Transfer Ribonucleic Acid Methylases of Bone

STUDIES ON VITAMIN A AND D DEFICIENCY

By DAVID S. BRADFORD,* BRUCE HACKER and IRWIN CLARK†

*Departments of Orthopaedic Surgery and Biochemistry, College of Physicians and Surgeons,
Columbia University, New York, N.Y. 10032, U.S.A.,*

and

*Department of Pharmacology and Toxicology, Division of Oncology,
University of Rochester School of Medicine, Rochester, N.Y. 14620, U.S.A.*

(Received 6 August 1971)

Methods were devised for the assay of tRNA methylases of rat bone. The activities of bone tRNA methylases are similar to those from other mammalian tissues. However, unlike reports on liver methylases, no inhibitors were found in the supernatant fraction from pH 5 precipitate of bone extracts. The effects of vitamins A and D on the methylation of tRNA by cell-free extracts of rat bone were studied. Deficiency of either vitamin resulted in a decrease in the rate and extent of tRNA methylation, whereas the administration of vitamin A to hypovitaminotic-A rats and vitamin D to hypovitaminotic-D rats increased the rate and extent of tRNA methylation. These effects appear to be apart from changes in ribonuclease activity or in concentrations of calcium or magnesium. No evidence of inhibitors of tRNA methylases was found in bone extracts from vitamin-deficient rats nor of activators in bone extracts from deficient rats given vitamin A or D. The pattern of tRNA methylation under conditions of vitamin A or D deficiency was not changed, suggesting a generalized cellular deficiency. It was of significance to find that the specificity for methylation of specific bases in tRNA was different after the administration of vitamin A as contrasted with the effects of vitamin D. The possible significance of tRNA methylation to the biochemical action of the vitamins on bone is discussed.

The tRNA methylases are a unique group of enzymes that transfer methyl groups from methyl-S-adenosylmethionine to specific locations on bases of tRNA at the macromolecular level (Fleissner & Borek, 1963). These enzymes are species- and base-specific (Srinivasan & Borek, 1963) and recognition of substrate appears to involve a sequence rather than single nucleotides (Baguley & Staehelin, 1969). The function of the methylated bases in tRNA is at present not known. It is known, however, that changes in tRNA methylases, both in extent and specificity of methylation, occur in tissues undergoing differentiation and shifts in control mechanisms (Wainfan *et al.*, 1966; Kerr & Dische, 1970; Hancock *et al.*, 1967; Kaye *et al.*, 1966; Simon *et al.*, 1967).

Bone is an unusual tissue in that it undergoes continued cellular differentiation and architectural remodelling throughout life. These activities are influenced by vitamins A and D; the molecular basis of their action, however, is not clear. It is well estab-

lished that excessive amounts of vitamin D cause increased bone resorption (Harris & Innes, 1931; Follis, 1950; Clark & Bassett, 1962) and at the same time there are increased amounts of collagen and mucopolysaccharides in the skeleton (Clark & Bassett, 1962; Clark & Smith, 1964). More recent work has shown that vitamin D increases the rate of collagen synthesis (Canas *et al.*, 1969), which may account for the increased amounts of collagen found in bones of hypervitaminotic-D rats (Clark & Smith, 1964). Deficiency of this vitamin produces the clinical condition known as rickets, which is characterized by growth cessation, a widened epiphyseal plate and an inhibition of mineralization. Despite these disturbances in ossification, collagen synthesis is also increased in rachitic bone (Paterson & Fourman, 1968).

Excessive amounts of vitamin A hasten bony maturation and remodelling in the young rat (Wolbach, 1947) and lead to bone rarefaction, fragility and matrix destruction in older rats (Moore & Wang, 1945; Clark & Bassett, 1962; Clark & Smith, 1964; Belanger & Clark, 1967). Bone growth is disturbed in avitaminosis A resulting in bones that are shorter but normal in shape (Wolbach, 1947). However, it has not been completely resolved whether the

* Present address: Department of Orthopaedic Surgery, University of Minnesota Medical Center, Minneapolis, Minn. 55455, U.S.A.

† Present address: University of North Carolina School of Medicine, Orthopaedic Research Laboratories, Chapel Hill, N.C. 27514, U.S.A.

disturbance is in endochondral (Wolbach, 1947) or appositional growth (Mellanby, 1944).

It is apparent that both of these vitamins are of crucial importance in maintaining normal growth, maturation and protein synthesis in bone. This work was undertaken for the purpose of studying the tRNA methylases of normal bone and the role of these vitamins during the processing of tRNA at the level of enzymic methylation. Results are presented in the present paper demonstrating that vitamins A and D affect both the rate and extent of tRNA methylation by extracts from rat bone, and that these vitamins may mediate the methylation of specific bases.

Part of this work was presented at the Orthopaedic Research Society Meetings in San Francisco, January 1971.

Materials and Methods

Animals and diets

Male weanling Holtzman rats were fed on a diet deficient in vitamins A and D. The diet contained 24% vitamin-free casein, 60% Cerelease, 10% Mazola oil, 2% Alphacel and 4% salt mix, which contained all essential trace elements. The salt mix was prepared such that the final diet contained 0.8% calcium, 0.4% phosphorus, 0.36% sodium, 0.12% potassium and 0.25% magnesium. All known vitamins including choline chloride were added in maintenance dosages to the diet with the exception of vitamins A and D. The vitamin D-deficient rats were fed on 125 i.u. of vitamin A weekly and the vitamin A-deficient rats were given 75 i.u. of vitamin D. The control rats were given 125 i.u. of vitamin A and 75 i.u. of vitamin D. All rats received their vitamins orally three times weekly. Deficiency states produced by depletion in vitamin A or D were evidenced by decreased weight gain as well as by both gross and microscopic appearance of the bones and cartilaginous growth plates. The experimental groups consisted of: (1) vitamin D-deficient rats, (2) control rats, pair-fed to the vitamin D-deficient ones, (3) vitamin A-deficient rats, (4) control rats pair-fed to the vitamin A-deficient ones, (5) vitamin D-deficient rats which were fed 1000 i.u. of vitamin D orally for 3 days, (6) vitamin A-deficient rats which were fed 1000 i.u. of vitamin A orally for 3 days, (7) control group fed *ad libitum*.

Materials

[¹⁴C]RNA (specific radioactivity 59.6 μ Ci/mg) and [¹⁴C]methyl-S-adenosylmethionine (specific radioactivity 30–50 mCi/mmol) were purchased from Schwarz BioResearch Inc., Orangeburg, N.Y., U.S.A. tRNA species from yeast and rat liver were obtained from General Biochemicals Inc., Chagrin Falls, Ohio,

U.S.A. Ribonuclease-free sucrose was purchased from Mann Research Laboratories Inc., New York, N.Y., U.S.A. Bentonite was prepared by the method of Barlow & Mathias (1966).

Preparation of enzyme

Tibial bone and femoral bone were obtained from experimental animals after decapitation. Soft tissue and periosteum were dissected from the bones along with a major portion of articular cartilage in a cold room at 4°C. The epiphysis was carefully prised away from the metaphysis at the cartilaginous zone of hypertrophy and the metaphysis was sharply divided from the diaphysis. The three portions were minced into smaller pieces and irrigated copiously with 0.25 M-sucrose under suction to remove marrow elements. Histological preparations of the irrigated bone fragments confirmed the relative purity of the preparation, i.e. very few marrow elements were noted in comparison with the number of skeletal cells. By using 10 strokes of a metallic hand crusher (Thermovac Industries Corporation), the bone chips were broken into small particles, which were then suspended in 4 vol. of 0.25 M-sucrose containing 0.5% bentonite, stirred mechanically, and centrifuged at 600g for 10 min at 4°C. The supernatant fraction was centrifuged at 105 000g for 60 min at 4°C and the enzyme extract obtained by puncturing the bottom of the centrifuge tube and allowing all the supernatant except the lipid cap to drain through the hole. The lipid fraction has been shown by Kaye & Leboy (1968) to inhibit methylase activity in addition to contributing to high blank values. The methylase activity of the supernatant fraction was stable at –15°C for several months. From results of preliminary experiments, bone methylases were most active when prepared in sucrose and bentonite without additional ions or reducing agents.

Liver extracts were prepared by homogenizing fresh liver in 3–4 vol. of 0.25 M-sucrose containing 0.5% bentonite in a Potter–Elvehjem homogenizer at 4°C. The homogenate was centrifuged at 600g for 10 min at 4°C. The supernatant fraction was collected, centrifuged at 105 000g for 60 min at 4°C and stored at –15°C.

Assay of tRNA methylase activity

Methylation *in vitro* was carried out by the method of Fleissner & Borek (1963). *E. coli* B tRNA showed superior capacity as compared with rat liver or yeast tRNA in accepting [¹⁴C]methyl groups catalysed by tRNA methylases from epiphyseal extracts. tRNA from *E. coli* accepted 7 times as many methyl groups as yeast tRNA and 70-fold more methyl groups than rat liver tRNA. With enzymes from rat liver, Kaye & Leboy (1968) have reported similar findings.

In all experiments dealing with bone methylases, tRNA from *E. coli* B was used.

The standard incubation mixture contained in a total volume of 0.6ml: 60 μ mol of tris-HCl buffer, pH 8.2; 3 μ mol of $MgCl_2$; 5 μ mol of 2-mercaptoethanol; 200 μ g of tRNA from *E. coli* B; 0.5 μ Ci of [^{14}C]methyl-S-adenosylmethionine; various amounts of enzyme protein. The reaction mixture was incubated at 37°C for 30min and then 1ml of 0.1M-sodium pyrophosphate was added to remove acylamino acids from the tRNA. Incubation was continued for another 10min and, after the addition of 1mg of yeast tRNA as carrier, an equal volume of water-saturated phenol (88%) was added. The mixture was shaken vigorously for 30min and the aqueous layer removed quantitatively after centrifugation for 20min at 1500g. The aqueous layer was extracted with phenol to remove the last traces of protein and the tRNA was precipitated with 1.5ml of 10% (w/v) trichloroacetic acid in an ice bath. The precipitate was collected on glass-fibre filter (Whatman GF/C, 2.4cm), repeatedly washed with 5% (w/v) trichloroacetic acid, and dried under an i.r. lamp. The filter containing the precipitate was suspended in 10ml of toluene containing 0.5% 2,5-diphenyloxazole and 0.03% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene and the radioactivity was counted in a liquid-scintillation spectrometer. Together with each reaction mixture, the radioactivity of a blank consisting of all components except the *E. coli* B tRNA was determined. With the extraction technique described above, the blank value was usually 50–60 c.p.m. above background.

For the vitamin experiments, extracts prepared from the distal femoral and proximal tibial, epiphyseal and metaphyseal bones together with the intervening growth plate were combined in an attempt to cancel out any variations that might arise from imprecise cleavage at the cartilaginous growth plate, particularly in the vitamin D-deficient animals. The extent of methylation was also determined in each extract to assure that there was excess of enzyme in the reaction mixture.

Ribonuclease activity was assayed by incubating 10 μ Ci of [^{14}C]RNA in solution containing 60 μ mol of tris-HCl buffer, pH 8.2, 3 μ mol of $MgCl_2$, 5 μ mol of 2-mercaptoethanol and various amounts of enzyme protein in a final volume of 2.0ml. The mixture was incubated at 37°C and 0.5ml samples were removed after 0, 10, 20 and 30min. Yeast tRNA (1mg) was added as a carrier and the tRNA precipitated at 0°C with 1ml of 10% (w/v) trichloroacetic acid. This was then collected on a glass-fibre filter (Whatman GF/C, 2.4cm) and washed with 5% trichloroacetic acid. The dried filter was assayed for radioactivity by the procedure described above for methylated tRNA.

Magnesium and calcium were determined with a Perkin-Elmer Atomic Absorption Spectrometer.

Protein concentrations were determined by the procedure of Lowry *et al.* (1951), with bovine albumin as the reference standard.

Assay of inhibitor activity

To determine if the high-speed supernatant fraction contained inhibitors of tRNA methylases, the pH of the supernatant fraction was adjusted to pH 5.0 by the addition of 1M-acetic acid and centrifuged at 10000g for 15min at 4°C as described by Kerr (1970). The supernatant fraction was removed, neutralized and tested as a source of inhibitor. The precipitate was washed once with 0.25M-sucrose containing 2mmol of EDTA, pH 5.0, and was suspended in 10mm-tris-HCl buffer, pH 8.2, containing 2mmol of EDTA and 5mmol of 2-mercaptoethanol. Care was taken not to dilute or concentrate the precipitate, and volumes of buffer equal to the original extract were used.

Analysis of methylated nucleosides

Large-scale incubations were carried out at saturation amounts of each enzyme in a manner similar to that described for assay of enzyme activity with the following modifications. The amounts in the reaction mixture were increased 10–15-fold. After incubation at 37°C, 6ml of 0.1M-sodium pyrophosphate was added and the incubation continued for an additional 10min. Nucleosides were isolated after enzymically treating the radioactive tRNA products, and characterized by the Celite partition procedures described by Hacker & Mandel (1969).

Results

Effect of incubation time on rate of tRNA methylation of E. coli by bone extracts

Extracts of epiphyseal bone were prepared as described above and incubated for periods up to 1h. A progressive increase in [^{14}C]methyl groups incorporated was noted during this period as long as tRNA was in excess and not rate-limiting (Fig. 1). This linear rate of incorporation of [^{14}C]methyl groups into tRNA is similar to those reported by other workers using methylases from sources other than bone (Kerr, 1970; Hacker, 1969; Leboy, 1970).

Methylase activity of epiphyseal, metaphyseal and diaphyseal rat bone extracts

In this experiment, the distal femoral epiphyses and metaphyses of nine rats weighing 400–500g were used. Fig. 2 shows the comparison between the different areas of bone. In rats of this size, metaphyseal and epiphyseal bone had the greatest methylating activity and diaphyseal bone had the least. Enzyme

saturation of the substrate (a measure of the extent of methylation) was achieved only in epiphyseal bone under conditions of the assay. Less protein per ml of

Fig. 1. Effect of incubation time on the incorporation of [¹⁴C]methyl groups from [¹⁴C]methyl-S-adenosyl-methionine into *E. coli* B tRNA

Preparation of bone extract and method of assay are described in the Materials and Methods section.

enzyme extract was recovered from diaphyseal bone than from metaphyseal or epiphyseal bone, and this would be expected since the diaphysis is the least cellular part of bone. It is of note that liver homogenates from this group of animals had less methylase activity/mg of protein than did epiphyseal or metaphyseal extracts (Fig. 2). The reason for this is not clear. It is possible that greater amounts of non-enzyme protein were extracted from liver than from bone, and thus attempts to compare methylase activity/mg of protein between these two tissues might give misleading conclusions. It is conceivable also that extracts from bone contain activating substances that stimulate methylase activity; however, an inherently greater bone methylase activity cannot be ruled out.

Stimulation of bone methylase activity in vitro

Alterations in polyamine content in newborn tissue (Rosenthal & Tabor, 1956), regenerating liver (Janne, 1967) and tumours (Neish & Key, 1967) have been found; conditions in which tRNA methylases are known to show increased activity. Ammonium ions also have been shown to stimulate tRNA methylation by liver extracts and undialysed extracts of KB and HeLa human carcinoma cells (Kaye & Leboy, 1968; Rodeh *et al.*, 1967; Zeleznick, 1967). It

Fig. 2. Comparison of methylating activity in extracts from different areas of bone and liver

Original protein concentrations of extracts were (mg/ml): epiphyseal bone (●), 5.47; metaphyseal bone (○), 5.96; diaphyseal bone (▲), 1.88; liver (■), 42.0. Preparation of extracts and assay conditions are described in the Materials and Methods section.

was therefore decided to determine whether ammonium ions and polyamines had similar effects on bone methylase activity. To obtain sufficient enzyme, extracts of distal femoral epiphyses and metaphyses were combined with those from proximal tibial epiphyses and metaphyses from three rats, weighing 380–400 g. Ammonium acetate (0.36M) in the reaction mixture resulted in a 4–5-fold rise in methylating activity and the polyamines putrescine (20mmol) or spermidine (5mmol) resulted in approximately a twofold rise in activity.

Effects of vitamins A and D on tRNA methylase activity

The bones of vitamin A-deficient rats and particularly those of the vitamin D-deficient animals showed a marked decrease in tRNA methylase activity. This effect was not the result of inanition since the same trend was not observed in the pair-fed animals. In four separate experiments the administration of vitamin A or D to the corresponding vitamin-deficient group partially, but consistently, restored the loss of enzymic activity (Fig. 3).

Since the liver is a site of the metabolic conversion of vitamin D into an 'active factor' (DeLuca, 1969) and the principal storage place of vitamin A (Moore, 1931), it was decided to evaluate the effects of these vitamins on liver tRNA methylase activities. The response to vitamin A and D deficiency resulted in elevated liver methylase activity, which was the opposite pattern to that found in bone cells (Fig. 4). After treatment with the vitamins there was, however, a trend in values approaching that found in control animals.

Ribonuclease activity in bone extracts was assayed in all groups to exclude the possibility of tRNA degradation, which might interfere with methylation of tRNA. No significant ribonuclease activity was detected in any of the preparations.

Effects of vitamins A and D on the extent of tRNA methylation

Because of the differences noted in the rate of tRNA methylation in vitamin A and D deficiency it was decided to determine the effects of these vitamins

Fig. 3. Rate of tRNA methylase activity of bones from vitamin A- and vitamin D-deficient, pair-fed and control rats

The reaction mixture contained 200 μ g of *E. coli* B tRNA as substrate, 60 μ mol of tris-HCl buffer, pH 8.2, 3 μ mol of mercaptoethanol, 0.5 μ Ci of [14 C]methyl-S-adenosylmethionine (specific radioactivity 24 mCi/mmol) and various amounts of enzyme protein to a final volume of 0.6 ml. Incubations were carried out at 37°C for 30 min. (a) Vitamin D series; (b) vitamin A series. $\circ$, Control rats; $\blacktriangle$, vitamin pair-fed rats; $\bullet$, vitamin-deficient rats; $\blacksquare$, vitamin-deficient-treated rats.

on the extent of tRNA methylation. Preliminary experiments were carried out to determine the maximal transfer of [^{14}C]methyl groups to tRNA in a

Fig. 4. Rate of tRNA methylase activity from the rat livers of the seven study groups

Incubations were carried out as described in the Materials and Methods section.

1 h period where the enzyme was in excess. Table 1 shows that bone extracts from animals deficient in vitamin D and which had been treated with 1000i.u. of vitamin D for 3 days had a 2.6-fold increase in methylase activity. In contrast, extracts from animals deficient in vitamin A and treated

Table 1. Extent of methylation with undialysed rat bone extracts

E. coli tRNA (20 μg) was incubated in the presence of excess amounts of enzyme from experimental rat bones. Protein concentrations (mg/ml) of enzyme extracts were; control, 1.43; vitamin D deficiency, 1.79; vitamin D deficiency-treated, 2.08; vitamin A deficiency, 1.99; vitamin A deficiency-treated, 2.12. [^{14}C]Methyl-S-adenosylmethionine (specific radioactivity 49mCi/mol) (2nmol) was used for each experiment. The deficiency-treated rats were given 1000i.u. of the missing vitamin for 3 days before preparation of the bone extracts.

Treatment	Incorporation of [^{14}C]methyl groups (pmol)
Control	51.4
Vitamin D deficiency	19.8
Vitamin D deficiency-treated	51.4
Vitamin A deficiency	44.4
Vitamin A deficiency-treated	57.3

Table 2. Distribution of [^{14}C]methyl nucleosides derived from enzymic hydrolysis of radioactive methylated *E. coli* B tRNA and the effects of vitamins A and D

Large-scale tRNA methylase assays were prepared as described in the Materials and Methods section. Radioactive nucleosides, obtained after enzymic hydrolysis of *E. coli* B tRNA with snake venom phosphodiesterase and bacterial alkaline phosphatase, were characterized by using partition column and paper chromatography techniques (Hacker & Mandel, 1969). The values in the table are expressed as percentages of recovered radioactivity.

		Radioactivity (% of total)				
Partition column fraction	Nucleosides	Control	Vitamin D deficiency	Vitamin D deficiency-treated	Vitamin A deficiency	Vitamin A deficiency-treated
Purines						
I	<i>N</i> ¹ -Methyladenosine	6.69	6.81	9.62	7.20	9.00
II	<i>N</i> ⁶ -Methyladenosine	8.79	3.33	4.63	3.83	3.55
V	<i>N</i> ⁷ -Methylinosine	3.35	22.0	13.7	20.9	24.3
IV	<i>N</i> ² -Methylguanosine	45.6	28.2	33.7	34.4	35.4
	<i>N</i> ² <i>N</i> ² -Dimethylguanosine	13.0	13.8	14.8	13.5	1.02
	<i>O</i> ² -Methylguanosine	13.4	10.7	8.37	8.10	10.5
	Total	90.8	84.8	84.8	87.9	83.8
Pyrimidines						
III	5-Methyluridine	1.26	4.22	5.71	3.06	2.28
VI	5-Methylcytidine	7.53	10.4	8.95	8.65	13.70
	Total	8.8	14.6	14.7	11.7	16.0

with 1000 i.u. of vitamin A gave only a 20% increase in tRNA-methylating enzyme specific activity.

Composition of [^{14}C]methyl nucleosides in radioactive tRNA products

The results presented in Table 2 summarize the composition of the methylated nucleosides isolated from each tRNA product formed by using methylases from bone cells of rats. Methylase for N^1 -methylguanosine, N^7 -methylguanosine and 3-methylcytidine were not detected in any enzyme preparations.

In general, changes in the methylation were essentially the same in vitamin A and D deficiency (Table 2). Although there was an overall decrease in methylation of the purine derivatives, particularly with regards to N^6 -methyladenosine, an increase of about 7-fold was found in N^7 -methylinosine formed. No significant changes were found in N^1 -methyladenosine or N^2 -dimethylguanosine. On the other hand, methylation of pyrimidine derivatives was increased in the vitamin-deficient animals, in particular the amount of 5-methyluridine.

Although similar changes were noted in the methylation of tRNA by extracts of bones from vitamin A- and D-deficient rats, the administration of the missing vitamin had rather anomalous effects on the patterns of methylation. Both vitamins increased methylation of N^1 -methyladenosine and N^2 -methylguanosine. Although N^1 -methyladenosine may be converted into N^6 -methyladenosine under alkaline conditions, it occurs only to a limited extent under enzymic hydrolysis (Hacker & Mandel, 1969). Confirmation of the existence of N^6 -methyladenosine in mammalian tRNA has been obtained by using acid hydrolysis. However, vitamin D increased the amount of N^6 -methyladenosine and N^2 -dimethylguanosine formed about 15% and 8% of their control values. Also, vitamin D decreased the methylation of N^7 -methylinosine and O^2 -methylguanosine,

whereas vitamin A increased it. Differences were also noted in the methylated pyrimidine derivatives. Vitamin D treatment increased methylation of 5-methyluridine but decreased methylation of 5-methylcytidine whereas vitamin A had the opposite effects.

The possibility that changes in Ca^{2+} or Mg^{2+} concentrations induced by vitamin deprivation might be a factor in altering total tRNA methylase activity was investigated. No correlation could be found between the concentrations of these ions in the enzyme extracts and methylase activity. These findings suggest that the changes in methylating activity were not related to alterations in the total calcium or magnesium concentrations of the medium.

Studies on bone methylase inhibitors

Rodeh *et al.* (1967) have found that the pattern of methylation changed as crude enzymes were purified and Baguley & Staehelin (1969) have implied that increased methylation found in purified extracts was the result of elimination of inhibitors. Kerr (1970) demonstrated an inhibitor of tRNA methylases in adult rabbit and rat organs which is non-diffusible, heat-labile and sensitive to trypsin digestion. In attempts to determine whether normal bone preparations contained inhibitors of low molecular weight, the 105000g supernatant was dialysed at 4°C against two 4-litre changes of 0.25M-sucrose in 5mM-tris-HCl buffer, pH 8.2. After 1 h no significant decrease in methylating activity was found in the non-diffusible material, but after a further 2 h dialysis against two further 4-litre changes of buffered sucrose there was a 20% decrease in methylating activity; 24 h dialysis against two additional buffer changes resulted in an 80% loss in activity (Table 3). Ca^{2+} concentration in the extract was determined before and after dialysis and found to be unchanged. Mg^{2+} removal would not appear to be a factor since this ion is added to the

Table 3. *Effect of dialysis and pH5 supernatant on bone methylase activities*

Enzyme fractions were prepared and assayed as described in the Materials and Methods section. The pH5 precipitate was taken up in a volume of buffer equal to the original volume and incubations were carried out for 30 min at 37°C. All assays contained 200 μg of tRNA, 20 μmol of [^{14}C]methyl-S-adenosylmethionine, and the volume of enzyme assayed was 0.2 ml.

Enzyme preparation	Protein (mg/ml)	Incorporation of [^{14}C]methyl groups (nmol/mg of protein)
Original extract (105000g supernatant)	1.87	64
After dialysis (1 h)	1.93	63
After dialysis (3 h)	1.95	45
After dialysis (24 h)	1.95	12
pH5 precipitate	1.34	68
Re-neutralized pH5 supernatant	0.51	3
pH5 precipitate + pH5 supernatant		71

assay system. These experiments suggest that dialysis does not remove inhibitors but rather may remove activators or necessary cofactors or perhaps may inactivate the enzymes since methylases in general are relatively unstable.

To determine if the high-speed supernatant fraction of normal bone extracts contained inhibitors of tRNA methylases similar to those found in liver, an extract was prepared by the method of Kerr (1970). No appreciable increase in methylating activity of the pH5 precipitate over the 105000g supernatant fraction could be found (Table 3). This may perhaps be the result of instability of certain base-specific methylases whose lability has been demonstrated in other systems (Kerr, 1970). Addition of the re-neutralized pH5 supernatant to the pH5 precipitate did not reveal inhibitor activity. A small amount of activity was found in the neutralized pH5 supernatant, which could have been from protein not completely removed by precipitation. From these experiments it would appear that there is no appreciable amount of inhibitor in bone extracts.

In view of the consistent alterations shown for tRNA methylase activity in bone brought about by vitamin A and D deficiency, both with respect to the

rate and extent of methylation, further studies were undertaken to determine the presence or absence of activating or inhibiting substances. The results shown in Table 4 indicate that methylase activity in the presence of extracts from bones of untreated control animals with or without preparations from vitamin-deficient animals was about the same. This suggests that inhibiting or activating substances were not present in the bones of animals fed on vitamin-deficient diets. Enzyme extracts from bones of vitamin D-deficient and vitamin A-deficient rats were added in different proportions to enzyme extracts from vitamin-deficient animals that had been treated with the missing vitamin. Again the values were additive, thus demonstrating the absence of inhibition or activation.

When enzyme extracts from the vitamin A- and D-deficient rat bone were precipitated at pH5 by the method of Kerr (1970), the presence of inhibitor substances was not detected (Table 5). The apparent inability to increase bone enzyme specific activity of the vitamin A- and D-deficient animals in the pH5 precipitate above that found in the 105000g supernatant is in agreement with results obtained with normal bone, again suggesting lability of certain methylases. Only traces of activity were found in the

Table 4. *Absence of methylase inhibitors or activators in bone extracts from vitamin A- or D-deficient rats*

Enzyme extracts from control bone were mixed in equal proportions with extracts from vitamin A-deficient and vitamin D-deficient rat bone. The reaction mixture contained 60 μ mol of tris-HCl buffer, pH 8.2, 3 μ mol of MgCl₂, 5 μ mol of 2 mercaptoethanol, 20 μ mol of [¹⁴C]methyl-S-adenosylmethionine (specific radioactivity 25 mCi/mmol) and 200 μ g of *E. coli* B tRNA in a final volume of 0.6 ml. Incubations were carried out at 37°C for 30 min. Protein concentrations were (mg/ml): control, 1.69; vitamin D deficiency, 2.44; vitamin A deficiency, 1.86.

Amount of enzyme extract and source (ml)					Incorporation of [¹⁴ C]methyl groups (pmol)
Control	Vitamin D- deficient bone	Vitamin D deficiency- treated bone	Vitamin A- deficient bone	Vitamin A deficiency- treated bone	
0.2					31.7
	0.2				21.4
0.2	0.2				50.7
			0.2		26.6
0.2			0.2		55.3
	0.2				25.4
		0.1			14.1
	0.2	0.1			40.5
		0.2			
	0.2	0.2			50.7
	0.1	0.2			41.2
			0.2		24.5
				0.1	14.4
			0.2	0.1	37.9
				0.2	28.7
			0.2	0.2	52.5
			0.1	0.2	40.2

Table 5. *tRNA methylase activities of pH5 precipitates and supernatants*

Enzyme fractions were prepared as described in the Materials and Methods section. Standard assay conditions were used. Protein concentrations (mg/ml): vitamin D-deficiency extract, 1.79; vitamin A-deficiency extract, 1.86.

Enzyme fraction	Volume used (ml)		Incorporation of [14 C]methyl groups (pmol/mg of protein)	
	Vitamin D deficiency	Vitamin A deficiency	Vitamin D deficiency	Vitamin A deficiency
105000g supernatant	0.2		51.1	
pH5 precipitate	0.2		50.1	
Re-neutralized pH5 supernatant	0.2		2.3	
pH5 precipitate+supernatant	0.4		60.8	
105000g supernatant		0.2		61.9
pH5 precipitate		0.2		63.6
Re-neutralized pH supernatant		0.2		3.8
pH5 precipitate+supernatant		0.4		69.3

re-neutralized pH5 supernatant fraction. Moreover, addition of re-neutralized pH5 supernatant fraction to the precipitate resulted in a greater [14 C]methyl-labelling of the tRNA than that found by using the original 105000g supernatant fraction. Although the differences were not very large, this perhaps suggests that the pH5 supernatant fraction of the deficiency groups contained some stimulatory material but there was no evidence of any inhibitory material.

Discussion

A fundamental role has been postulated for tRNA in the translation of the genetic code (Smith *et al.*, 1966; Carbon *et al.*, 1966; Wainwright & Wainwright, 1967). Although indirect evidence suggests that quantitative and qualitative alterations of specific tRNA species play an important role in differentiation, neoplasia and during metabolic homeostasis and that structural modifications of tRNA can markedly alter their function in translation, little in the way of definite evidence has been forthcoming to indicate the precise role of the tRNA species (for review of literature see Söll, 1971).

It was therefore noteworthy in the present study that deficiencies of vitamins A and D as well as feeding with the missing vitamins affect both the rate and extent of tRNA methylation by rat bone extracts. The results suggest that these effects are not related to inhibitors or activators of bone methylases since neither type of activity could be demonstrated, at least by the method of Kerr (1970).

At present the molecular basis of action of vitamin A or D is not known and it is not clear whether they affect primarily the transcription or translation process. However, it has been demonstrated that vitamin D is involved in the synthesis of certain proteins (Canas *et al.*, 1969; Paterson & Fourmann, 1968;

Wasserman & Taylor, 1963; DeLuca, 1969; Zull *et al.*, 1966). It has also been demonstrated that the nucleus is the major subcellular repository for radioactive 25-hydroxycholecalciferol, an active metabolite of vitamin D₃. Further, increased template activity for DNA-directed RNA synthesis in chromatin isolated from intestinal mucosal cells of animals treated with vitamin D has also been reported (Hallick & DeLuca, 1969), implying that vitamin D may have some role during the transcription of DNA. Also, vitamin A deficiency (Wolbach, 1947; Mellanby, 1944) or hypervitaminosis A results in increased bone protein formation or turnover (Belanger & Clark, 1967; Clark & Bassett, 1962; Clark & Smith, 1964), indicating the involvement of this vitamin during protein synthesis.

In general, most vitamin deficiencies are similar in that they hinder growth in young animals. The decreased methylase activity of the skeleton during vitamin A or D deficiency may simply reflect an inhibition of growth as a consequence of cellular deficiency and implies that tRNA methylases are an important class of enzymes during protein synthesis associated with skeletal growth. With the decrease in growth there is a marked fall in the methylation of *N*⁶-methyladenosine and *N*²-methylguanosine but an increase in the modified pyrimidines 5-methyluridine and 5-methylcytidine. Further, the ratio of methylated adenosines to the methylated guanosines fell in the deficiency state whereas the methylated uridines/methylated cytidines ratio rose. The significance of these altered ratios is not apparent at present. On the other hand, the feeding of vitamin A or D for only 3 days to the deficient rats reflects short-term changes in the cell. The results show that the effects of vitamins A and D on methylation of tRNA are different. This is particularly evident among the pyrimidine ratios where vitamin D increases the

methyluridine/methylcytidine ratio, whereas vitamin A restored the ratio to that found in the normal animal. These studies suggest that vitamins A and D may influence some part of translation (control). It has been suggested by others that vitamin A directly or indirectly is involved in protein synthesis at the translational level (DeLuca *et al.*, 1969). In this connexion, it is possible that the effects of these vitamins during differentiation, growth and development in certain bone cells may be regulated by the extent of tRNA processing, i.e. methylation.

Translation control affected by mammalian hormones has been suggested by Korner & Gumbley (1965) and Gorski & Palnos (1965). In addition it has been shown by Hacker (1969) that tRNA methylases in the chick oviduct are modulated by treatment with oestrogen. Work by Ilan *et al.* (1970) would imply that control of gene expression by juvenile hormone from *Tenebrio molitor* is at the translational level and involves the appearance of new tRNA and activating enzyme. It is noteworthy in this regard that variations in amounts of specific tRNA species have been shown to regulate the rate of protein synthesis at the translational level (Anderson, 1969). It has been shown that in the mammary gland increased methylating enzyme activity in response to hormone stimulation *in vitro* as well as during development *in vivo* paralleled closely a relative increase in tRNA (Turkington, 1969). Thus vitamins A and D may have similar influences to hormones at the translational level. The findings by Hallick & DeLuca (1969) of increased template activity of intestinal mucosal chromatin in animals treated with vitamin D would not rule out a translational mechanism of control. The acceptor site for these vitamins could be DNA or a precursor tRNA with subsequent appearance of a specific tRNA, which could then arise from vitamin-DNA interaction or alternatively the occurrence of a structural modification (e.g. methylation) of pre-existing or precursor tRNA species.

This work was supported in part by NIH Grant AM04041-11, Atomic Energy Commission Contract AT (30-1) 2530 and TIAM 5408 of NIAMD to I. C., GRSG Research Grant from the Public Health Service and Grant IN-18K from the American Cancer Society to B. H. The authors are indebted to Dr. P. R. Srinivasan, College of Physicians and Surgeons, Columbia University, for his suggestions and assistance during this study. D. S. B. was a postdoctoral trainee of the National Institute of Arthritis and Metabolic Diseases.

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