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# DECREASE IN TYROSINE HYDROXYLASE SYNTHESIS IN CULTURED ADRENAL MEDULLA BY $N^6$-METHYLADENOSINE

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**Abstract**—Explants of adrenal medullae were cultured in defined media for up to 22 hr, during which time the tissue remained histologically intact. Addition of $N^6$-methyladenosine to the medium led to a diminution in the activity of tyrosine hydroxylase (EC 1.14.16.2) in the tissue. The enzyme activity was inversely proportional to the concentration of $N^6$-methyladenosine in the culture medium. The extent of loss of tyrosine hydroxylase, as measured by immunochemical titration, corresponded to the degree of loss in enzyme activity under the same conditions. The decreased amount of enzyme protein was due to a decreased rate of synthesis of tyrosine hydroxylase. A significant decrease in the relative rate of tyrosine hydroxylase synthesis indicates the selectivity of this effect of $N^6$-methyladenosine. The rate of enzyme degradation was not affected by this compound. Neither adenosine, $N^6$-cyclohexyladenosine, nor several other methylated nucleosides including $N^1$-methyladenosine, $N^7$-methylguanosine and $N^2$-methylguanosine had an effect on the enzyme. However, two other $N^6$-substituted adenosines, $N^6$-dimethyladenosine and $N^6$-$\gamma\gamma$-dimethylallyladenosine, were effective in reducing tyrosine hydroxylase. The results are consistent with the view that specific substitutions at the $N^6$ position of adenosine could play a role in regulation of levels of tyrosine hydroxylase by altering its rate of biosynthesis.

The activity of adrenal tyrosine hydroxylase (TH)‡ (tyrosine 3-monooxygenase; EC 1.14.16.2) may be regulated by several mechanisms. Regulation occurs as an acute response of the enzyme to end product inhibition [1]. A second short-term mechanism of enzyme regulation entails transformation of TH to a more active form through direct phosphorylation of the enzyme [2, 3]. A third mechanism involves long-term alterations in amounts of enzyme protein [4] by induction [5, 6] or repression [7] of enzyme synthesis. While the investigations have provided some understanding of mechanisms for short-term regulation of TH, little is known about the biochemical mechanisms underlying long-term regulation of TH.

Methylation of nucleosides is known to play a role in regulation of synthesis of a number of proteins [8, 9]. We have shown recently that methylation of adenosine may also play a role in regulating the synthesis of a catecholamine-synthesizing enzyme, phenylthanolamine *N*-methyltransferase (PNMT; EC 2.1.1.28) [10]. In this paper we report the effects of four methylated nucleosides ($N^6$-methyladenosine, $m^6A$; $N^1$-methyladenosine, $m^1A$; $N^7$-methylguanosine, $m^7G$; and $N^2$-methylguanosine, $m^2G$) as well as adenosine and a number of $N^6$-substituted adenosines on the activity of TH in cultured rat adrenal medullary explants. The effect of $m^6A$ on the amount and rates of synthesis and degradation of TH was also determined with specific antibody to rat adrenal TH [11].

## MATERIALS AND METHODS

Adenosine, $m^6A$, $m^7G$, $m^1A$, $N^2$-methylguanosine, $N^6$-dimethyladenosine and $N^6$-$\gamma$-diethylallyladenosine were obtained from the Sigma Chemical Co., St. Louis, MO. $N^6$-Cyclohexyladenosine was from Research Biochemicals Inc., Wayland, MA. [$^{14}$C]Leucine (344 mCi/mmole) and [$^3$H]tyrosine (52 Ci/mmole) were obtained from the New England Nuclear Corp., Boston, MA. Medium 199 with glutamine and Earles salts was purchased from Flow Laboratories, Rockville, MD.

*Explant culture.* Male rats (Holtzman) weighing 90–100 g were used in all experiments. Before being killed, animals were housed five or six to a cage under controlled conditions of lighting and humidity in a room maintained at 21.0 ± 0.5°. Purina rat chow and water were supplied *ad lib.*

Animals were killed by a blow to the head and decapitated. The right adrenal gland was removed rapidly, and the medulla was dissected free of cortex under a dissecting microscope. The tissue was cultured by a modification of the method of MacDougall and Coupland [12]. Each medulla was cut in half,

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‡ Abbreviations: TH, tyrosine hydroxylase; PNMT, phenylethanolamine *N*-methyltransferase; MAO, monoamine oxidase; $m^6A$, $N^6$-methyladenosine; $m^1A$, $N^1$-methyladenosine; $m^7G$, $N^7$-methylguanosine; $m^2G$, $N^2$-methylguanosine; CHA, $N^6$-cyclohexyladenosine; and $N^6$-methyl-SAH, $N^6$-methyladenosine homocysteine.

and the sections were placed on lens paper floated in a culture dish containing 3 ml of medium 199 buffered to pH 7.4 with 0.02 M sodium *N*-tris-(hydroxymethyl)methyl-2-aminoethane sulfonic acid (TES; Sigma Chemical Co.). Penicillin and streptomycin were added to provide concentrations of 100 units/ml and 0.2 mg/ml respectively. Ascorbic acid was added to provide a total concentration of 5.7 mM. Compounds to be tested were added to the medium at concentrations noted for each experiment. The tissue was dispensed so that one-half of the same medulla served as a matched control for the experimental piece. Incubations were at 37° in an atmosphere of 95% oxygen–5% carbon dioxide for a maximum of 22 hr. Medullae cultured in medium 199 in the presence or absence of 1.0 mM $m^6A$ were fixed in 10% formalin, sectioned, stained with hematoxylin eosin, and examined by light microscopy. Viability of the cultured explants was also determined using the 0.1% trypan blue dye exclusion method of Sawicki *et al.* [13].

*Preparation of homogenates for enzyme assays.* At the end of the culture period, tissue was homogenized in 0.5 ml of 0.5 M potassium-phosphate buffer, pH 6.0, containing 0.2% Triton X-100. After centrifugation at 12,000 *g* for 10 min, the supernatant fraction was taken for TH assay.

*Enzyme assays.* TH activity was assayed by a modification of the methods of Reis *et al.* [14] and Lerner *et al.* [15]. In summary, the assay mixture contained: 50 μl of enzyme; 10 μl of 0.5 M potassium-phosphate, pH 6.64; 10 μl of 0.2 M ascorbic acid; 10 μl of 1 mM ferrous ammonium sulfate; 2400 units of catalase; 15 μl of 10 mM 6 methyl-5,6,7,8-tetrahydropterine (reduced with $PtO_2$ and hydrogen gas in 0.01 N HCl immediately prior to assay); 10 μl of 10 mM *m*-hydroxybenzylhydrazine; 2 μCi of L-[3,5-$^3$H]tyrosine (52 Ci/mmole) (dried with a stream of $N_2$ prior to assay); and water to make the total volume of the assay mixture 125 μl. The reaction was initiated by the addition of 50 μl of supernatant fraction and was stopped after 15 min with 5 ml of 0.5 M potassium-phosphate buffer, pH 8.5, containing 30 mg $Na_2S_2O_5$, 100 mg disodium ethylenediaminetetraacetic acid and 1 μg of carrier L-dopa. The product was adsorbed immediately onto 200 mg of alumina [16] by shaking for 5 min. The alumina was then washed three times with 10-ml aliquots of water. The [$^3$H]dopa was then eluted by shaking the alumina with 3 ml of 0.4 N perchloric acid for 5 min. Two milliliters of the eluate was then counted in phase combining system (PCS) counting fluid in a Beckman scintillation spectrometer. The values were not corrected for recovery [17]. The unit of enzyme activity reported for TH corresponds to the formation of 1 nmole of radioactive product/hr.

*Immunochemical titration of TH.* Explants of adrenal medullae were homogenized and centrifuged at 12,000 *g*. An aliquot of the supernatant fraction was taken for measurement of protein content by the method of Lowry *et al.* [18]; the remainder was used to measure the relative concentrations of TH. To stabilize enzyme activity during incubation with the antibody, 38,000 units of catalase were added to 1 ml of this supernatant fraction. Antibody to rat adrenal TH was prepared and judged to be specific according to a method previously described [4, 11]. This antibody was used to titrate [4, 19] the amount of enzyme in soluble extracts from rat medullae that had been incubated previously for 20 hr in either the presence of absence of 1.0 mM $m^6A$. Portions of the soluble extract measuring 5–50 μl were added to tubes containing 15 μl of antibody. The volume of each tube was adjusted to 65 μl with 0.15 M NaCl. This mixture was allowed to stand at 4° with occasional swirling, for 60 min, after which the tubes were centrifuged at 6000 *g* for 10 min.

A 50-μl aliquot of the supernatant fraction was removed from each tube, and the activity of TH was determined. From a graph of enzyme activity in the supernatant fraction versus addition of medullary extract, the equivalence point between enzyme and antibody was determined as previously described [4, 14].

*Measurement of rates of synthesis and degradation of TH.* Opposite halves from thirty-two adrenal medullae (sixteen pairs in each medium) were cultured for 10 hr at 37° in medium 199 in either the presence of absence of 1.0 mM $m^6A$. The medullae were transferred to fresh leucine-free medium to which 40 μCi of [$^{14}$C]leucine was added and cultured for an additional 4 hr. For determination of the relative rate of TH synthesis, half of the medullae were removed from each culture dish and each pair of medullae was homogenized in 310 μl of 5 mM potassium-phosphate, pH 7.0, containing 0.2% Triton X-100. Aliquots were precipitated with 0.4 N perchloric acid for determination of [$^{14}$C]leucine incorporation into total protein and protein content of the tissue. The rest of the homogenate was centrifuged at 15,000 *g* for 15 min. To 200 μl of the supernatant fraction, 20 μl of antibody to TH was added, and the mixture was incubated for 60 min at room temperature and overnight at 4°. To precipitate the total TH antibody complex, 150 μl of secondary antibody, goat antirabbit IgG, was added to the mixture. The mixture was incubated for 90 min with occasional shaking, and the TH antibody secondary antibody complex was collected by centrifugation. The $^{14}$C-labelled protein complex as washed twice with 500 μl of 0.15 N NaCl, once with 0.4 N perchloric acid, and then dissolved in 200 μl of NCS tissue solubilizer and counted in 20 ml of OCS liquid scintillation fluid with 10 μl acetic acid to prevent photon interference with counting.

To determine the rate of degradation of TH, the remaining pairs of medulla were transfered to fresh medium of the same composition without [$^{14}$C]leucine and maintained for an additional 8 hr. The radioactive leucine remaining in TH was measured by the method described above.

*Measurement of the specific activity of the medullary leucine pool.* Opposite halves from ten medullae were cultured for 10 or 20 hr at 37° in medium 199 in either the presence or absence of 1.0 mM $m^6A$. The medullae were transferred to fresh leucine-free medium to which 20 μCi of [$^{14}$C]leucine was added and cultured for an additional 4 hr. The tissue was homogenized in 100-μl aliquots of methanol to precipitate protein [20] and centrifuged at 20,000 *g* for 30 min. The amount of [$^{14}$C]leucine incorporated into protein and the protein content of the tissue

were determined. The concentration of methanol in the supernatant fraction was decreased to 50% with the addition of $H_2O$. Aliquots of the supernatant fraction were then counted in 10 ml of PCS to determine the amount of free [$^{14}$C]leucine and its metabolites in the soluble fraction. To determine the percent of total counts which represented [$^{14}$C]leucine, unlabelled leucine and *o*-phthaldealdehyde reagent were added to this soluble fraction, and the leucine peak was collected from the high pressure liquid chromatography column and counted in PCS. A correction was made for the 91.6% recovery of [$^{14}$C]leucine from the column. Aliquots of 100 μl of the supernatant fraction were then added to 100 μl of *o*-phthaldealdehyde reagent [20] concentrated five times to reduce the volume needed for the reaction. After derivitization, the amount of leucine in the soluble fraction was determined using high pressure liquid chromatography with electrochemical detection according to a method previously described [20, 21]. The mobile phase was 40% methanol in a 0.1 M sodium-phosphate buffer, pH 7.0.

*Evaluation of results.* Statistical comparisons of means of two independent samples were made using Student's *t*-test. Paired data were compared by the *t*-test, difference method.

## RESULTS

Prior to culture, the TH activity in medulla was 2.01 ± 0.03 units/mg protein; after 20 hr of culture this decreased to 1.08 ± 0.06 and thereafter was stable with a value of 1.10 ± 0.02 at 42 hr.

Exposure of adrenal medullary tissue to 0.5 mM m$^6$A for 20 hr resulted in a 32% decrease in TH activity (Table 1, Expt. 1). As the concentration of m$^6$A in the medium was increased over a 10-fold range, there was a corresponding decrease in TH activity in homogenates of cultured explants (Table 1, Expt. 1). This effect was not due to a direct effect of m$^6$A on TH. $N^6$-Methyladenosine when added directly to the reaction mixture at a final concentration of 1 mM produced only a small decrease of 13% in TH activity (control 1291 ± 11 dpm × $10^{-3}$/adrenal vs m$^6$A: 1125 ± 17). At the same concentration in the medium, m$^6$A produced an 87% decrease in the TH (Table 1, Expt. 1).

To determine the specificity of the effect on TH, adenosine, a nonmethylated analog, was tested at concentrations up to 1.0 mM. This nonmethylated purine nucleoside had no effect on TH activity during a 20-hr culture (Table 1, Expt. 2). Similarly, other methylated purine nucleosides including m$^1$A, m$^2$G and m$^7$G had no effect on TH after culture (Table 1, Expt. 3). To ascertain whether the $N^6$ position was critical for this effect, we tested three $N^6$-substituted adenosine analogues. The two compounds containing methyl groups at the $N^6$ position, $N^6$-γγ-dimethylallyladenosine and $N^6$-dimethyladenosine, were as effective as m$^6$A in reducing TH activity when added to the culture medium. However, $N^6$-cyclohexyladenosine (CHA) had no effect on TH activity (Table 2).

To determine whether this effect of m$^6$A was mediated through adenosine receptors, we attempted to block the effect of m$^6$A on TH with the adenosine receptor ligand CHA. Even at micromolar concentrations the cyclohexyl-derivative had no effect on the m$^6$A-elicited reduction in TH activity (Table 3, Expt. 1). Because CHA is structurally similar to m$^6$A but itself has no effect on TH, we sought to determine whether this compound in concentrations large enough to affect the metabolism of m$^6$A could alter the effect of m$^6$A on TH. At a concentration of 0.5 mM, CHA reversed the m$^6$A repression of TH.

We have shown previously [10] that in tissue cultured in the presence of 1.0 mM m$^6$A for 48 hr the activities of medullary monoamine oxidase (MAO) and acid phosphatase were not affected. To further establish the viability of tissue cultured in the presence or absence of 1.0 mM m$^6$A, several parameters

Table 1. Effects of m$^6$A and other nucleosides on adrenal medullary enzymes in cultured explants*

| Expt. | Compound added | Tyrosine hydroxylase (units/mg protein) |
|---|---|---|
| 1 | None | 0.83 ± 0.05 |
| | 0.5 mM $N^6$-Methyladenosine | 0.56 ± 0.08† |
| | 1.0 mM $N^6$-Methyladenosine | 0.11 ± 0.04‡ |
| | 5.0 mM $N^6$-Methyladenosine | 0.01 ± 0.003‡ |
| 2 | None | 0.67 ± 0.11 |
| | 1.0 mM Adenosine | 0.79 ± 0.14 |
| 3 | None | 0.82 ± 0.11 |
| | 1.0 mM $N^1$-Methyladenosine | 0.94 ± 0.05 |
| | 1.0 mM $N^7$-Methylguanosine | 1.14 ± 0.08 |
| | 1.0 mM $N^2$-Methylguanosine | 1.13 ± 0.20 |

* Adrenal medullary explants were cultured in medium 199 containing the compounds listed at the concentration indicated. Homogenates of these explants were assayed for TH as outlined under Materials and Methods. Each mean (±S.E.) is derived from five replicate cultures.

† $P < 0.025$.

‡ $P < 0.001$.

Table 2. Effects of other $N^6$-substituted adenosines on TH activity in cultured adrenal medullae*

| Compound added | Tyrosine hydroxylase (units/mg protein) |
|---|---|
| None | 1.08 ± 0.06 |
| 1.0 mM $N^6$-Cyclohexyladenosine | 0.86 ± 0.29 |
| 1.0 mM $N^6$-$\gamma$ $\gamma$-Dimethylallyladenosine | 0.02 ± 0.001† |
| 1.0 mM $N^6$-Dimethyladenosine | 0.01 ± 0.002† |

* Adrenal medullary explants were cultured for 20 hr in medium 199 or medium 199 containing the compound listed at the concentration indicated. Homogenates of these explants were assayed for TH as outlined under Materials and Methods. Each mean (±S.E.) is derived from four replicate cultures.
† $P < 0.001$.

were measured. The tissue was structurally intact as verified by examination of stained sections under light microscopy. Although 5–10% of the tissue took up trypan blue, this was due to tissue damage during dissection. There was no difference in uptake of this vital stain between control and m$^6$A-treated tissue. Neither the degradation rate of medullary protein (control: 0.69 ± 0.05 vs 1.0 mM m$^6$A treated: 0.7 ± 0.23) nor that of TH (see Table 5) was increased by m$^6$A.

The decrease in TH activity could be due to a change in amount of TH enzyme protein or to a shift to a less active form. To segregate these mechanisms, immunochemical titration was carried out. The equivalence point of extracts of medullae cultured in the presence of 1.0 mM m$^6$A for 20 hr was shifted to 1.2 $\mu$g protein from 0.35 $\mu$g observed for control explants (Fig. 1). This 71% diminution in relative amount of TH agreed with the 77% loss in measurable TH activity in the same extract of tissue incubated with m$^6$A (767 dpm/$\mu$g protein) compared to tissue incubated in basal medium (3389 dpm/$\mu$g protein).

To determine whether the decrease in amount of TH in m$^6$A-treated medullae was due to a decrease in rate of TH synthesis or an increased rate of degradation or both, the incorporation of [$^{14}$C]leucine into TH at 14 hr and the [$^{14}$C]leucine remaining in TH at 22 hr were determined. When 1.0 mM m$^6$A was present in the culture medium, there was an 87% decrease in the incorporation of [$^{14}$C]leucine into TH at 14 hr compared to the control culture (Table 4). Although there was a decrease in incorporation of [$^{14}$C]leucine into total protein (control: 24,407 ± 1,103 dpm/$\mu$g protein vs 1 mM m$^6$A: 4,542 ± 960), the relative rate of TH synthesis ([$^{14}$C]leucine into TH/[$^{14}$C]leucine into total protein × 100) was significantly ($P < 0.05$) decreased by 24% (control: 2.95 ± 0.24 vs 1 mM m$^6$A: 2.24 ± 0.27). The decrease in incorporation of [$^{14}$C]-leucine into TH and protein was not due to a decreased availability of [$^{14}$C]leucine since after 10 hr of culture with 4 hr additional exposure to [$^{14}$C]-leucine the total amount of [$^{14}$C]leucine taken up was greater in the m$^6$A-treated tissue (control: 188 ± 16 dpm/$\mu$g protein vs 1.0 mM m$^6$A: 275 ± 23; $P < 0.05$). In the control tissue, 50.1 ± 0.4% of this was present as leucine; in the m$^6$A-treated tissue, 73.3 ± 0.1% was leucine. This decreased incorporation of [$^{14}$C]leucine into TH and protein in m$^6$A-treated tissue was also not due to a decrease in specific activity of the intracellular leucine pool since the specific activity of this pool after 20 hr of culture with 4 hr exposure to [$^{14}$C]leucine was increased by m$^6$A (control: 34.4 ± 2.0 nCi/nmole vs 1.0 mM m$^6$A: 109.9 ± 5.8; $P < 0.001$). In contrast to the rate

Table 3. Effect of CHA on m$^6$A repression of TH in cultured adrenal medullae*

| Expt. | Compound added | TH (units/mg protein) |
|---|---|---|
| 1 | 1.0 mM $N^6$-Methyladenosine | 0.22 ± 0.05 |
| | 1.0 mM $N^6$-Methyladenosine + 1.0 $\mu$M $N^6$-cyclohexyladenosine | 0.17 ± 0.05 |
| 2 | 1.0 mM $N^6$-Methyladenosine | 0.02 ± 0.005 |
| | 1.0 mM $N^6$-Methyadenosine + 0.5 mM $N^6$-cyclohexyladenosine | 0.45 ± 0.007† |

* Adrenal medullary explants were cultured for 20 hr in medium 199 or medium 199 containing the compound listed at the concentration indicated. Homogenates of these explants were assayed for TH as outlined under Materials and Methods. Each mean (±S.E.) is derived from four replicate cultures.
† $P < 0.001$.

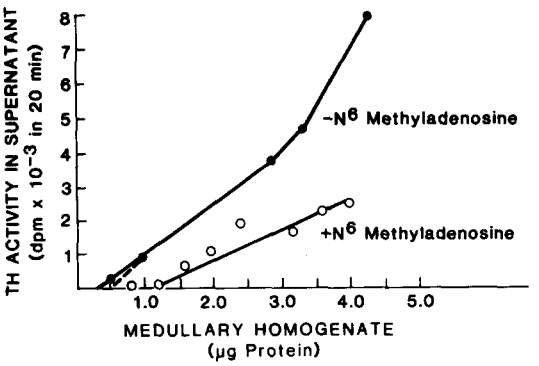


Fig. 1. Amount of immunotitratable TH in medullary explants cultured in the presence or absence of $m^6A$. Explants of adrenal medullae were cultured for 20 hr at 37° in medium 199 in either the presence or absence of 1 mM $m^6A$. At the end of the incubation period, tissue was homogenized and centrifuged at 12,000 *g*. The resulting soluble portion of the homogenate was added to specific antibody to TH, and the activity of the remaining unprecipitated enzyme was determined.

of synthesis, the rate of degradation, which was determined by measurement of [$^{14}$C]leucine remaining in TH at 22 hr, was not changed from control values when medullae were cultured in the presence of $m^6A$ (Table 5).

## DISCUSSION

Investigations into the mechanisms for short-term regulation of TH by direct end product inhibition [1, 22] or by allosteric activation through phosphorylation [2, 3] have greatly increased our knowledge in this area. It is also known that the long-term regulation of TH involves induction of new enzyme protein [4] through an increase in its rate of synthesis [5]. However, the molecular mechanisms underlying long-term regulation of TH synthesis are unknown. We have shown recently that the methylation of adenosine could play an important role in regulation of PNMT, another catecholamine-synthesizing enzyme. Because TH and PNMT appear to be closely linked on the genome [23], it was of interest to determine whether substitutions at the $N^6$ position of adenosine could also play an important role in the regulation of TH synthesis.

Table 4. Effect of $m^6A$ on synthesis of TH in cultured adrenal medullae*

| $N^6$-Methyladenosine (mM) | [$^{14}$C]TH (dpm/μg protein) |
|---|---|
| 0 | 723 ± 76 |
| 1.0 | 96 ± 16† |

* Explants of adrenal medullae were cultured for 10 hr at 37° in medium 199 in either the presence or absence of $m^6A$. Tissue was transferred to fresh medium of the same composition but without leucine. [$^{14}$C]Leucine (40 μCi) was added to each culture dish. After 4 hr of additional incubation, the tissue was removed for determination of [$^{14}$C]leucine incorporation into TH (see Materials and Methods). Each mean (±S.E.) is derived from four replicate cultures.

† $P < 0.001$.

Addition of $m^6A$ to adrenal medullary explant cultures in concentrations similar to those which have been used in cell-free systems [8] led to diminished levels of TH. Immunochemical titration showed that this decrease in activity was due to a decrease in amount of enzyme protein rather than a direct inhibition of TH by the nucleoside. The decrease in the amount of TH, in turn, was entirely due to a decrease in the rate of synthesis of the enzyme with no change in the rate of degradation. The relative rate of TH synthesis compared to total protein synthesis was also decreased. This observation, together with the fact that two other medullary enzymes were not affected even after 48 hr of exposure to $m^6A$ [10], indicates that the effect, although not specific, was selectve and not due to destruction of tissue.

Although there was a decrease in the incorporation of [$^{14}$C]leucine into total protein and a rapid turnover of protein in $m^6A$-treated tissue, neither total protein content nor the activities of MAO or acid phosphatase [10] were affected even after 48 hr of exposure to $m^6A$. These results indicate that we are measuring synthesis of only a small family of proteins with a short half-life. Assuming an average mass

Table 5. Effect of $m^6A$ on degradation of TH in cultured adrenal medullae*

| $N^6$-Methyladenosine (mM) | [$^{14}$C]TH (dpm/μg protein) At 14 hr | At 22 hr | [$^{14}$C]TH (At 22 hr/at 14 hr) |
|---|---|---|---|
| 0 | 1252 ± 100 | 872 ± 198 | 0.68 ± 0.10 |
| 1.0 | 38 ± 44 | 258 ± 92 | 0.71 ± 0.18 |

* Explants of adrenal medullae were cultured for 10 hr at 37° in medium 199 in either the presence or absence of $m^6A$. Tissue was transferred to fresh medium of the same composition but without leucine. [$^{14}$C]Leucine (40 μCi) was added to each culture dish. After 4 hr of additional incubation, half the tissue from each dish was removed for determination of [$^{14}$C]leucine incorporation into TH (see Materials and Methods). The remaining tissue was placed in fresh nonradioactive medium of the same composition and maintained for an additional 8 hr after which the [$^{14}$C]leucine remaining in TH was determined. Each mean (±S.E.) is derived from two replicate cultures.

fraction of leucine in medullary proteins of 10% [24], only 11.7% of the total protein content is being synthesized at 20–24 hr and 1.7% of the medullary protein is being synthesized at 10–14 hr. Our results indicate that $m^6A$ selectively affects this small family of rapidly turning over proteins. This selectivity is due to the fact that most of the medullary proteins are being synthesized very slowly or not at all. In addition, the fact that the relative rate of TH synthesis was decreased indicates that not all of the rapidly turning over proteins were affected by $m^6A$. We have identified two highly regulated [4, 25] catecholamine-synthesizing enzymes, TH and PNMT [10]m which are selectively affected in this group. Other catecholamine enzymes which are closely linked on the genome [23] as well as other rapidly turning over proteins may belong to this family. Lajtha *et al.* [26] have also reported only partial labeling of proteins after up to 5 days of incorporation of $^{14}C$-labeled amino acid. Rapid as well as more slowly turning over families of proteins in the same tissue have been described [26].

Several other methylated nucleosides, including $N^1$-methyladenosine, had no effect on TH. This suggests that the $N^6$ position on adenosine is critical. Substitutions on the adenosine molecule at the $N^6$ position with methyl, dimethyl or $\gamma\gamma$-dimethylallyl but not with cyclohexyl were effective. This indicates that the structure of the substitution is important for the effect on TH synthesis.

The mechanism of this effect is not certain, however; there are a number of possibilities. Both $m^7G$ and $m^6A$ are present in 5′-terminal cap structures of many cellular and viral mRNAs [27, 28]. Methylation of mRNA has been shown to be required for translation of a variety of proteins in cell-free systems. Hickey *et al.* [8] have shown that 7-methylguanosine 5′-monophosphate inhibits translation of rabbit globin mRNA in a wheat germ protein-synthesizing system. Similar results have been obtained for several other proteins [9, 29]. It has been postulated that methylation of 5′-terminal caps provides a recognition factor for subsequent binding of mRNA to ribosomes [30]. By analogy one mechanism to explain the effect of $m^6A$ on TH synthesis could be that translation of TH mRNA is blocked at the ribosomal recognition site by $m^6A$ or by its phosphorylated form. Second, it is possible that a metabolite of $m^6A$ could mediate this effect. This view is supported by the fact that CHA, in concentrations which are known to be effective for enzyme inhibitors in this tissue [7, 31], blocked the effect of $m^6A$ on TH. This suggests that, at least in part, the effect of $m^6A$ may be mediated by a metabolite of this compound. One such chemical, $N^6$-methyladenosine homocysteine ($N^6$-methyl-SAH), is known to inhibit a number of mRNA methyltransferases including guanine-7-methyltransferase [32]. This could, in turn, alter the methylation of the 5′ cap structure necessary for translation of mRNAs. In addition, it has been suggested that methylation of certain tRNAs may be necessary for their function [33], and a tRNA methyltransferase is also inhibited by $N^6$-methyl-SAH [34]. The presence of the enzymes *S*-adenosylhomocysteine hydrolase (EC 3.3.1.1), which converts $m^6A$ to $N^6$-methyl-SAH, has been demonstrated in bovine liver by Guranowski *et al.* [35] and in bovine adrenal medulla (our unpublished observation). The above two mechanisms, competitive binding to the recognition site and inhibition of methyl group transfers necessary for efficient protein translation, are not necessarily exclusive and could be activated at different concentrations of $m^6A$ in the tissue. The fact that this effect is selective for catecholamine-synthesizing enzymes and that $N^6$-dimethyladenosine is not a substrate for the hydrolase [35] favors the first mechanism. However, that general protein synthesis was also affected is consistent with the view that other terminal caps of mRNAs or methylated tRNAs required for synthesis of several proteins could be affected by a metabolite of $m^6A$ such as $N^6$-methyl-SAH. Finally, $N^6$-substituted adenosines are known to bind to adenosine receptors which alters cyclic nucleotide levels in the cell [36]. Cyclic nucleotides, in turn, have been shown to alter activities of catecholamine-synthesizing enzymes [37]. This mechanism seems unlikely in the adrenal medulla for the following reasons. Millimolar concentrations of $m^6A$ were required for this effect while Snyder *et al.* [36] have shown that tissue concentrations of adenosine receptor are in the picomole/gram range. Neither adenosine, an A2 receptor agonist, nor CHA, an A1 receptor ligand, had any effect on TH. Further, micromolar concentrations of CHA, which has nanomolar affinity for adenosine receptors [36, 38], did not reverse the effect of $m^6A$ on TH.

It should be noted that, although methylated nucleosides, including $m^6A$, occur as post-transcriptional modifications of mRNA as well as other RNA species in mammalian tissues [27, 39, 40], there has been no report of endogenous $m^6A$ in adrenal medullary tissue. However, there are high concentrations of both *S*-adenosylmethionine [41] and adenosine [42], precursors of $m^6A$, in this tissue. This data and the fact that millimolar concentrations of CHA blocked the effect of $m^6A$ suggest, although do not prove, that altered methylation of nucleosides could be a naturally occurring mechanism for regulation of TH synthesis.

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