II fractions can be concentrated by ethanol precipitation at pH 5.5 and then dialyzed first against water and then against 0.01 M Tris-HCl (pH 7.5)–0.001 M $MgCl_2$.

The total amount of complex isolated by the above technique is approximately 50 A_{260} units/pound of thyroid gland (wet weight). Because of the even lower amounts of the subfractions, chemical analyses were carried out on the entire complex II fraction rather than on each subfraction. However, various experiments concerned with the biological role of the complex could be examined with either IIa+b or IIc or both.

Preliminary Chemical Analysis of Complex II. The chemical analyses, methods of analyses and calculated composition of complex II are shown in Table I. The complex contains phosphorus, carbohydrate in addition to ribose, protein, and iodine and is orcinol reactive. DNA, as judged by the diphenylamine reaction, is absent. Based on the assumption that RNA is 9.4% phosphorus by weight, it is evident there is close agreement between the phosphorus content of complex II and its orcinol reactivity. Additional calculations show that there is more carbohydrate present than could be accounted for based solely on ribose constituting 40% of the total weight of RNA. The nature of the additional carbohydrate is unknown at the present but we are considering that it might be present as a glycoid unit attached to either the peptide or nucleotide portions of the complex.

Table IB shows the per cent composition, daltons per component, and the numbers of residues per mole of complex. The calculations are based on the assumptions that the complex contains only the components listed in Table IA and has an average molecular weight of 20,000 daltons (estimated by Sephadex chromatography). By examination of these figures the fraction could be considered to be composed of an iodinated glycoprotein (peptide) portion and an oligonucleotide (RNA) portion.

Amino Acid Content of Complex II. The amino acid content of the complex is presented in Table II and varies from that reported previously (Kull and Soodak, 1971) with respect to absolute quantitation, but as previously reported the porcine complex is low in histidine, arginine, and the sulfur amino acids; tyrosine and phenylalanine are virtually absent. In addition, acidic residues are in greater abundance than basic residues.

Pronase digests of the purified complex II were carried out as previously described (Kull and Soodak, 1971) and in the Experimental Section.

By these experiments thyroxine was established as part of the complex and is present in isolatable amounts. This result confirms our earlier observations on the calf thyroid complex, but differs in that diiodotyrosine was absent in hydrolysates from the purified porcine complex II fraction.

Effects of Complex II on Aminoacylation Reactions. Studies concerning possible biological functions of the calf thyroid complex were hindered because it could not be separated from tRNA (Kull and Soodak, 1971). These complications, in part, have been overcome by the successful separation of pig thyroid complex II from all or the bulk of tRNA.

Apparent aminoacylation of complex IIa+b and IIc is shown in Table III. In experiment 1 complex IIa+b was tested under aminoacylation conditions with a mixture of aminoacyl-tRNA synthetases active in aminoacylation of 15 separate amino acids. Porcine thyroid tRNA free of complex (as judged by absence of 320-nm absorbance) served as a control for establishment of the presence of active enzymes and for comparative purposes. Incorporation of amino acid into cold trichloroacetic acid form was observed for seven amino acids. Complex IIc was able to accept the same seven amino acids.

In expt 2 (Table III) results are shown which were obtained when aminoacylation reactions were carried out under conditions in which tRNA was limiting. In this experiment it can be seen that complexes IIa+b and IIc incorporate different amounts of the seven amino acids tested. In an attempt to rationalize the apparent aminoacylation of complex II with the severe conditions required to effect its elution from BD-cellulose it is possible to theorize that the complex II fraction contained aminoacylated tRNA which could be tightly adsorbed to BD-cellulose and not elute under normal conditions. The aminoacylation we observed could then be explained as the result of an exchange reaction between the cold aminoacyl-tRNA and radioactive amino acid. To test this hypothesis fraction IIa+b was again deacylated with 0.5 M Tris-HCl (pH 8.8) and rechromatographed on BD-cellulose as described in Figure 1. The results obtained after repeating the aminoacylation experiment were the same as before. In addition, similar results were obtained when 0.2 M glycine buffer (pH 10.3) was used instead of Tris-HCl for the deacylation step.

A separate experiment performed in the presence or absence of pancreatic ribonuclease showed the incorporation of the seven amino acids tested in expt 2 (Table III) to be RNA dependent. In addition, radioactivity was not trichloroacetic acid soluble which tends to rule out the possibility of non-ribosomal-mediated incorporation into peptide linkage as has been described from various organisms and tissues including sheep and bovine thyroid (Kaji *et al.*, 1965; Soffer, 1966; Strycharz, 1969). While the possibility of RNA-dependent incorporation into hot acid-soluble peptides cannot be excluded at this time, it should be pointed out that tRNA was not required for incorporation of amino acids into hot acid-insoluble linkage by the non-ribosomal, bovine, thyroid system studied by Strycharz.

Table IV compares the apparent aminoacylation of complex II with complex-free tRNA using the two amino acids most actively incorporated into complex II; tyrosine and leucine. Both aminoacylation of tRNA and incorporation into complex II responded qualitatively alike to all variables or additions tested which suggests both reactions follow similar paths. The major differences were in the degree of magnesium ion dependence and the degree of inhibition by inorganic pyrophosphate. Inorganic pyrophosphate at 3.3 mM inhibited the incorporation of leucine only 6–10%, however, at 10 mM the incorporation of tyrosine was essentially eliminated. Finally, in both experiments decreasing the 2-mercaptoethanol concentration to 0.83 mM showed incorporation into complex II to be decreased relative to that into tRNA.

TABLE III: Aminoacylation of Porcine Thyroid tRNA and Apparent Aminoacylation of Complex II Fractions.[a]

	pmol/A_{260}				
	Expt 1		Expt 2		
Amino Acid	tRNA	Complex IIa+b	tRNA	Complex IIa+b	Complex IIc
Ala	32.80	0.80			
Arg	57.20	4.70	13.20	7.45	25.00
Asp	29.7	0.62			
Glu	19.6	0.44			
Gly	54.2	0.73			
His	24.2	4.33	7.13	0	4.10
Ile	22.7	0.89			
Leu	28.1	29.60	7.55	2.90	9.30
Lys	44.5	2.50	8.40	8.50	12.40
Phe	41.8	11.45	11.60	8.55	13.70
Pro	20.6	0.95			
Ser	41.7	2.96	11.4	10.35	5.15
Thr	8.7	0.67			
Tyr	48.0	32.80	14.3	22.8	15.10
Val	15.6	1.35			

[a] Incubations were for 20 min at 25° and were as described in the Experimental Section. In expt 1, 2.0 A_{260} units of tRNA (peak 2, Figure 1; free of 320-nm absorbance) or 1.2 A_{260} units of complex IIa+b was present. In expt 2, tRNA or complex was present at 0.326 A_{260} unit/incubation. The L-[^{14}C]amino acids all had equal specific activities of 50 Ci/mol and counting efficiency was 50%.

Possible effects of complex II on the aminoacylation of tRNA were examined as described in the Experimental Section and Table V. Reactions were carried out to compare aminoacylation of tRNA alone and tRNA in the presence of complex II at a ratio of A_{260} of tRNA:A_{320} complex II of 15:1; that present prior to BD-cellulose chromatography. Table V shows that the aminoacylation of all but three amino acids was affected by the presence of complex IIa+b. Aminoacylation of eleven amino acids was inhibited greater than 10%; in the case of aspartic acid inhibition was nearly 50%. In contrast the aminoacylation of isoleucine was significantly stimulated.

It should be pointed out that the conditions for aminoacylation were not necessarily optimal for each of the amino acids tested. Therefore, there could be differences in degree or even sign under optimal conditions.

Table VI shows the effect of different concentrations of complex IIa+b on the aminoacylation of threonine and isoleucine to tRNA. In agreement with the results of Table V there was no obvious effect of complex IIa+b on the aminoacylation of threonine at even the highest concentration of the complex. However, all levels of the complex stimulated the

TABLE IV: A Comparison of the Requirements for the Aminoacylation of Porcine Thyroid tRNA and Incorporation of Tyrosine and Leucine into Complex II.[a]

	pmol/A_{260}			
	L-[^{14}C]Tyrosine		L-[^{14}C]Leucine	
	tRNA	Complex II	tRNA	Complex II
Complete	2.26	1.61	7.80	4.23
Complete + inorganic pyrophosphate	0.26	0.24	7.35	3.80
Complete + ribonuclease A	0.08	0.12	0.12	0.12
−Synthetase	0.45	0.20	0.09	0.06
−tRNA or complex II	0.33	0.00	0.09	0.43
$-MgCl_2$	1.18	0.35	1.54	0.81
−ATP	0.53	0.43	0.44	0.35
−2-Mercaptoethanol[b]	2.67	1.43	8.05	3.65

[a] Incubations (60 min at 25°) were as described in the Experimental Section and contained either 0.150 A_{260} unit of tRNA (peak 2, Figure 1; free of 320-nm absorbance) or 0.150 A_{260} unit of unfractionated complex II in a volume of 0.075 ml. Inorganic pyrophosphate was present in one series from initiation of reaction at a final concentration of 10 mM (tyrosine) or 3.3 mM (leucine). In another series ribonuclease A was added after 45 min to a final concentration of either 257 μg (tyrosine) or 200 μg (leucine) per ml. The specific activities of the L-[^{14}C]tyrosine and L-[^{14}C]leucine were 460 and 311 Ci per mol, respectively. Counting efficiency was 50%. [b] A small amount of 2-mercaptoethanol was introduced into this series with the partially purified synthetases (final concentration 0.83 mM).

TABLE V: Effect of Complex IIa+b on the Aminoacylation of Porcine Thyroid tRNA.[a]

	pmol/A_{260}		
Amino Acid	tRNA	tRNA + Complex IIa+b	% Control
Ala	43.2	33.6	78
Arg	69.0	61.0	88
Asp	25.7	13.5	53
Glu	18.5	15.2	82
Gly	69.5	50.0	72
His	25.7	24.1	94
Ile	8.6	12.7	148
Leu	4.9	4.7	96
Lys	44.5	40.0	90
Phe	46.4	33.2	72
Pro	22.4	16.2	72
Ser	55.7	42.4	76
Thr	12.4	12.2	98
Tyr	62.7	52.8	84
Val	36.0	22.2	62

[a] Incubations (20 min at 25°) were as described in the Experimental Section and contained either 2.0 A_{260} units of tRNA (peak 2, Figure 1; free of 320-nm absorbance) or 2.0 A_{260} units of tRNA and 0.133 A_{320} unit of complex IIa+b. The specific activities of the amino acids were all 50 Ci/mol and counting efficiency was 50%.

aminoacylation of isoleucine over that obtained with tRNA alone. At a ratio of tRNA:complex IIa+b of 15:1, stimulation in the latter case is 400% that of the control. This result (in which the same synthetase fraction used in the experiment in Table V was used) and other experiments is suggestive that the stimulation is related to the "age" of the synthetase fraction. Complex IIc also affects the aminoacylation of isoleucine and stimulates both the rate and extent of its aminoacylation.[3]

Discussion

Amino acids and/or peptides have been reported to be found in close association with purified DNA and RNA from yeast, *E. coli*, chick embryo, bovine pancreas, and human spleen, as well as thyroid (Akashi *et al.*, 1958; Prokof'ev *et al.*, 1960; Bendich *et al.*, 1964; Hall, 1964; Olenick and Hahn, 1964; Holoubek, 1966; Levine and Van Vunakis, 1966; Bichowsky-Slomnicki, 1969; Ishikura *et al.*, 1969; Kongsvik and Messineo, 1970; Marzloff *et al.*, 1972; Hall, 1965; Hall and Chedda, 1965; Kull *et al.*, 1965; Kull, 1967; Kull and Soodak, 1971).[2] In some of these cases, amino acids have been reported to be linked to nucleic acids by other than aminoacyl bonds (Bogdanov *et al.*, 1962; Hall, 1964; Ishikura *et al.*, 1969). The biological role of most of these complexes has not been investigated. However, workers have suggested possible roles for peptidyl or amino acid containing nucleic acids in: genetic function (Bendich *et al.*, 1964), protein recyclization (Harris and Wisemann, 1962), codon–anticodon interaction (Ishikura *et al.*, 1969), and derepression (Balis *et al.*, 1964).

Our study of RNA–iodoproteinaceous complexes found associated with the tRNA fraction of bovine, ovine, and porcine thyroids have added other examples of amino acids or peptides associated with nucleic acids. Definitive study of the thyroid complexes has been hindered up until now because of difficulty in separation of the complexes from tRNA by nondegradative methods. Whether this close association is a result of some form of interaction of the complexes with tRNA or is merely a result of close similarities between the two with respect to physical properties is not known. However, present evidence suggests that the association is probably the result of a combination of both these factors.

The method used for chromatography of the thyroid tRNA fraction on BD-cellulose has an advantage over other methods we have attempted in not only permitting the preparation of good quantities of tRNA free of complex, but also yields the complexes in reproducible quantities and high purity.

It is apparent that there are pronounced differences in the affinities of tRNA and the complexes for the BD-cellulose. These differences could be a result of the interaction of the proteinaceous portion of the complex with the adsorbent, perhaps through thyroxine residues, or through interaction of some modified base component of the RNA portion of the complex (Thiebe and Zachau, 1965; Fink *et al.*, 1971). Differential affinities of the various subfractions of complex II for BD-cellulose allowed their initial observation and appear to

[3] M. I. Levy and F. J. Kull, unpublished observations.

be the result of hydrophobic interactions rather than differences in ionic character. All fractions remain adsorbed to the BD-cellulose at high NaCl concentrations (1.2 M) in the presence of 20% ethanol. Furthermore, complex IIa+b, which can be eluted from the adsorbent by 1.5 M NaCl in 25% ethanol, is not fractionated by DEAE-Sephadex A-50, but is partially separated by G-50 Sephadex into two fractions.

The complex II fraction appears to contain at least three subfractions. We do not know at this time whether the subfractions are the result of: (1) autolytic or artifactual breakdown taking place during the lengthy preparative procedure, (2) minor differences in a class of related molecules, or (3) that the various subfractions represent stages of metabolism of a single molecule. Because of the results obtained in our experiments concerned with effects of the complex under aminoacylating conditions and on the aminoacylation reaction we favor the second alternative.

It should be stressed that the chemical analysis is only preliminary and was performed using the entire complex II fraction. However, the analysis is strongly suggestive that we are dealing with RNA–proteinaceous complexes of complicated makeup that are closely associated with tRNA. In addition the complexes could contain thyroxine as part of their protein components. To our knowledge this is the first example of an important animal hormone isolated from complexes such as we have described.

The possibility of contamination of the tRNA fraction by protein has been discussed previously (Kull, 1967; Kull and Soodak, 1971) and though considered unlikely remains a serious consideration. In addition to the reasons cited in our previous studies (Kull, 1967; Kull and Soodak, 1971),[2] we consider nonspecific contamination by protein unlikely for the following additional reasons. The tRNA fraction always contains complex II no matter which method of tRNA preparation we employ. Also, we consistently obtain very similar yields of the complex using the rigorous methods we have outlined here.

Both complex IIa+b and complex IIc fractions incorporate some amino acids into RNA-dependent, cold trichloroacetic acid insoluble–hot trichloroacetic acid soluble form. The possibility of contamination by traces of tRNA or aminoacyl-tRNA tightly adsorbed to BD-cellulose remains a possibility. However, we feel such an explanation is not very probable. Our strongest argument against this possibility is the data presented in Table III (expt 2). Under conditions where tRNA is limiting, the degree of incorporation seen with the complexes is greater than that of the tRNA alone in all but one case, histidine. In addition, repeated deacylation of the complex fraction or deacylation under different conditions of buffer, pH, and molarity followed by rechromatography on BD-cellulose, and the fact that most of the amino acids incorporated are not aromatic, argue strongly against the possibility that apparent aminoacylation of the complex II fractions is the result of contamination by tRNA or aminoacyl-tRNA.

We have not ruled out the possibilities that tRNA is bound to the proteinaceous component so tightly that the two behave as single molecules or that the apparent activity is a function of the complex II *per se*. Both of the latter possibilities seem reasonable.

The varied and pronounced effects that complex II exerts on aminoacylation of tRNA at low levels was unexpected and interesting. At present we cannot offer conclusive data differentiating between the possibilities that the effects we have observed are related to interactions between the complexes and tRNA, the aminoacyl-tRNA synthetase, some component in the incubation mixture (Mg^{2+}, ATP, etc.) or are due to the combination of these possibilities. Present data concerning the effect on the aminoacylation of isoleucine tends to implicate an interaction with the synthetase, but is far from conclusive.

TABLE VI: Effect of Complex IIa+b on the Aminoacylation of Porcine Thyroid tRNA with Threonine and Isoleucine.[a]

Amino Acid	pmol of Amino Acid/A_{260} of tRNA			
		A_{260} of tRNA:A_{320} of Complex IIa+b		
	tRNA	15:1	10:1	5:1
Threonine	12.00	11.30	10.00	12.10
Isoleucine	2.76	11.20	3.42	5.60

[a] Incubations were as described in the Experimental Section and contained 1.0 A_{260} unit of tRNA (peak 2, Figure 1; free of 320 nm absorbance) or 1.0 A_{260} unit of tRNA and the appropriate amount of complex IIa+b. The specific activities of threonine and isoleucine were 50 Ci/mol. Counting efficiency was 50%.

The observation that hormone-containing complexes, such as we have described here, have effects on the aminoacylation of most amino acids suggests a mechanism by which thyroxine could exert control of protein biosynthesis at the translational level as has been suggested by others (Unsworth and Cohen, 1968).

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Specificity of Metal Ion Interaction with Concanavalin A†

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ABSTRACT: The interaction of metal ions with concanavalin A was measured by equilibrium dialysis at 4°, pH 5.2. The transition metal binding site (S1) binds Mn^{2+}, Co^{2+}, Ni^{2+}, Zn^{2+}, and Cd^{2+} ions. The values of the binding constants are in the order $K_{Mn^{2+}}$ (0.2×10^4 M^{-1}) < $K_{Co^{2+}}$ (1.6×10^4 M^{-1}) < $K_{Ni^{2+}}$ (12×10^4 M^{-1}) > $K_{Zn^{2+}}$ (1.4×10^4 M^{-1}) < $K_{Cd^{2+}}$ (6.2×10^4 M^{-1}). Ca^{2+} and Mg^{2+} ions are not bound to S1. These results suggest that S1 contains one or more nitrogenous ligands. The calcium binding site (S2) binds Ca^{2+} and Cd^{2+} equally well ($K_{Ca^{2+}} = 3.0 \times 10^3$ M^{-1}, $K_{Cd^{2+}} = 1.5 \times 10^3$ M^{-1}), Sr^{2+} very weakly, and Mg^{2+}, Ba^{2+}, Mn^{2+}, Ni^{2+}, and Sm^{3+} not at all. This suggests that S2 is a rigid site which can accommodate only divalent metal ions of radii very nearly 1 Å.

Concanavalin A, a saccharide-binding protein from Jack bean, contains metal ions in its native state (Sumner and Howell, 1936a). On removal of metal, transition metal ions can be bound at a site designated S1. On occupation of S1, a new metal binding site, S2, is formed. The saccharide binding site is created when the two metal binding sites, S1 and S2, are occupied (Kalb and Levitzki, 1968). Concanavalin A, mol wt 55,000, is composed of two identical subunits (Kalb and Lustig, 1968; Greer *et al.*, 1970). The molecule contains two of each of the metal binding sites, S1 and S2, and two saccharide binding sites (Yariv *et al.*, 1968).

In this publication we show that only divalent metal ions which have an affinity for nitrogen ligands are bound to site S1. The interaction with S2 is restricted to divalent metal ions whose radii are in a narrow range around 1 Å. In this case, it is not required that the ion should have an affinity for nitrogen.

Materials and Methods

Buffer Solutions. The solvent used in this investigation was 0.05 M sodium acetate–acetic acid buffer, pH 5.2, containing 0.2 M NaCl or, in some cases, 0.2 M $NaNO_3$, made with column-deionized, distilled water and treated with a metal-chelating resin (Chelex 100).

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