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SYNTHESIS OF RNA IN THE PINEAL GLAND DURING *N*-ACETYLTRANSFERASE INDUCTION

THE EFFECTS OF ACTINOMYCIN D, α-AMANITIN AND CORDYCEPIN

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Abstract—The biosynthesis of poly(A) containing RNA in the cultured rat pineal was monitored during the isoproterenol-induced increase in *N*-acetyltransferase activity in the presence of various inhibitors of RNA synthesis. The induction of *N*-acetyltransferase in the pineal gland by the β-agonist isoproterenol was found to be inhibited by actinomycin D and α-amanitin, but relatively insensitive to cordycepin. The concentration of actinomycin D which inhibits the induction process 50 per cent is on the order of 5 μg/ml, whereas only 0.1 μg/ml is needed to inhibit poly(A)-containing RNA synthesis by 50 per cent. Cordycepin, which inhibits the addition of poly(A) into newly synthesized messenger RNA, inhibited poly(A)-containing RNA synthesis by 85 per cent but inhibited the induction of *N*-acetyltransferase by isoproterenol only 15 per cent. The mushroom toxin α-amanitin, which should preferentially inhibit messenger type RNA synthesis, reduced poly(A)-containing RNA synthesis 50 per cent at 10 μg/ml of toxin, and inhibited enzyme induction 50 per cent at 40 μg/ml. While these results support the participation of an RNA species in the apparent induction of *N*-acetyltransferase in the pineal gland, they suggest that the induction stimulus may not be exerting its effect by simply causing an increase in the synthesis of messenger RNA containing a poly(A) terminus.

In the companion paper [1] to this one, we had observed no significant changes occurring in cellular RNA synthesis in the cultured pineal gland during the β-agonist induction of *N*-acetyltransferase activity. RNA synthesis is required for enzyme induction as evidenced by the actinomycin D sensitivity of the process [1–4]. This investigation focuses on the effects of the RNA synthesis inhibitors actinomycin D, cordycepin and α-amanitin on poly(A)-containing messenger RNA and the (*l*)-isoproterenol-induced increase in *N*-acetyltransferase activity in the cultured pineal gland.

MATERIALS AND METHODS

Materials and methods are exactly as described in the companion paper [1] except that actinomycin D, cordycepin and α-amanitin were purchased from Sigma Chemical, St. Louis, MO. Cordycepin was obtained from CalBiochem. LaJolla, CA, and α-amanitin was purchased from Boehringer Mannheim, Indianapolis, IN.

RESULTS

Effect of actinomycin D on N-*acetyltransferase induction.* Previous workers [2–4] have found the induction of the *N*-acetyltransferase by (*l*)-isoproterenol is inhibited by actinomycin D. The concentration of actinomycin D utilized was generally 10 μg/ml. The effects of a wide range of inhibitor concentrations on pineal RNA synthesis and enzyme induction were examined in cultured pineals incubated with (*l*)-isoproterenol in media containing [^{3}H]uridine and various amounts of actinomycin D. A concentration of actinomycin D of 0.5 μg/ml was found to inhibit total RNA synthesis approximately 70 per cent while inhibiting enzyme induction approximately 15 per cent (see Fig. 1). At 5 μg/ml of actinomycin D there was a 90 per cent inhibition of [^{3}H]uridine incorporation into total RNA with a 50 per cent reduction in enzyme induction. The 10 μg/ml dose of actinomycin D effectively abolished both RNA synthesis and the appearance of enzyme activity. Actinomycin D at a concentration of 0.05 μg/ml reduced RNA synthesis only about 30 per cent and had little effect on enzyme induction. The labeled RNA's in these experiments were extracted with phenol and separated by agarose gel electrophoresis. As seen in Fig. 2, the low doses, i.e. 0.05 μg/ml of actinomycin D, seem to preferentially inhibit 18 and 28s ribosomal RNA synthesis without appreciably affecting the 4s transfer RNA or 5s ribosomal RNA. The synthesis of the 4s transfer RNA and 5s ribosomal RNA is inhibited at the 0.5 and 5.0 μg/ml concentrations of actinomycin D. This is consistent with the relative effects of actinomycin D on cell culture systems [5, 6]. The inhibition of poly(A)-rich messenger RNA by actinomycin D was gradual and paralleled the inhibition of total RNA synthesis. At 0.05 μg/ml of antibiotic there were a 30 per cent inhibition of total RNA synthesis (indicative of a 75 per cent inhibition of 28s and 18s ribosomal RNA and little effect on 4s transfer RNA) and a 30 per cent inhibition of poly(A)-rich messenger RNA. This effect of low doses of actinomycin D on putative messenger RNA precursor synthesis (i.e. heterogeneous RNA) has been observed by a number of investigators [7–10]. The major consensus is that

low actinomycin D concentrations preferentially inhibit ribosomal RNA synthesis and there is some effect on potential messenger RNA synthesis. It is interesting that, when the synthesis of poly(A)-rich messenger RNA was inhibited by more than 90 per cent by 5 μg/ml of actinomycin D, the induction of *N*-acetyltransferase was inhibited only 50 per cent.

Effect of cordycepin on N-*acetyltransferase induction.* Cordycepin (3-deoxy-adenosine) is a primary inhibitor of poly(A) addition to messenger RNA and it inhibits the eventual appearance of this messenger RNA in the cytoplasm [5, 11, 12]. Different concentrations of this inhibitor were tested for effects on the synthesis of total RNA, poly(A)-rich RNA, and *N*-acetyltransferase induction in the cultured pineal. As seen in Fig. 3, a progressive increase in the cordycepin concentration inhibits both total RNA synthesis and poly(A)-rich messenger RNA synthesis. The reduction in total RNA synthesis seems to represent a uniform decrease in the synthesis of the 28s, 18s and 5s ribosomal RNA and 4s transfer RNA (see Fig. 5). The poly(A)-rich messenger RNA is more effectively inhibited than the total RNA. In contrast, the appearance of the enzyme *N*-acetyltransferase in response to (*l*)-isoproterenol is not appreciably affected by cordycepin. At concentrations of cordycepin which inhibit poly(A)-rich RNA synthesis approximately 65 per cent (i.e. 50–100 μg/ml of cordycepin), enzyme induction is inhibited only about 20 per cent. At a concentration of 200 μg/ml of cordycepin, presumed messenger RNA synthesis is inhibited about 85 per cent while *N*-acetyltransferase induction is reduced only about 15 per cent.

Effect of α-amanitin on N-*acetyltransferase induction.* Eukaryotic systems appear to have three forms of DNA-dependent RNA polymerase (types I, II and III) [13–15] which may be distinguished *in vitro* by their relative sensitivity to the fungal toxin α-amanitin [15, 16]. In general, polymerase II, which is responsible for messenger RNA synthesis, is preferentially inhibited by low levels of α-amanitin [15–17]. In order to resolve the difference observed between actinomycin D and cordycepin on *N*-acetyltransferase induction, we tested various concentrations of this inhibitor on the synthesis of total RNA, poly(A)-rich RNA, and enzyme induction in the cultured pineal. As seen in Fig. 4, increasing concentrations of toxin inhibit both total cytoplasmic RNA synthesis and poly(A)-rich RNA synthesis. The reduction in total RNA biosynthesis results from a decreased ribosomal RNA synthesis (see Fig. 5) as well as an inhibition of poly(A)-rich messenger RNA synthesis. As with the cordycepin, the α-amanitin inhibited poly(A)-rich messenger RNA synthesis more effectively than total RNA synthesis. In contrast to cordycepin, however, the α-amanitin was found to inhibit the (*l*)-isoproterenol induction of *N*-acetyltransferase at high concentrations of toxin. At 25 μg/ml, α-amanitin inhibited poly(A)-rich RNA synthesis by about 70 per cent while enzyme induction was not significantly affected. When the α-amanitin level was raised to 50 μg/ml, the poly(A)-rich RNA synthesis was inhibited approximately 80 per cent and the β-agonist induction of *N*-acetyltransferase was decreased by almost 60 per cent.

Effect of cordycepin and α-amanitin on total RNA synthesis. The RNA synthesis inhibitors cordycepin and α-amanitin are generally thought to specifically affect poly(A)-containing RNA [5, 11, 12] and polymerase II-derived messenger RNA [16, 17] respectively. In Figs. 3 and 4 it was observed that these inhibitors were also affecting total RNA synthesis.

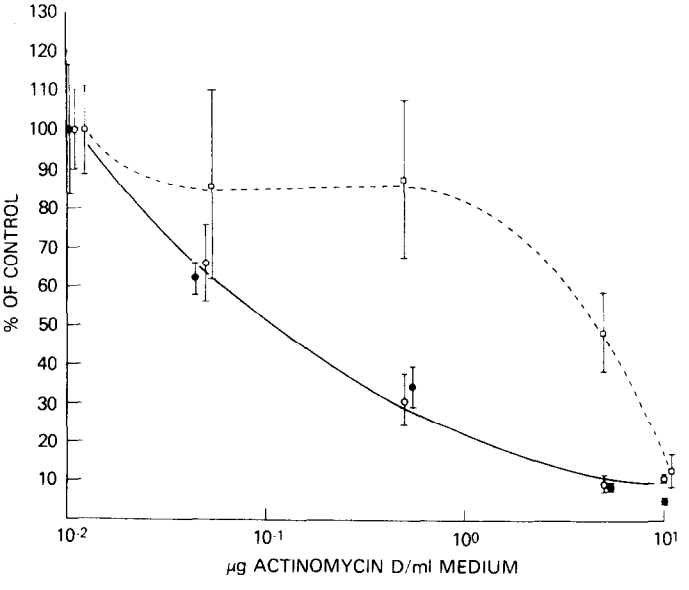

Fig. 1. Effect of actimomycin D on pineal RNA synthesis and *N*-acetyltransferase induction by (*l*)-isoproterenol. Pineals were cultured in medium containing 2 μM (*l*)-isoproterenol and 80 μCi/ml of [^{3}H]-uridine with the indicated concentrations of actinomycin D for 6 hr. Glands were removed and assayed for *N*-acetyltransferase activity (□——□) or for total RNA (○——○) and poly(A)-rich RNA (●——●) synthesis as described. Data shown represent eight to twelve determinations ± standard error of the mean.

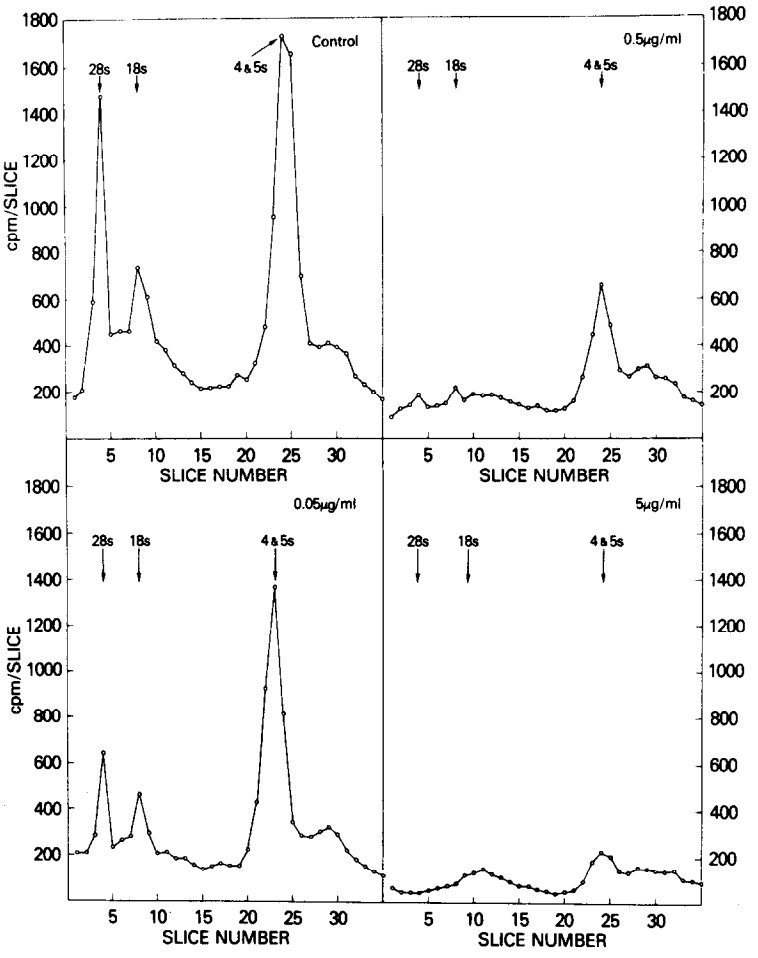

Fig. 2. Effect of actinomycin D on pineal RNA synthesis. The total labeled cytoplasmic RNA from the actinomycin D-treated pineals in Fig. 1 was extracted with phenol and electrophoresed on agarose gels as described.

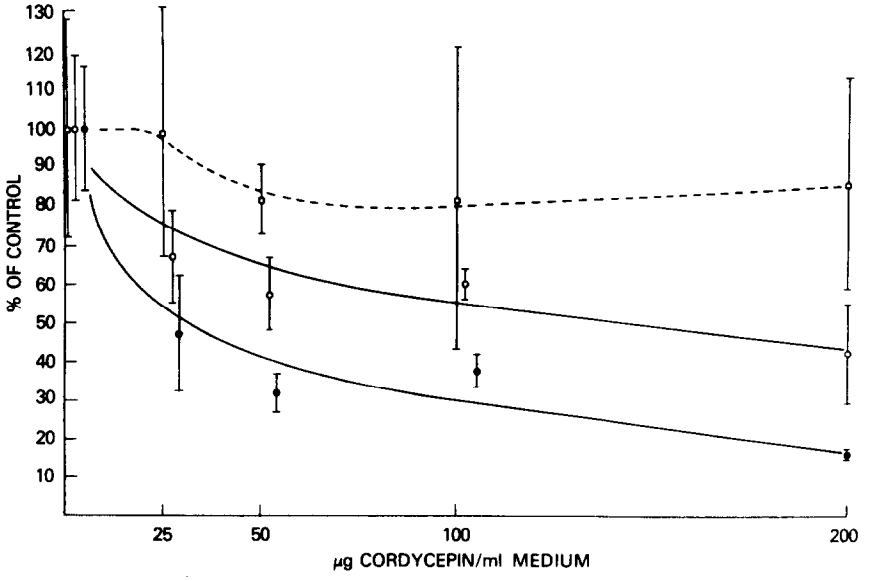

Fig. 3. Effect of cordycepin on pineal RNA synthesis and *N*-acetyltransferase induction by (*l*)-isoproterenol. Pineals were cultured in a medium containing 2 μM (*l*)-isoproterenol and 80 μCi/ml of [^{3}H]uridine with the indicated concentrations of cordycepin (3-deoxyadenosine) for 6 hr. Glands were removed and assayed for *N*-acetyltransferase activity (□——□) or for total RNA (○——○) and poly(A)-rich RNA (●——●) synthesis as described. Data shown represent eight to twelve determinations ± standard error of the mean.

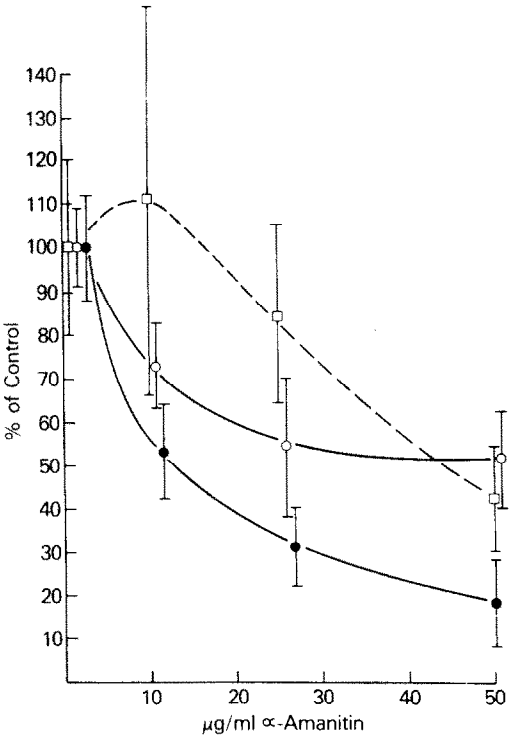

Fig. 4. Effect of α-amanitin on pineal RNA synthesis and *N*-acetyltransferase induction by (*l*)-isoproterenol. Pineals were cultured in a medium containing 2 μM (*l*)-isoproterenol and 80 μCi/ml of [^{3}H]uridine with the indicated concentrations of α-amanitin for 6 hr. Glands were removed and assayed for *N*-acetyltransferase activity (□——□) or for total RNA (○——○) and poly(A)-rich RNA (●——●) synthesis as described. Data shown represent eight determinations ± standard error of the mean.

Examination of the total cytoplasmic RNA fraction that was labeled with [^{3}H]uridine in the presence of 100 μg/ml of cordycepin or 50 μg/ml of α-amanitin revealed that both inhibitors affect ribosomal RNA and transfer RNA synthesis (Fig. 5). Cordycepin has been found to inhibit total RNA synthesis over a wide range of concentrations in cultured mouse fibroblasts [18] and in Reuber H-35 hepatoma cells [19] as well as preferentially inhibiting messenger RNA synthesis. Similarly, α-amanitin has been found to inhibit ribosomal RNA synthesis in intact cells [20–23]; however, α-amanitin will not inhibit ribosomal RNA synthesis in isolated nuclei [16, 17, 22]. Over the 6-hr period of inhibitor treatment in the intact pineal gland, the primary effects of cordycepin and α-amanitin seem to be directed toward messenger RNA synthesis and secondarily toward ribosomal and transfer RNA synthesis.

DISCUSSION

The effect of actinomycin D on the (*l*)-isoproterenol induction of pineal *N*-acetyltransferase may be more complex than generally thought [2–4]. Concentrations of actinomycin D causing a 50 per cent inhibition of RNA synthesis in the cultured pineal are: 0.05 μg/ml for ribosomal RNA, 0.1 μg/ml for total cytoplasmic RNA and poly(A)-rich messenger RNA, and 0.4 μg/ml for 4s transfer and 5s ribosomal RNA. By comparison, approximately 5 μg/ml of actinomycin D is required to inhibit *N*-acetyltransferase induction by 50 per cent. At this concentration of actinomycin D, essentially 90 per cent of the total cycloplasmic RNA and poly(A)-rich messenger RNA synthesis was inhibited. If a messenger RNA species is indeed required for overall enzyme induction, then its synthesis is extremely resistant to the action of actinomycin D.

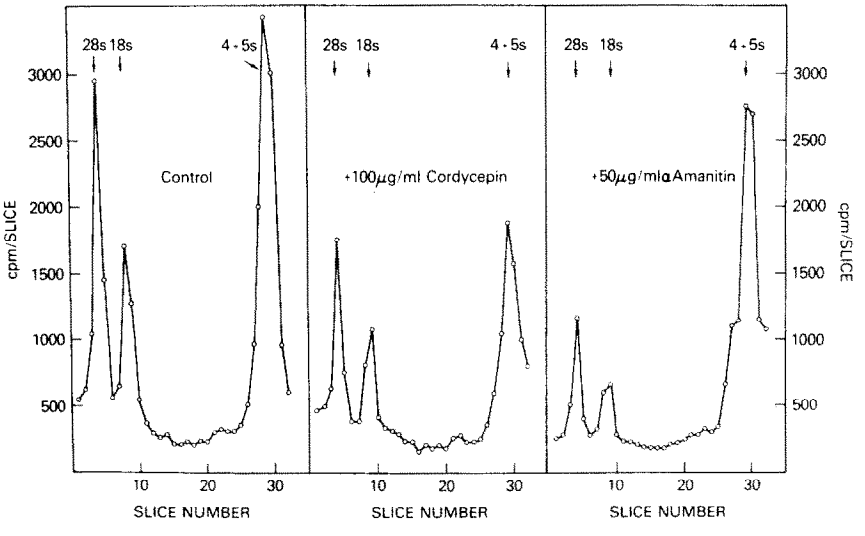

Fig. 5. Effect of cordycepin or α-amanitin on pineal RNA synthesis. The total labeled cytoplasmic RNA from pineals incubated with 2 μM (*l*)-isoproterenol ± 100 μg/ml of cordycepin or ±50 μg/ml of α-amanitin was extracted with phenol and electrophoresed on agarose gels as described.

Similar results were found with α-amanitin. High concentrations of the toxin were required to block the induction of *N*-acetyltransferase. A 50 per cent inhibition of poly(A)-rich RNA synthesis was achieved at 10 μg/ml of α-amanitin while about 40–50 μg/ml of inhibitor was needed to affect enzyme induction by (*l*)-isoproterenol. The synthesis of total RNA was also less sensitive to α-amanitin than was the poly(A)-rich RNA synthesis. Again, as with the actinomycin D study, a 70 per cent inhibition of apparent messenger RNA synthesis had little significant effect on *N*-acetyltransferase induction while an 80 per cent inhibition of poly(A)-rich RNA synthesis did inhibit enzyme induction.

The apparent lack of an effect by cordycepin (3-deoxyadenosine), an inhibitor of poly(A) addition to messenger RNA, on the induction of pineal *N*-acetyltransferase activity is most interesting. A 50 per cent inhibition in the synthesis of total cytoplasmic RNA was achieved by a concentration of approximately 150 μg/ml of cordycepin. The inhibition of poly(A)-rich messenger RNA synthesis by 50 per cent was accomplished with considerably less of the drug (i.e. 25 μg/ml). Cordycepin did not appreciably inhibit the induction of *N*-acetyltransferase although the standard errors of mean enzyme levels were broadened (see Fig. 3). As mentioned, cordycepin inhibits the addition of poly(A) during the maturation of messenger RNA [5, 11, 12]. The net result is an inhibition of transport of newly synthesized messenger RNA from the nucleus into the cytoplasm for eventual translation [24]. Therefore, the inhibition of pineal *N*-acetyltransferase induction by actinomycin D and α-amanitin and the lack of inhibition by cordycepin are paradoxical. Either the required RNA is not a messenger RNA or it is a member of a class of newly discovered messenger RNA molecules which lack poly(A) [25–30]. The poly(A) minus messenger RNA contains polynucleotide sequences different from those of the poly(A)-containing messenger RNA [25, 26], and these two different messenger RNA classes also appear to synthesize different proteins in heterologous cell-free systems [29]. It was recently shown that mRNA extracted from mouse sarcoma cells codes for two major polypeptides in heterologous systems and that the message for one of these polypeptides contains poly(A) whereas the other message does not contain poly(A) [30]. This suggests that the poly(A)-containing and poly(A)-minus messenger RNA codes for different proteins and may have functional significance. Histone messenger RNA does not contain poly(A) [31, 32], and cordycepin does not appear to inhibit the transport of the histone messenger RNA from the nucleus into the cytosol in HeLa cells [33]. Cordycepin at 50 μg/ml will inhibit the appearance of poly(A)-containing messenger RNA into HeLa cytosol by 95 per cent while inhibiting the appearance of poly(A)-minus messenger RNA by 60 per cent [26], suggesting that the synthesis of the poly(A)-minus messenger RNA may not be completely free of primary or secondary inhibition by cordycepin. Approximately 60 per cent of the rat mammary casein messenger RNA does not bind to oligo dT-cellulose [34], and some translatable trout testis protamine messenger RNA has been found to contain poly(A) whereas some lacks poly(A) [35]. In addition, cordycepin inhibits the synthesis of only 45 per cent of the rapidly labeled RNA in rabbit brain [36] but is an effective inhibitor of the induction of some enzymes in neural-related tissues [37, 38].

It is not known if all the messenger RNA originally possessed poly(A) at some point in the history of the molecule and then lost the poly(A) segment after entry into the cytosol or if the lack of appreciable poly(A) segments represents relatively old messenger RNA molecules [39]. If all messenger RNA molecules did originally have poly(A), then one would naively expect cordycepin to inhibit the eventual appearance of all messenger RNA into the cytosol. Finally, it is not known if a 90 per cent or better inhibition of poly(A)-containing RNA synthesis by cordycepin in the pineal rather than the observed 85 per cent inhibition reported in this investigation would appreciably affect *N*-acetyltransferase induction. Enzyme induction in the presence of actinomycin D or α-amanitin was significantly affected only after the poly(A)-containing RNA synthesis was inhibited 90 or 80 per cent, respectively, whereas a 70 per cent inhibition by either drug seemed not to influence significantly the induction of *N*-acetyltransferase by isoproterenol. Since both actinomycin D and α-amanitin seem to affect RNA synthesis in general [i.e. no specific inhibition of poly(A)-containing apparent messenger RNA synthesis], a specific induction of an *N*-acetyltransferase messenger by the β-agonist (*l*)-isoproterenol cannot be absolutely supported but it remains a strong possibility. The apparent insensitivity of enzyme induction to cordycepin suggests but does not unambiguously prove that pineal *N*-acetyltransferase synthesis initiated by the β-agonist isoproterenol requires an RNA species which may lack poly(A).

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