Transfer Ribonucleic Acid Dependent but Ribosome-Independent Leucine Incorporation into Rat Brain Protein†

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ABSTRACT: An unusual type of posttranslational modification has been observed in a rat brain in vitro system. It consists in leucine addition to a preformed protein in such a way that the added leucine is not located at either the NH_2 or the COOH terminus of the acceptor protein. The incorporation reaction requires ATP, ATP-generating components, and tRNA. It is inhibited by aurintricarboxylic acid but does not require the presence of ribosomes or GTP. The incorporated leucine has a free NH_2 group, and it is not released by leucine aminopeptidase or carboxypeptidase A. It is linked to the acceptor protein through a bond that is too alkali labile and too hydroxylamine labile to be a peptide bond. The simplest interpretation of the results consists in proposing that an ester bond is formed between the leucine and the side chain of a serine, threonine, or tyrosine in the acceptor protein.

It is known that protein synthesis, defined as amino acid incorporation into polypeptides, is *not* the monopoly of ribosomes. Some short polypeptide antibiotics and cell wall constituents are assembled in step-by-step amino acid activation and condensation catalyzed by specific enzymes in the absence of mRNA and ribosomes (Lipmann, 1971; Strominger, 1970). Several examples of posttranslational amino acid incorporation into preformed polypeptides are also known. The enzyme transglutaminase favors the displacement of the amide ammonia in Gln by the ϵ-amino group of Lys; the result is a branching point in an otherwise linear polypeptide chain (Folk, 1979). There exists a derivative of tubulin in which a Tyr residue is added in peptide linkage to the -COOH terminus of its α chain in the absence of ribosomes, mRNA, and tRNA (Barra et al., 1980). In eukaryotes one finds an enzyme that adds Arg in peptide linkage to the α-NH_2 terminus of several acceptor proteins. The Arg donor is arginyl-tRNA but the reaction is ribosome independent (Soffer, 1974; Deutch et al., 1978). A similar enzymatic activity is found in bacteria, except that the amino acids transferred are Leu, Met, and Phe (Scarpulla et al., 1976).

In this paper we report an unusual type of Leu incorporation in a rat brain in vitro system. This Leu incorporation is tRNA dependent but ribosome independent. It consists of Leu addition to a preformed protein in such a way that the incorporated Leu has a free NH_2 group and is linked to the protein acceptor through a *nonpeptidic*, alkali-labile bond. This mammalian Leu incorporating activity differs in several other respects (e.g., resistance to puromycin) from the Leu incorporation directed by the bacterial leucyl-tRNA transferase (Soffer, 1974).

Materials and Methods

Animals. Male rats, either Sprague-Dawley or Fisher 344, were used. No significant difference was seen between enzyme preparations from either breed.

Chemicals. Chloramphenicol and aurintricarboxylic acid were purchased from Sigma. Calf liver tRNA, *Escherichia coli* tRNA, yeast tRNA, yeast $tRNA^{Phe}$, poly(U), ATP (disodium salt), GTP (disodium salt), creatine phosphate (disodium salt), all other enzymes, and antibiotics were purchased from Boehringer Mannheim. Dipeptides were from Vega Biochemicals, Tucson, AZ. Spermine and Spermidine were from Aldrich. Radioactive [^{3}H] leucine (50 Ci/mmol) was obtained from New England Nuclear. Rat liver tRNA synthetase was a gift by Dr. L. Kleiman and had been prepared according to Ortwerth (1971).

Buffers. Buffer A contained 7.5 mM phosphate, 0.4 mM spermidine, 0.3 mM spermine, 50 mM KCl, 50 mM potassium acetate, 5 mM NH_4Cl, 0.5 mM $CaCl_2$, 5 mM magnesium acetate, and 1 mM dithiothreitol, pH 7.5. Buffer B consisted of buffer A without Mg^{2+} and Ca^{2+} but with 0.5 mM EDTA. The homogenization buffer was buffer A with 0.25 M sucrose. Buffer C consisted of buffer A with 10% sucrose.

Cell-Free Extracts. Brains were quickly removed and rinsed in ice-cold homogenization buffer. All procedures were performed at 0-4 °C unless otherwise noted. The brains were homogenized (0.5 g wet weight/mL of homogenization buffer) with six strokes of a Dounce homogenizer (Bellco). The homogenate was centrifuged at 2500*g* for 3 min after which the supernatant was centrifuged at 20000*g* for 20 min. The resulting cell-free extract was preincubated 20 min at 37 °C in homogenization buffer containing 1 mM neutralized GTP, 1 mM neutralized ATP, 10 mM creatine phosphate, and 50 μg/mL creatine kinase. This was followed by dialysis against buffer A or B (hence the names S20A or S20B in the remainder of the text), centrifugation for 10 min in the Eppendorf microcentrifuge, separation of the supernatants into aliquots, and storage at -80 °C.

In order to make comparative studies with the *E. coli* leucyl-tRNA transferase activity, a S30 was prepared from *E. coli* K12 (Staehelin et al., 1969). After the cells were ground in alumina and the extract was centrifuged twice at 30000*g* for 30 min, the S30 was preincubated and dialyzed against buffer B as described for the preparation of the brain extract.

In Vitro Leucine Incorporation. The basic reaction mixture contained, in 100 μL, 25 μL of S20, 5 μg of creatine kinase, 10 mM creatine phosphate, 1 mM ATP, 1 mM GTP, 7.5 mM dithiothreitol, 0.2 mg of unfractionated calf liver tRNA, 10 μCi of [^{3}H]leucine, and 27.5 μM leucine. The other chemicals and their concentrations were those of buffer A or B, depending on the buffers against which the S20 had been dialyzed. The final pH was 7.3. Incubations were routinely

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* Recipient of a research career development award from the Conseil de la Recherche en Santé du Québec.

0006-2960/82/0421-5694$01.25/0

Table I: Types of Leucine Incorporation Encountered in This Paper

types of leucine incorporation[a]	sensitivity or resistance of the incorporation to: cycloheximide (1 mM) and puromycin (0.1 mM)	sensitivity or resistance of the incorporation to: pancreatic RNase
RNA dependent		sensitive
ribosome directed or cycloheximide sensitive	sensitive	sensitive
cycloheximide resistant	resistant	
transferase directed	resistant	sensitive
RNA independent	resistant	resistant

[a] The RNA-dependent incorporation is the sum of the ribosome-directed and the transferase-directed incorporations. The cycloheximide resistant incorporation is the sum of the transferase-directed and the RNA-independent incorporations.

performed for 30 and 90 min at 37 °C (the incorporation does not cease until after 2 h). The reactions were stopped by the addition of 3 mL of TCA (a solution containing 5% trichloroacetic acid and 1% casamino acids). The resulting precipitate suspensions were heated for 45 min at 82 °C and collected on Whatman GF/A glass filters under vacuum. The tubes were rinsed twice with 3 mL of TCA and the filters were subsequently washed 3 more times with 5 mL of TCA. Finally, each filter was washed with 6 mL of 95% ethanol, dried at 90 °C for 40 min and counted with 5 mL of Omnifluor scintillation fluid.

The nature of this work makes it necessary to distinguish clearly between various types of Leu incorporation. These are presented in Table I and in order of dominance. It follows from Table I that in a S200 (deprived of ribosomes), the transferase-directed incorporation and the tRNA-dependent incorporation become exact synonyms.

All the data presented in the tables and Figure 3 are reported as percent ± standard error of the mean of three to five experiments.

[^3H]Leucyl-tRNA Levels. The formation of leucyl-tRNA was measured as for in vitro Leu incorporation except that cold TCA was added to the samples, the hot TCA treatment was omitted, and filtration was done in the cold room.

Preparation of Leucyl-tRNA. The reaction mixture contained, per milliliter, 12 μmol of magnesium acetate, 950 μg of calf liver tRNA, 0.5 mCi of [^{3}H]leucine, and 2.8 mg of rat liver tRNA synthetase. The other components were as in Yang & Novelli (1971). After a 30-min incubation at 30 °C, the mixture was extracted with phenol–chloroform, and the aqueous layer was shaken with 4% isoamyl alcohol in chloroform. The [^{3}H]leucyl-tRNA was washed free of any residual [^{3}H]amino acids by repeated ethanol precipitation and ethanol washing of the precipitate (Kaji et al., 1965). The final precipitate was dissolved in 25 mM sodium acetate, pH 4.5, and stored at −80 °C.

Periodate Oxidation of tRNA. Sodium metaperiodate pretreatment of tRNA was performed as previously described (Millar & Steiner, 1965) at a concentration of 10 mg of tRNA/mL and a pH of 6.0. The dialysis was against water.

NH_2-Terminal Analysis. For the determination of NH_2-terminal amino acid the phenyl isothiocyanate procedure of Edman was used (Fraenkel-Conrat et al., 1955). The method of DeLange et al. (1968) was followed except for converting the NH_2-terminal amino acid thiazolinone to the corresponding phenylthiohydantoin, which was done according to the procedure of Momose & Kaji (1966).

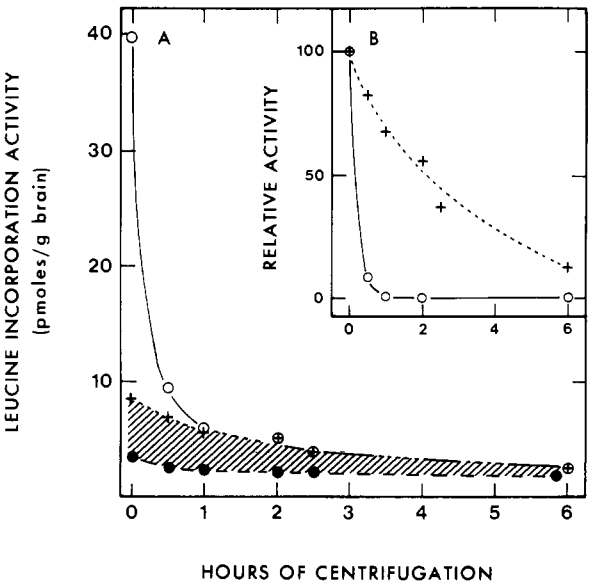

FIGURE 1: 0.45 mL of S20A (Materials and Methods) was layered over 0.22 mL of buffer C in a 5 × 41 mm Beckman ultraclear centrifuge tube. After centrifugation at 200000*g* (45 000 rpm in the SW 50.1 rotor at 4 °C) for the indicated periods of time, the top 0.45 mL (supernatant or S200) was assayed for Leu incorporation (i) in the presence of 1 mM cycloheximide, 0.1 mM puromycin, and 40 μg/mL pancreatic RNase [(A) (●)], (ii) in the presence of 1 mM cycloheximide and 0.1 mM puromycin [(A) (+)], and (iii) in the absence of antibiotics and RNase [(A) (○)]. In (B) the ribosome-directed incorporation activity (○) and the transferase-directed incorporation activity (+) are plotted on a relative scale: both activities are arbitrarily set at 100 at time 0 for comparative purposes. The continuous line of (B) is obtained by going to (A) and subtracting the dotted line from the continuous line. The dotted line of (B) is obtained by subtracting the dashed line from the dotted line in (A). Identical results were obtained when a S20B was centrifuged and assayed in the absence of Mg^{2+} and Ca^{2+}, except that the cycloheximide-sensitive incorporation activity was greatly reduced.

Assays with Carboxypeptidase A and Leucine Aminopeptidase. The incorporated product was incubated for 30 min at 37 °C in the presence of either 1 mg/mL leucine aminopeptidase or 2.5 mg/mL carboxypeptidase A. In the aminopeptidase experiment, the ionic conditions were those of buffer A. In the carboxypeptidase experiment, the ionic conditions were those of buffer A with 0.9 M NaCl (Petra, 1970). After the incubation, the samples were precipitated with TCA and processed as described for in vitro Leu incorporation.

Reaction with $HgCl_2$. The incorporated product was incubated for 10 min at 37 °C in the presence of 30 mM $HgCl_2$. After the incubation, the samples were precipitated with TCA and processed as above.

N-Ethylmaleimide (NEM) Labeling. An S20B was reacted with 8 mM NEM as previously described (Laughrea & Moore, 1978) except that the reaction buffer was buffer A deprived of dithiothreitol and that the dialysis buffer was buffer A supplemented with 6 mM dithiothreitol.

Results

An RNA-Dependent, but Ribosome-Independent, Leucine Incorporation Activity. A S20 was obtained from a rat brain homogenate and was centrifuged at 200000*g* for designated periods of time over a 10% sucrose cushion. The Leu incorporation activity left in the supernatant was measured (Figure 1). From the known density and sedimentation coefficient of mammalian ribosomes (van Holde & Hill, 1974) it was computed that the supernatant incorporation activity should vanish within the first hour of centrifugation if the whole of rat brain protein synthesis was ribosome directed. In Figure

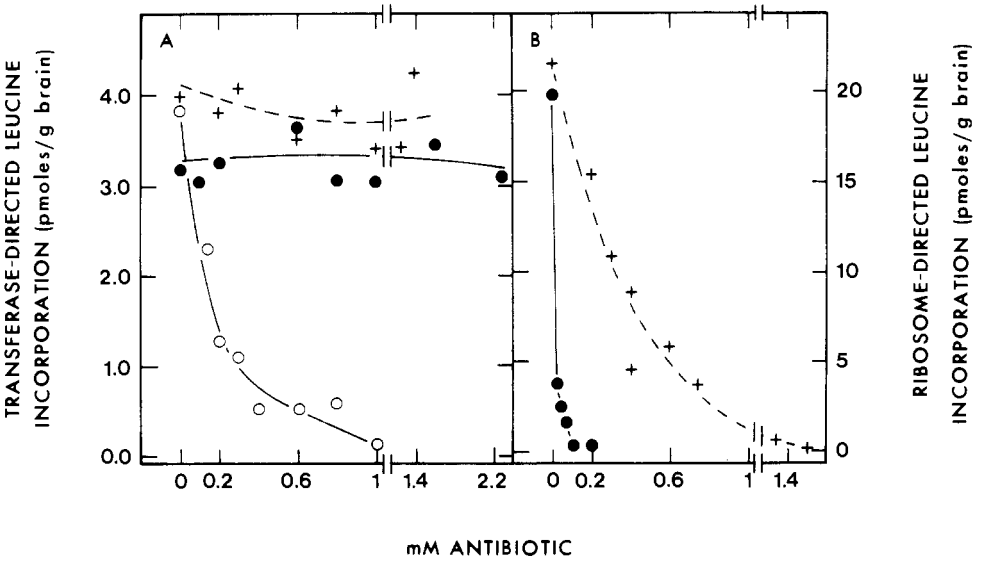

FIGURE 2: Comparative effects of antibiotics on rat brain leucine transferase and on ribosome-directed Leu incorporation activity. (A) A S20A (Materials and Methods) was centrifuged at 200000*g* for 60 min as described in Figure 1. The supernatant was assayed for RNA-dependent Leu incorporation as a function of increasing amounts of puromycin (●), tetracyclin (+), and aurintricarboxylic acid (○). Similar results were obtained if the supernatant was replaced by a S20B containing 1 mM cycloheximide. (B) The original S20A was assayed for ribosome-directed Leu incorporation as a function of increasing amounts of puromycin (●) and tetracyclin (+).

1A (continuous line) it is seen that a rapid decrease in incorporation activity is followed by a slow decrease at times greater than 1 h of centrifugation. This suggests that, as expected, most of the Leu incorporation activity is ribosome directed but that some of it (about 20% in Figure 1A) is ribosome *independent*. Proof is provided by the dotted line in Figure 1A, which represents the incorporation obtained in the presence of 1 mM cycloheximide and 0.1 mM puromycin, two powerful inhibitors of the elongation process during ribosome-directed synthesis (Vazquez, 1979; Nathans, 1967). The subtraction of the dotted-line activity from the continuous-line activity yields the ribosome-directed incorporation activity (Figure 1B, continuous line), which indeed is found to disappear from the supernatant within the first hour of centrifugation and to leave a wholly ribosome independent activity after centrifugation for more than 1 h. (This ribosome-independent incorporation, which is operationally defined as the incorporation observed in the presence of 1 mM cycloheximide and 0.1 M puromycin, will be referred to as "cycloheximide resistant incorporation" in the remainder of the text).

It was found that the addition of pancreatic RNase to the incorporation mixture reduces the cycloheximide resistant incorporation activity by 60%, leading us to divide this activity into two categories: (i) an RNA-independent activity and (ii) a RNA-dependent, or RNase-sensitive, activity (the shaded area in Figure 1A). This paper focuses on this second category of incorporation, to be thereafter called "transferase-directed incorporation". The putative enzymatic activity responsible for this type of incorporation will be called by the generic names "leucine transferase activity", "leucine transferase", or, simply, "transferase". The RNA-independent Leu incorporation has not been studied to any significant extent because it was not specific for Leu: the level of [^{3}H]Ile, [^{3}H]Val, or [^{3}H]Leu incorporated through the RNA-independent pathway were similar. On the contrary the transferase-directed Leu incorporation activity was at least 5 times larger than the activity observed in the presence of either [^{3}H]Ile or [^{3}H]Val.

The leucine transferase directed incorporation activity is obtained by subtracting the RNA-independent activity from the cycloheximide resistant incorporation activity. Figure 1B makes clear the macromolecular nature of the transferase-directed activity (it is largely sedimented after 6 h at 200000*g*) but also stresses that it is about 1 order of magnitude less sedimentable than the ribosome-directed Leu incorporation activity.

Transferase Activity Is Resistant to Puromycin and Tetracyclin but Sensitive to Aurintricarboxylic Acid. Figures 2 and 3 present some functional characteristics of the leucine transferase activity present in an S200 or an S20 extract. The activity is resistant to puromycin and tetracyclin (two antibiotics that prevent aminoacyl-tRNA binding to the ribosome), but it is sensitive to aurintricarboxylic acid (Figure 2A). Aurintricarboxylic acid preferentially inhibits many, but not all, nucleic acid binding proteins (Apirion & Dohner, 1975; Igarashi & Zmean, 1975; Bina-Stein & Tritton, 1976; Blumenthal & Landers, 1973). Control experiments show that under the same ionic conditions ribosome-directed Leu incorporation is 90% inhibited by 0.04 mM puromycin or 0.8 mM tetracyclin (Figure 2B). The transferase activity is also resistant to 0.3 mM chloramphenicol and 10 mM cycloheximide (higher concentrations were not tested). This resistance of the transferase activity to several ribosome inhibitors suggests a simple and efficient way to assay it. Instead of removing physically the ribosomes by centrifugation, they can be functionally removed by assaying the S20 in the presence of 1 mM cycloheximide and 0.1 mM puromycin. As an extra benefit, one gains significantly in activity since the transferase activity is slowly sedimentable (Figure 1B).

Requirement for ATP and ATP-Generating Components: Absence of Requirement for GTP. Figure 3 presents data obtained with an S20 extract. The transferase activity requires ATP and ATP-generating components but is left unchanged by the omission of GTP or Mg^{2+} (Figure 3). Control experiments showed that the omission of GTP or Mg^{2+} reduces ribosome-directed protein synthesis 5- and 30-fold, respectively, but does not significantly affect tRNA charging. The addition of a mixture of amino acids (400 μM of each except Leu) does not change the amount of incorporated radioactivity. Because of the presence of a leucylphenylalanylmethionyl-tRNA transferase in bacteria (Scarpulla et al., 1976) and of an arginyl-tRNA transferase in eukaryotes (Deutch et al., 1978;

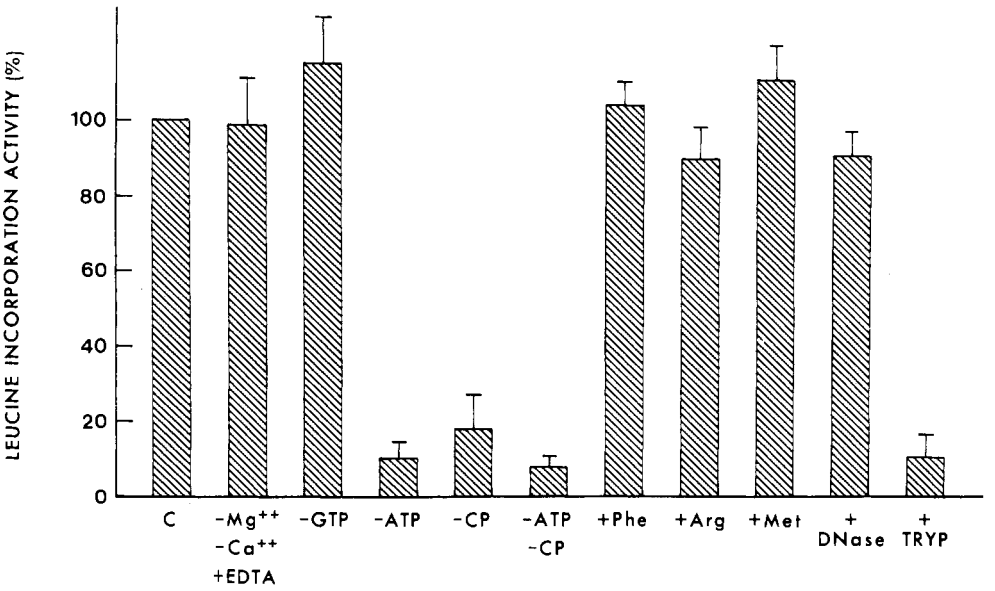

FIGURE 3: Requirements for the transferase-directed Leu incorporation. Transferase-directed incorporation was assayed as described under Materials and Methods. The control incorporation (C) obtained with a S20A in the presence of Mg^{2+}, Ca^{2+}, GTP, ATP, creatine phosphate (CP), etc. (see Materials and Methods) was arbitrarily set at 100 for comparative purposes. (100 represents 5.5 pmol of Leu incorporated/g of brain). A minus (−) means that the indicated component was absent from the reaction mixture, whereas a plus (+) means that it was added to the reaction mixture at the beginning of incubation. Trypsin (TRYP) and DNase I were used at concentrations of 200 and 40 μg/mL, respectively. EDTA, Phe, Arg, and Met were added at the respective concentrations of 0.5, 10, 2, and 20 mM. Similar results were obtained in experiments where the S20 was replaced by a S200 free of ribosomes.

Soffer, 1974), the possible effects of Phe, Met, and Arg on the incorporation were studied in more detail. No reduction in incoporated radioactivity was observed in the presence of 2 mM Arg, 10 mM Phe, and 20 mM Met, indicating that the leucine transferase is specific for Leu incorporation and that Phe, Arg, and Met do not have any competitive activity against Leu incorporation (Figure 3) (the radioactivity incorporated was also found to be left unchanged by the presence of the related amino acids Val and Ile at a concentration of 1 mM).

Intact Eukaryotic tRNA Is Required for the Transferase Activity. The specificity of the RNA requirement of the transferase-directed reaction was also studied. This is shown in Table II. Poly(U), fragments of calf liver tRNA, *E. coli*, tRNA, and yeast $tRNA^{Phe}$ were not adequate cofactors for the transferase activity. On the other hand, the addition of bulk yeast tRNA resulted in about half the control transferase activity. It is interesting to consider the capacity of these tRNAs to be leucylated by the rat brain in vitro system. *E. coli* tRNA and yeast $tRNA^{phe}$ are hardly leucylated at all whereas yeast tRNA gets leucylated to a considerable extent (Table II). These results, in addition to showing that rat brain leucine tRNA synthetase is quite specific for eukaryotic tRNAs, suggest that the substrate of the transferase activity may be leucyl-tRNA. Consistent with this idea is the fact that the transferase is inhibited by aurintricarboxylic acid. We have found rat brain leucyl-tRNA synthetase to be 50% inhibited by 180 μM aurintricarboxylic acid: this same concentration also reduces the transferase activity by 50% (Figure 2). So that the possibility of an involvement of leucyl-tRNA in the transferase activity could be tested further, [^{3}H]leucyl-tRNA was prepared and added to the brain extracts in the presence of 2 mM cold Leu under conditions where ribosome-directed incorporation is impossible. Hot TCA-precipitable incorporation did occur (Table II), showing that the leucine transferase can use leucyl-tRNA as a substrate. In confirmation, we found that periodate-oxidized tRNA (which cannot be aminoacylated because of a cleavage at its 3′-end ribose) lost most of its ability to stimulate the transferase activity (Table II).

Table II: RNA Specificity of the Leucine Transferase Activity[a]

exogenous RNA added	transferase act. (%)	[^{3}H]Leu-tRNA level (%)
calf liver tRNA (control)	100	100
poly(U)	−6 ± 9	
RNase-degraded calf liver tRNA	2.5 ± 3	
E. coli tRNA	13 ± 8	0.3
yeast $tRNA^{Phe}$	7 ± 6	1
yeast tRNA	49 ± 8	19
$NaIO_4$-oxidized calf liver tRNA	17 ± 9	2
[^{3}H]leucyl-tRNA	21 ± 7	

[a] Leucine transferase activity was assayed as described under Materials and Methods by using a S20B. However, some minor modifications were needed. (i) Only the control reaction mixtures contained calf liver tRNA; the other reaction mixtures contained other types of exogenous RNA as specified under "exogenous RNA added". (ii) In the RNase-degraded tRNA experiments, the leucine transferase activity was measured by subtracting the incorporation in the absence of calf liver tRNA and the presence of RNase from the incorporation in the presence of calf liver tRNA and 40 μg/mL RNase. (iii) In all the other experiments, including the control, the leucine transferase activity was measured in the absence of RNase by subtracting the incorporation in the absence of exogenous RNA from the incorporation in the presence of exogeneous RNA. (iv) In the [^{3}H]leucyl-tRNA experiment, 2 mM cold Leu was added to the reaction mixtures in order to dilute any [^{3}H]leucine that might have been released during the incubation period. [^{3}H]Leucyl-tRNA was added to the incubation mixture instead of the usual free [^{3}H]leucine. The transferase activity was measured by subtracting the incorporation at time 0 from the incorporation at time 90 min. 500 000 cpm of [^{3}H]leucyl-tRNA were added to the reaction mixtures because this was equal to the number of cpm present as [^{3}H]leucyl-tRNA at the end of the incubation period in the control reaction mixture. All exogenous RNAs were added at a concentration of 2 mg/mL except for the [^{3}H]leucyl-tRNA, which was present at a concentration of 2.3 mg/mL.

Leucine Is Incorporated into a Protein. The site of incorporation of the transferase-directed [^{3}H]leucine was first analyzed by studying whether proteases, DNases, or RNases can

Table III: Nature of the Product into Which Leucine Is Incorporated by the Transferase[a]

treatment	leucine incorporated (% of controls)
(a) (1) DNase	98 ± 9
(2) RNase	94 ± 12
(3) tyrpsin	16 ± 5
(4) proteinase K	24 ± 7
(5) 1 N HCl	97 ± 6
(6) 0.02 N KOH	67 ± 15
(7) 0.05 N KOH	16 ± 8
(b) trypsin + proteinase K	14 ± 3
(c) (1) carboxypeptidase A	104 ± 17
(2) leucine aminopeptidase	101 ± 15
(d) (1) ether	124 ± 19
(2) hexane–2-propanol	94 ± 20

[a] S20Bs were assayed for transferase-directed Leu incorporation as described under Materials and Methods. The incubation time was 90 min (control). Three types of treated samples were studied. In treatment a, the 90-min incubation was followed by a 20-min postincubation at 37 °C in the presence of 40 μg/mL DNase, 40 μg/mL pancreatic RNase (this reduces the [^{3}H]leucyl-tRNA level of the reaction mixture by 98% within the first minute of postincubation), 200 μg/mL trypsin, 200 μg/mL proteinase K, 1 N HCl, 0.02 N KOH (this concentration of KOH reduces the [^{3}H]-leucyl-tRNA level of the reaction mixture to 5% of its original value), or 0.05 N KOH. In treatment b, the postincubation lasted only 5 min in the presence of 100 μg/mL trypsin and 100 μg/mL proteinase K. Treatment c is described under Materials and Methods. Treatment d: after the GF/A filters had been washed (Materials and Methods) they were placed in 9 mL of ether or 9 mL of hexane-2-propanol, 3:2 (v/v), and incubated for 20 min at 37 °C. After this incubation, the filters were washed with ethanol, dried, and counted.

render the incorporated product acid soluble. Table III shows that DNase I or pancreatic RNase, added to the incubation product, had no effect on the incorporated radioactivity. On the other hand, the incorporated radioactivity is readily rendered acid soluble upon addition of trypsin or proteinase K, indicating that the Leu is incorporated into a protein. Consistent with this idea, it was found that the product is soluble neither in ether nor in hexane–2-propanol, 3:2 (v/v) (Radin, 1981).

Incorporated Leucine Has a Free NH_2 Terminus. There are several ways in which Leu can theoretically get incorporated into a protein, and it is not the purpose of this paper to identify without ambiguity the nature of the linkage between Leu and the acceptor protein. Nevertheless, a few possibilities can be ruled out or at least made unlikely on the basis of simple experiments.

First, the incorporated product was subjected to a single-step Edman degradation in order to identify whether the incorporated radioactivity had a free amino group or not (and to verify whether the incorporated tritium belonged in fact to leucine). After this degradation procedure, 90% of the radioactivity incorporated by the transferase was found to be soluble in the organic solvent ethylene dichloride. [The identification of this soluble radioactivity was performed by paper chromatography (Momose & Kaji, 1966). The radioactivity moved faster than valine thiohydantoin, slower than proline thiohydantoin, and at the same speed as leucine thiohydantoin.] On the contrary, when ribosome-directed Leu incorporation was studied, more than 90% of the radioactivity incorporated was found to be insoluble in ethylene dichloride. Furthermore, no radioactivity was released after incubation of the products in the presence of carboxypeptidase A, under conditions that solubilized much of the ribosome-directed Leu incorporation (Table III).

Incorporated Leucine Is Not Linked to the Acceptor Protein through a Peptidic Bond. Since the transferase-directed Leu incorporation is not likely to occur at the COOH terminus of the protein acceptor and since the incorporation does not involve the NH_2 group of [^{3}H]leucine, one might imagine that the Leu incorporation involves a peptide bond formation between the COOH group of Leu and the NH_2 terminus of the protein acceptor. Four types of experiments make that proposition unlikely. (i) Incubation of the incorporated products in the presence of leucine aminopeptidase does not solubilize any radioactivity (Table III). Under the same conditions, 57 ± 9% of the Leu incorporated by the *E. coli* leucyl, phenylalanyltRNA protein transferase was made TCA soluble by the aminopeptidase. (ii) The bond uniting the transferase-directed Leu to its protein acceptor is labilized by a 20-min incubation at 37 °C in the presence of as little as 0.05 N KOH (Table III). (As a comparison, none of the Leu incorporated by the *E. coli* leucylphenylalanyl-tRNA transferase is rendered TCA-soluble by a 30-min incubation at 37 °C in the presence of as much as 0.5 N KOH). (iii) A 20-min incubation of the transferase-directed incorporation product in the presence of 0.5 M NH_2OH at pH 7.3 suffices to render TCA soluble more than half the incorporated radioactivity. (On the contrary, 105 ± 12% of the Leu incorporated by the *E. coli* transferase remains TCA insoluble after a 30-min incubation at 37 °C in the presence of 1 M NH_2OH at pH 7.3.) (iv) If the [^{3}H]leucine was getting incorporated at the NH_2 terminus of the protein acceptor, one could expect, as was observed with the mammalian arginyl-tRNA transferase and the *E. coli* leucylphenylalanyl-tRNA transferase (Soffer, 1973a,b), that the addition of appropriate TCA-soluble dipeptides to the incubation mixture might compete with the TCA-precipitable protein acceptor and reduce the TCA-precipitable Leu incorporation. Leucine transferase activity was assayed in the presence of 10 mM of any one of the following dipeptides: Ala-Asp, Arg-Ala, Arg-Pro, Asp-Ala, Glu-Gly, Gly-Asn, His-Ser, Ile-Ala, Lys-Phe, Met-Val, Phe-Ala, Pro-Ala, Thr-Phe, and Trp-Ala. Depending on the dipeptides, the incorporation activities were either unchanged or differed by at most 22% from those of controls incubated in the absence of dipeptides.

Transferase Activity Is NEM Sensitive but the Incorporated Leucine Is Not Linked to the Acceptor Protein through a Classical Thiol Ester Bond. It may be hypothesized that the incorporated Leu is linked to the acceptor protein through a thiol ester bond between the side chain of a Cys residue and the COOH group of the Leu. To test this hypothesis, we incubated the incorporated product at 37 °C in the presence of 30 mM $HgCl_2$. None of the incorporated leucine got released into a TCA-soluble form. [Thiol esters, such as acetyl coenzyme A, acetoacetyl coenzyme A, and thioaspirin (Bender, 1960; Lynen et al., 1951), are known to undergo hydrolysis almost instantaneously in the presence of stoichiometric amounts of mercuric ion salts.] On the other hand, the S20 lost more than 95% of its transferase activity upon reaction with *N*-ethylmaleimide. These results, taken together, suggest that while the active site(s) of the transferase involve(s) one or several SH groups, the covalent bond linking the incorporated leucine to the acceptor protein does *not* contain any sulfur.

Discussion

Posttranslational modifications of proteins involve a myriad of reactions. It has been estimated that if all the proteins from a given organism were subject to careful hydrolysis of only the peptide bonds, several hundred different amino (imino)

acids would be released (Wold, 1981). We have identified, in a rat brain in vitro system, a Leu incorporation activity that is tRNA dependent but ribosome independent. This incorporation activity belongs to the category of posttranslational modification of proteins and represents Leu addition to a protein at a site most likely other than the NH_2 terminus or the COOH terminus. The incorporated leucine has a free NH_2 group, and it is linked to its acceptor protein through a link that is resistant to Hg^{2+} but too alkali labile to be a peptide bond. The incorporated leucine is labilized by a 20-min incubation in the presence of 0.5 M NH_2OH or 0.05 N KOH (Table III). In contrast, peptide bonds are not significantly labilized in the presence of hydroxylamine at neutral pH (Nuenke & Cunningham, 1961; Raacke, 1958) or in the presence of 1 N KOH, at 37 °C, for a few hours (Warner, 1942; Hammel & Glasstone, 1954; Leach, 1953).

The simplest interpretation of the data is that the leucine is linked through an ester bond to the hydroxyl group of some serine(s), threonine(s), or tyrosine(s) in the acceptor protein. The nature of the acceptor protein(s) is unknown. The addition to the incubation mixture of bovine serum albumin, cytochrome *c*, hyaluronidase, cellulase, aprotinin, or phosphoglucomutase neither inhibits nor stimulates the rate of incorporation.

The activity that we have characterized here differs from the bacterial leucylphenylalanyl-tRNA transferase and the mammalian arginyl-tRNA transferase activity in several respects. It differs from the bacterial enzyme by its greater resistance to puromycin (Kaji et al., 1965; Momose & Kaji, 1966; Leibowitz & Soffer, 1970) and its unreactivity with such dipeptides as Arg-Ala and Arg-Pro (Soffer, 1973b). It differs from the mammalian enzyme by its inability to transfer [^{3}H]leucine to the acceptors Asp-Ala, Glu-Gly, and bovine serum albumin (Soffer, 1973a; Soffer & Horinishi, 1969). It differs from both the bacterial enzyme and the mammalian enzyme by its specificity for Leu, the site of attachment of the amino acid to the acceptor protein [the bacterial leucylphenylalanyl-tRNA transferase and the mammalian arginyl-tRNA transferase transfer their cognate amino acid to the NH_2 terminus of the acceptor proteins (Soffer, 1974)] and the ester-like alkali and hydroxylamine lability of the bond that links leucine to the acceptor protein.

Posttranslational addition of amino acids could play an important regulatory role in, for example, cellular proliferation and aging processes (Kaji, et al., 1980). In mouse liver, chromosomal proteins can accept Arg through a tRNA-dependent but ribosome-independent mechanism (Kaji, 1976). Moreover, arginyl-tRNA transferase activities can be used as an aging marker both in human cultured fibroblasts and in rats (Lamon & Kaji, 1980; Kaji et al., 1980). In *E. coli*, a mutant has been identified that lacks leucyl-tRNA transferase activity. This mutant had an altered rate of proline catabolism, an abnormal cell morphology, and a reduced lactose operon expression (Freedman & Deutch, 1981).

Further work will be needed in order to determine whether the posttranslational addition of leucine described in this paper occurs in vivo and plays a positive role in brain metabolism.

Acknowledgments

I am grateful to Drs. L. Brakier-Gingras, L. Kleiman, J. Perdue, and H. Schulman for critical reading of the manuscript. Sprague-Dawley rats were a gift from Dr. Claude Côté. I am indebted to J. Latulippe for skillful technical assistance and to H. Karam for typing the manuscript.

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A Naturally Occurring Pyrimidodiazepine in *Drosophila*: Chemical and Spectral Properties and Relationship to Drosopterin†

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ABSTRACT: The structure of an intermediate, in drosopterin biosynthesis, as 6-acetylpyrimidodiazepine has been confirmed by high-resolution mass spectra, ^{13}C NMR, chemical ionization mass spectra, and chemical properties. A trivial name of 6-acetylhomopterin is suggested and should replace the term "quench spot" used heretofore. The structure of drosopterin includes, in part, a pyrimidodiazepine, a compound that consists of a fused six- and seven-membered heterocyclic ring system. Earlier studies demonstrated that 6-acetylhomopterin strongly stimulated the enzymatic synthesis of drosopterin and related eye pigments by preparations from *Drosophila*. The occurrence in nature is quite limited for diazepines; drosopterin and homopterin are the first examples in eukaryotes.

The regulation of the synthesis of the red drosopterin eye pigments of *Drosophila* is being examined from biochemical and genetic viewpoints. The concentrations of the drosopterins and of a number of simple pterin derivatives in the heads of several mutants have been determined (Wilson & Jacobson, 1977). In the course of this study a substance was found that had properties not typical of pteridines but affected by the same mutations that affect the drosopterins. This substance was termed quench spot (QS)[1] since, in contrast to the pteridines, it failed to fluoresce under certain circumstances. Subsequently quench spot was found to be an apparent intermediate in the enzymatic biosynthesis of pteridines (Dorsett & Jacobson, 1982). Also, when Wiederrecht et al. (1981) were studying drosopterin biosynthesis, they noted that a heat-stable stimulatory factor is present in extracts of *Drosophila* heads. They isolated this factor and proposed that its structure was that of a diazepine ring fused to a pyrimidine, i.e., a pyrimidodiazepine.

Seven-membered rings such as the tropolones and diazepines are uncommon natural products. Diazepines have been obtained from a few microorganisms (Suhadolnik, 1979; Oakami, 1973), but among animals the only diazepines reported are those in *Drosophila*. One is the pyrimidodiazepine (Wiederrecht et al., 1981); the other is drosopterin (Theobold & Pfleiderer, 1978).

This report concerns the nature of quench spot, offers evidence for the pyrimidodiazepine structure, beyond those presented by Wiederrecht et al. (1981), and presents some of its properties.

Materials and Methods

Chromatography media were obtained as follows: CM-23 and CM-52, forms of carboxymethylcellulose, and cellulose (CC-31) were from Whatman. The Zorbax C8 reversed-phase column (0.46 × 25 cm) was from Du Pont. HPLC-grade methanol came from Burdick and Jackson or Fisher Scientific. The C_{18} Sep-PAK was from Waters Associates. Thin-layer cellulose sheets (No. 13255) were from Eastman Kodak.

The vacuum cleaner was a "Kenmore Hand Vac" (Model 208, 61110, from Sears, Roebuck and Co.).

The reagent gases for chemical-ionization mass spectrometry were obtained from the following sources: isobutane from Matheson Gas Products, Inc. (East Rutherford, NJ); D_2O (>98.5 atom % D) from Aldrich Chemical Co. (Milwaukee, WI); ND_3 (>99 atom % D from Merck and Co. (St. Louis, MO). *N,O*-Bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane was purchased from Pierce Chemical Co. (Rockford, IL).

Mass Spectrometry. Electron-ionization mass spectra were recorded by using a Varian MAT 731 mass spectrometer, with samples introduced by direct probe (ionizing energy 70 eV, ion source temperature 200 °C). Low-resolution mass spectra were acquired by magnetic scanning; high-resolution spectra were recorded photographically at a resolution of 16 000 by using Ionomet-evaporated AgBr plates. Standard mass com-

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‡ Supported by Genetics Training Grant GM 7438. Present address: Department of Virology, Weizmann Institute, Rehovot, Israel. The mass spectrometry experiments reported here were supported by National Institutes of Health Grant GM 29812 to J.A.M. This research was sponsored by the Office of Health and Environmental Research, U.S. Department of Energy, under Contract W-7405-eng-26 with the Union Carbide Corporation.

[1] Abbreviation: QS, quench spot.

0006-2960/82/0421-5700$01.25/0