polymerized at 37°C were broken down by the addition of 5 mM Ca^{2+}, 100 μM colchicine, or by reducing the temperature to 0°C. This suggested that the disks were at least temporarily stable under conditions in which tubules were not, and that they probably originated from the dissolution of the microtubules. (vi) The microtubules formed in the low speed extracts appeared to be in equilibrium with subunits, since the microtubules slowly broke down (within 1 hour at 37°C) when diluted 1 : 10 with PMEG solution, but did not break down when diluted 1 : 10 with high speed extract.

From these observations, we conclude that the assembly of microtubules in porcine brain extracts proceeds by a nucleation mechanism and that the nucleation center is most probably a disk-type structure. Determination as to whether the disks are intermediates in the assembly of microtubules, or represent subunits modified to pack into a stable ring, must await the isolation of the disk structures. An interesting possibility is that the disk structures may be the microtubule organizing units postulated to exist in dividing and differentiating cells (*7*).

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Coding Properties of Reticulocyte Lysine Transfer RNA's in Hemoglobin Synthesis

Abstract. *Two isoacceptor transfer RNA's for lysine were found in rabbit reticulocytes. The codon recognition properties of these isoacceptors were studied in hemoglobin synthesis in a cell-free system. The two isoacceptors transferred lysine into different sites in hemoglobin, but showed no preference for one chain over the other. Codon cross recognition was less than 4 percent.*

Isoacceptor transfer RNA's (tRNA) for lysine have been found in rabbit reticulocytes, and their codon recognition properties have been investigated by measuring trinucleotide-induced binding of isoacceptors to *Escherichia coli* ribosomes (*1*, *2*). Results from our laboratory show the existence of two lysine isoacceptor tRNA's, one of which binds to ribosomes exclusively in the presence of AAG (A, adenosine; G, guanosine) while the other has a preference for AAA over AAG, in agreement with results of Rudloff and Hilse (*2*).

We were interested in determining whether or not these isoacceptors had the same codon specficity in protein synthesis as in the ribosome binding assay. Each isoacceptor was acylated with [^{14}C]lysine and then added to a highly active, reticulocyte, cell-free system (*3*) synthesizing many globin chains per ribosome during the course of an incubation. The tryptic peptides of the products synthesized in the presence of [^{14}C]lysine-labeled isoacceptor tRNA's were analyzed to determine the amount of lysine transferred into each site.

The results show that reticulocyte $tRNA^{Lys}_{I}$ (binds to ribosomes in the presence of AAG) transfers lysine to about the same extent into ten lysine sites in the α chain and ten lysine sites in the β chain. The reticulocyte $tRNA^{Lys}_{II}$ (binds preferentially to ribosomes in the presence of AAA) transfers lysine into the two remaining sites in the α chain and the two remaining lysine sites in the β chain. There appears to be very little overlap in the recognition of lysine sites by these two isoacceptors. Also, both RNA's recognize an equal number of lysine sites in the α and β chains, in contrast to results of Rudloff and Hilse (*2*), who report that the isoacceptor tRNA that binds to ribosomes in the presence of AAG transfers lysine mainly into the α chain and that the isoacceptor tRNA that preferentially binds to AAA transfers mainly into the β chain. We present our results and explore the reasons for the disagreements between the two findings.

The tRNA, prepared by phenol extraction of reticulocytes from anemic rabbits (*4*), was acylated with [^{14}C]-lysine with a reticulocyte synthetase preparation and fractionated into two isoacceptor species on benzoylated DEAE–(B-D) cellulose (*5*) and Freon reversed-phase (*6*) chromatography. The ratio of lysine I to lysine II tRNA as measured by acceptor activity was about 60 to 40 after B-D cellulose chromatography. The binding specificity of the two isoacceptors was similar to that reported by Rudloff and Hilse for their two major peaks of lysine isoacceptor activity (reticulocyte $tRNA^{Lys}_{I}$, which binds to AAG, corresponds to Rudloff and Hilse's $tRNA^{Lys}_{II}$; and our reticulocyte $tRNA^{Lys}_{II}$, which binds preferentially to AAA, corresponds to their $tRNA^{Lys}_{IV}$) (*7*).

Each of the acylated isoacceptor tRNA's was incubated in a reticulocyte cell-free system for 1 hour at 35°C (*3*). The cell-free systems sustained a linear rate of protein synthesis for approximately 15 minutes and synthesized more than eight globin chains per ribosome during an incubation. Although the amount of lysine tRNA added was about 100 times greater than that in the cell-free system, the amount of protein synthesized was depressed only slightly (about 10 percent), and the duration of synthesis was the same (about 30 minutes) as in the absence of added tRNA (*7*). Amino acid transfer from acylated tRNA's into hemoglobin was essentially complete within 5 minutes. Conditions were such as to minimize enzymatic deacylation of the added tRNA (*8*), and, furthermore, the specific activity of [^{14}C]lysine deacylated from the tRNA would have been so reduced by the pool of free [^{12}C]lysine in the mixture (50 μM) that its incorporation would have been below the level of detection.

Immediately after the incubation, the ribosomes were sedimented by centrifugation, uniformly labeled [^{3}H]lysine hemoglobin (prepared by whole cell incubation) was added to the supernatant, and globin was prepared by acid acetone precipitation (2 ml of concentrated HCl per liter of acetone).

The α and β chains were separated on carboxymethyl-cellulose columns (*9*), and the chains were digested with trypsin and fractionated on Dowex-50 columns (Aminex A-5 resin) according to the method of Jones (*10*).

The results of the lysine transfer into globin peptides by each of the isoacceptor tRNA's are shown in Fig. 1. The tryptic peptide pattern for the [^{3}H]lysine globin shows ten major peaks for each chain. Several of these peaks contain more than one tryptic peptide. The [^{3}H]lysine from the uniformly labeled globin serves not only to mark the position of elution of lysine-containing peptides but also as an internal standard to correct for variable recoveries of tryptic peptides. The procedure for determining the extent of transfer of lysine into peptides in each peak is as follows: the ratio of [^{14}C]lysine (transferred by a particular tRNA) to the [^{3}H]lysine contributed by the carrier hemoglobin is calculated for each peak. For each tRNA, the ratios of those peaks that show apparent lysine transfer (high ratio of ^{14}C to ^{3}H) are averaged, and all the peaks are normalized to the average (Fig. 1). These normalized ratios are plotted as bar graphs in Figure 1.

A normalized ratio greater than 0.8 represents transfer by the tRNA into the peptides in the peak. Thus reticulocyte $tRNA^{Lys}_{II}$ transfers lysine into residues 99 (αT_{12}) and 139 (αT_{14}) in the α chain, and residues 82 (βT_9) and 144 (βT_{15}) in the β chain; whereas reticulocyte $tRNA^{Lys}_{I}$ transfers lysine into all of the remaining sites. Since most of the tryptic peptides are not homogenous at this stage, it is necessary to purify the peptides to homogeneity in order to quantify the extent to which an isoacceptor tRNA can recognize a codon at a specific site.

Four peptides were selected for further purification, representing transfer by one or the other isoacceptor tRNA's. The [^{14}C] and [^{3}H]lysine content of each of the purified peptides was determined. The peptide with the highest ratio of ^{14}C to ^{3}H from each tRNA was arbitrarily set equal to 100 percent, and the ratios of the other peptides were normalized to this value (Table 1). These results support our interpretation of the data in Fig. 1 and indicate that data obtained from the initial peptide separation is reliable as a rough index of codon recognition for a given tRNA. Peaks $\alpha 8$ for reticulocyte $tRNA^{Lys}_{I}$ and $\alpha 2$, $\alpha 4$, $\beta 6$, and $\beta 9$ for $tRNA^{Lys}_{II}$ which showed a low level of incorporation into a peak recognized by the other tRNA were chromatographed again on Dowex-50 $\times$ 2, and in each case the radioactivity was associated with contaminating material and not with the major peptide.

The reticulocyte $tRNA^{Lys}_{I}$ transfers lysine into three of the four purified peptides, αT_7 (residue 60), $\alpha T_{8,9}$ (residues 61 and 68) and βT_7 (residue 65). The $tRNA^{Lys}_{II}$ transfers lysine into the purified peptide βT_{15} (residue 144). The low levels of lysine transfer by reticulocyte $tRNA^{Lys}_{I}$ into βT_{15} and by $tRNA^{Lys}_{II}$ into αT_7, $\alpha T_{8,9}$ and βT_7 represent the limits of sensitivity of the method and are probably maximum values for lysine transfer into these sites; therefore, we conclude that the preference of each of these isoacceptors for the respective sites is greater than 25 to 1. This result also shows that transacylation between lysine isoacceptors was not a major problem in the system.

Before discussing the reasons for the differences between our results and

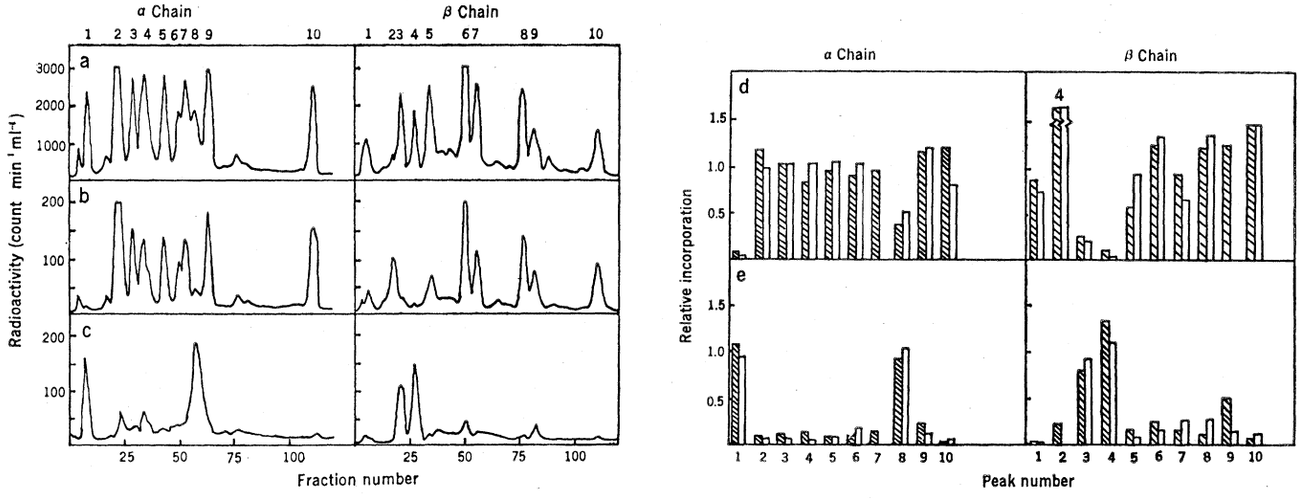

Fig. 1. Separation of lysine-containing tryptic peptides of rabbit hemoglobin on Aminex A-5 (Bio-Rad), and the relative incorporation of [^{14}C]lysine from reticulocyte isoacceptor tRNA's into globin peptides. Uniformly labeled [^{3}H]lysine hemoglobin was combined with the product from [^{14}C]lysine tRNA incubated in a cell-free supernatant from a reticulocyte cell system. The resulting product was separated into α and β chains (*9*); the chains were digested with trypsin, and the digests were placed on an Aminex A-5 column (0.9 by 15 cm) jacketed at 50°C (left side of figure) (*10*). The [^{14}C]lysine in each fraction was determined by drying a 1-ml portion on a planchet and counting it in a low background counter (Nuclear-Chicago, $^{14}C \simeq 30$ percent, $^3H < 0.02$ percent). The [^{3}H]lysine was determined by counting a 0.1- ml portion in a liquid scintillation counter (Nuclear-Chicago, $^{14}C \simeq 50$ percent, $^3H \simeq 10$ percent). (a) Uniformly labeled [^{3}H]lysine hemoglobin; (b) [^{14}C]lysine $tRNA^{Lys}_{I}$; and (c) [^{14}C]lysine $tRNA^{Lys}_{II}$. The [^{14}C]lysine transferred by an isoacceptor tRNA was measured relative to the [^{3}H]lysine from the uniformly labeled hemoglobin as described in the text (right side of the above figure). (d), $tRNA^{Lys}_{I}$; (e) $tRNA^{Lys}_{II}$. The peak numbers refer to the major lysine peaks shown in the left side of the figure, and the two bars, one clear and one shaded, for each peak represent the results of duplicate experiments. The high ratio for β peak 2 in the case of $tRNA^{Lys}_{I}$ is due to low amounts of a corresponding peak of [^{3}H]lysine from the uniformly labeled hemoglobin. The nature of this peptide is unknown. Peaks $\alpha 7$ and $\beta 9$ for $tRNA^{Lys}_{I}$ and $\alpha 7$ and $\beta 2$ for $tRNA^{Lys}_{II}$ were not sufficiently separated from adjacent peaks to obtain a reliable ratio. The $tRNA^{Lys}_{I}$ was acylated with [^{14}C]lysine (approximately 200,000 count/min; 10 A_{260}); $tRNA^{Lys}_{II}$ was acylated with [^{14}C]lysine (with approximately 100,000 count/min; 5 A_{260}).

those of Rudloff and Hilse (*2*), it is useful to consider how various properties of the system used limit the conclusions that can be drawn about codon recognition by tRNA's. (i) The system should be active enough to initiate several new chains per ribosome during the course of the incubation. If the system is carrying out primarily chain elongation and release with little initiation, then an increasing gradient of amino acid transfer will be observed from the NH_2-terminal to the COOH-terminal ends of the protein product (*11*). Thus, it would be difficult to compare the relative recognition of different sites in this protein. (ii) It should be shown that the added tRNA does not significantly perturb the system, and that the product synthesized in the presence and absence of added tRNA is identical to that synthesized in vivo. If the system meets the above criteria then specific sites of amino acid incorporation should be examined to determine whether different isoacceptor tRNA's demonstrate absolute or overlapping specificities in codon recognition.

We interpret our results to indicate that each lysine isoacceptor tRNA transfers equally into both α and β chain but at different sites, whereas Rudloff and Hilse interpret their results to indicate that one isoacceptor tRNA transfers predominantly into the α chain and the other mainly into the β chain. A comparison of the two sets of results in view of the criteria outlined above leads one to the conclusion that the cell-free system used by Rudloff and Hilse was not active enough to provide a valid test of the codon recognition properties of the lysine isoacceptor tRNA's, and that our results reflect the codon recognition properties of the two lysine isoacceptor tRNA's in hemoglobin synthesis.

First, the cell-free system Rudloff and Hilse used to test the isoacceptors is severely limited in its capacity to synthesize protein. On the assumption that 1.0 mg of ribosomes in 1.0 ml of solution gives an absorbancy at 260 nm (A_{260}) of 11.2 (*12*) and a molecular weight of approximately 4.5×10^6 for ribosomes, we calculate that the cell-free system incorporates only 4 to 5 pmole of lysine per 12 nmole of ribosomes, or less than one chain per 10^4 ribosomes. This would indicate that little or no chain initiation or elongation is occurring in this system, and that chains released during the incubation would have radioactivity only in lysine sites close to the COOH-terminal region.

Table 1. Transfer of [^{14}C]lysine into tryptic peptides in the α and β chains of rabbit hemoglobin by lysine isoacceptor tRNA's. Four peptides, α10, α7, β10, and β7, representing lysine transfer by reticulocyte $tRNA^{Lys}_{I}$ and reticulocyte $tRNA^{Lys}_{II}$, were rechromatographed on Dowex-50 $\times$ 2 (*10*). Each peptide was analyzed by cellulose thin-layer chromatography (*n*-butanol, pyridine, acetic acid, and water, 15 : 10 : 3 : 12); cellulose thin-layer electrophoresis at *p*H 1.9 (88 percent formic acid, acetic acid, and water, 50 : 150 : 800); and cellulose thin-layer electrophoresis at *p*H 5.6 (pyridine, acetic acid, and water, 4 : 1 : 995). If a peptide showed more than a single ninhydrin spot in any of the above systems, it was purified by that system on a batch scale, and its purity was rechecked. The peptides were then hydrolyzed with 6*N* HCl for 24 hours at 110°C and subjected to cellulose thin-layer chromatography (*n*-butanol, acetic acid, and water, 143.5 : 13 : 43.5—upper phase); the identity of each peptide was verified by its composition. The data are expressed as the percentage of [^{14}C]lysine transferred into the peptides, the number of counts per minute of [^{3}H]lysine was taken as the basis, and the peptide with highest ratio of ^{14}C to ^{3}H was considered 100 percent.

tRNA	[^{14}C]lysine transferred (%)			
	αT_7	$\alpha T_{8,9}$	βT_7	βT_{15}
Lys I	94	93	100	3
Lys II	3	4		100

Second, using carboxymethyl-cellulose columns to separate the α and β chains, Rudloff and Hilse found that their system incorporated about 4 times the radioactivity from unfractionated lysine tRNA into the α chain as into the β chain, and that in the case of the α chain the peak of radioactivity did not coincide with the peak of optical density for bulk α chain. These results suggest that (i) less β chain is being made in this system than α chain, and (ii) the product being synthesized in the case of the α chain may be something other than α chains synthesized in the cell. They were unable to look at tryptic digestions of their product due to the low levels of incorporation obtained with their system.

In contrast, we found the following. (i) The α and β chains were synthesized in approximately equal amounts. (ii) The radioactivity from the lysine transferred by an isoacceptor coincided exactly with the radioactivity from the uniformly labeled globin on CM cellulose (*7*). Further evidence that the product synthesized is globin is shown by the coincidence of tryptic peptides from uniformly labeled globin and the synthesized product. (iii) Transacylation between isoacceptors must have been minimal because cross recognition of the codons was less than 4 percent. Therefore we conclude that the two lysine isoacceptor tRNA's recognize different lysine sites in the hemoglobin message and that neither tRNA shows a preference for one chain over the other.

One of a number of possible explanations for the observed codon specificity of the two isoacceptor tRNA's is that $tRNA^{Lys}_{I}$, which recognizes the codon AAG has CUU (C, cytidine; U, uridine) for its anticodon, and $tRNA^{Lys}_{II}$, which recognizes AAA, has XUU for its anticodon, where X is any base that is restricted to base-pairing with A alone. Yoshida (*13*) has reported a 2-thiouridine derivative in the first position of the anticodon of a yeast glutamic acid isoacceptor tRNA that recognizes GAA alone. It is possible that a thiouridine base or one with similar base pairing properties occurs in the first position of the anticodon in $tRNA^{Lys}_{II}$, restricting recognition to the codon AAA. In this regard, it is of interest that Madison has found a thiouracil derivative in the first position of a yeast lysine isoacceptor tRNA (*14*).

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