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PROTECTIVE EFFECT OF ADENOSINE AND NICOTINAMIDE AGAINST AUDIOGENIC SEIZURE

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Abstract—Intraperitoneal injection of adenosine into Swiss albino mice, RB strain, sensitive to audiogenic convulsions, rapidly produces, in proportion to the dose administered: sedation, modification of the EEG, lowering of arterial pressure and protection against audiogenic seizures. Simultaneous injection of adenosine and nicotinamide, produces the most striking protection, the effect being independent of all the others. During the protective period to convulsions, there is an elevation in the energy rich compounds, ATP and phosphocreatine, which is attributable to a reduction in their rate of degradation. During the same period, a diminution in the cerebral level of noradrenaline takes place. The pharmacological effects of adenosine appear to oppose those of its structural analogue, caffeine, with regard to mobility, arterial pressure, basal metabolic rate and body temperature.

ELLIOT and Penfield[1] have reported the existence of a change in cerebral energetic metabolism before and after convulsive seizures. Sanders *et al.*[2] have shown that a lowering of the cerebral ATP level preceded convulsions induced by hydroxylamine or metrazol. We have tried to find out: (1) If it is possible to increase the level of cerebral NAD, and could such an increase augment the level of energy rich bonds. (2) Could this increase give protection against convulsive seizures.

An increase in the level of NAD after nicotinamide treatment has been demonstrated in the liver by Kaplan *et al.*,[3] in the kidney by Revel and Mandel[4] and in chicken brain by Doly and Mandel.[5] We have investigated the same phenomenon in mouse brain, and have tried to potentiate this effect by associating adenosine with nicotinamide. During this work, we have noticed that adenosine modifies the behaviour of mice; in particular it has a sedative effect. We have thus investigated the mechanism, and a possible parallelism between the sedative and anticonvulsivant effects. At the same time, we have sought to characterize the various pharmacological effects of adenosine, its association with nicotinamide, and, in particular, its effect on the cerebral levels of ATP, phosphocreatine, GABA, and noradrenaline.

To examine the effects of increased NAD levels and possibly the levels of energy rich bonds on convulsive seizures, we chose, as a model, the audiogenic seizure in Swiss albino mice of the RB strain, genetically sensitive to an acoustic stimulus.

The audiogenic seizure is a highly reproducible effect, and has an advantage over seizures produced by toxic agents which tend to produce secondary metabolic effects.

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Preliminary results of research into the effects of nicotinamide on audiogenic fits have been published previously.[6]

EXPERIMENTAL

Behavioural studies

Studies on the protection against audiogenic seizures. The experiments were performed on male mice of RB strain, sensitive to audiogenic convulsions, aged from 2·5 to 3 months, weighing about 30 g and were provided by the Laboratoire de Physiologie Acoustique (Jouy-en-Josas). At this age, the sensitivity to sound stimulus is at a maximum; later, it decreases. The experiments were carried out on randomly selected groups of at least fifteen animals.

To check their sensitivity, the animals were subjected to a sound stimulus of 7500 Hz at an intensity of 100 dB [C] 24 hr before the attempt to protect them by the substances under investigation. Adenosine was injected intraperitoneally in doses from 25 mg/kg up to 130 mg/kg. We chose 130 mg/kg injected as an aqueous suspension as it was equimolecular with the optimum dose of nicotinamide (400 mg/kg) as reported by Simler *et al.*[7] Each animal from an homogeneous group was submitted to a single stimulation during the experiment (7500 Hz, 100 dB [C]). In each case, the results were compared with those obtained from a control group of animals injected with physiological serum.

Sedative effect of adenosine. For each study, randomly selected animals were injected intraperitoneally with adenosine. The spontaneous activity of these animals (in groups of 10) was quantified by the following two methods:

(1) Recording personal observation, on an arbitrary scale from zero to four, every 30 sec.

(2) Recording the electromyogram of the neck muscles.

The results from the two methods were in agreement. Results, taken at the same time from a group of animals receiving physiological serum served as a reference. The sedative effect of adenosine was tested on Swiss Albino mice, the same effect was found in various other strains of mice and in Wistar rats.

Electroencephalographic recordings. The electroencephalographs were made with the aid of four enameled platinum electrodes (diameter 5–10 mm, 2 frontal, 2 occipital) placed at the cerebral surface after perforation of the cranium. The animal was anaesthetized with 100 mg/kg Nembutal and maintained in a stereotaxic apparatus. The electrodes were held in place by dental cement. The EEG were recorded 8 days after the implantation on the confined animal. Four recordings were taken from the animals which received adenosine (130 mg/kg) + nicotinamide (400 mg/kg) and were compared with those from two control animals which received physiological serum.

Measurement of the arterial blood pressure. The measurements were taken with the aid of apparatus from the EEM Instrument Company, Texas, equipped with a tail probe. The animals were placed in an enclosure maintained at 29° isolated by a Faraday cage.

Biochemical determinations

Enzymatic analyses of ATP, phosphocreatine, GABA and NAD. The assays were performed on the pooled extracts from three mouse brains. The treated and control

animals were killed by immersion in liquid nitrogen at various times after injection. The brains were removed whilst frozen, and homogenized in 3 ml ethanol–water (1:1, v/v) maintained at −20°. After centrifugation and reextraction twice with 3 ml of the same mixture, the supernatants were pooled, then acidified with 3·2 ml 2 N perchloric acid. After the final centrifugation, the pellet was washed twice with 0·3 N perchloric acid. The pooled supernatants were neutralized with 10 M, 1 M and finally 0·1 M KOH, using a pH meter. The suspension was left for 1 hr, then centrifuged. The resultant pellet was washed twice with ethanol–water, and the final volume was adjusted to 30 ml. All these operations were carried out at 4°. The assays were performed in a Zeiss fluorometer. For ATP, phosphocreatine and NAD the method of Lowry *et al.*[8] was used, and for GABA, the method of Scott and Jacoby.[9]

The incorporation of ^{32}P into ATP, phosphocreatine and NAD. The mice were sacrificed by immersion in liquid nitrogen 6 hr[10] after intraperitoneal injection of 200 μCi ^{32}P in 0·5 ml of physiological serum and 45 min after injection of the adenosine–nicotinamide mixture (130 mg/kg, 400 mg/kg).

After preliminary trials, we adopted the following procedures. The acid soluble material was extracted from twelve brains at a time. The next operations were carried out as previously described. The final extract was lyophilized, taken up in water and layered on a Dowex® column 1 × 8, H^+ from, ϕ 1·5 cm, I = 30 cm. The elution was carried out by the method of Davis and Minard[11] with the following modifications: A linear gradient of water (450 ml) formic acid (3·5 N) allowed good resolution of CMP, NAD, AMP and phosphocreatine. The column was then washed with formic acid 4 N–ammonium formate 0·4 N buffer, which permitted separation of ADP and ATP. The optical density at 260–280 nm and the radioactivity were recorded on 3-ml fractions of the eluate. The various peaks were identified by means of the ratio of the optical density at 280 and 260 nm.

The amounts of different nucleotides were evaluated by means of the molecular extinction coefficient determined by Bock *et al.*[12] and Colowick *et al.*[13] In the elution zone of phosphocreatine, inorganic phosphate was measured in each fraction after hydrolysis by the method of Briggs.[14] The relative specific activities were established by their relationship to the specific activity of acid soluble inorganic phosphate determined by Doty's method.[15]

Determination of the specific activity of the terminal phosphate of ATP. The fraction corresponding to ATP was lyophilized, and the formate was removed by sublimation. The material was taken up in 1 ml of 0·1 M Tris–HCl buffer, pH 7·3, containing 10 mM glucose, 5 mM $MgCl_2$, and 0·02 % serum albumin. Fifty microlitres of this solution was spotted on a fluorescent silica gel TLC plate (Merck). Another 50 μl sample was spotted after 30 min incubation at room temperature with 10 μg hexokinase. The chromatogram was developed for 3 hr in ammonia–water–methanol (10:10:60, by vol). The u.v. absorbing spots, ATP and ADP (about 0·02 μmole) were scraped off and eluted from the silica gel with 1 ml of 0·01 N HCl. The activity of the γ-phosphate was determined by the difference in the activities in aliquots of the ADP and ATP fractions.

Cerebral levels of noradrenaline. The mice were separated into four groups of eight animals. The first group received an intramuscular injection of physiological serum; the second, an injection of 50 mg/kg FLA 63, an inhibitor of dopamine-β-hydroxylase[16]; the third, an injection of FLA 63 + 70 mg/kg of caffeine; and the fourth

an injection 50 mg/kg FLA 63 + 130 mg/kg of adenosine. Nicotinamide was not injected because this experiment was only designed to study the relationship between adenosine-sedation and noradrenaline. After 90 min the animals were killed by immersion in liquid nitrogen. The brains were removed whilst frozen, then homogenized in ethanol–water–0·5 0·5 M HCl (74 : 16 : 20, by vol.) at 4°C.

The noradrenaline was separated on a Dowex® column according to the method of Pujol[17] and assayed by the method of Laverty and Taylor.[18] (Fla 63:*bis*[4-methyl-1-homopiperazinyl thiocarbonyl] disulfide came from A.B. Biotec.)

RESULTS

Behavioural studies

Protection against audiogenic seizures. Protection against audiogenic seizure was observed after administration of nicotinamide, adenosine, and a mixture of both compounds. The degree of protection as a function of time is represented in Fig. 1. Protection was maximum 45 min after injection. The protection afforded by nicotinamide alone was slight (20 per cent of the animals) ; that of adenosine was better (55 per cent), whereas a mixture of both substances induces protection in all the animals. However, 60 min after the injection, the protection was only 50 per cent; it stayed at this level for about 1 hr, then decreased.

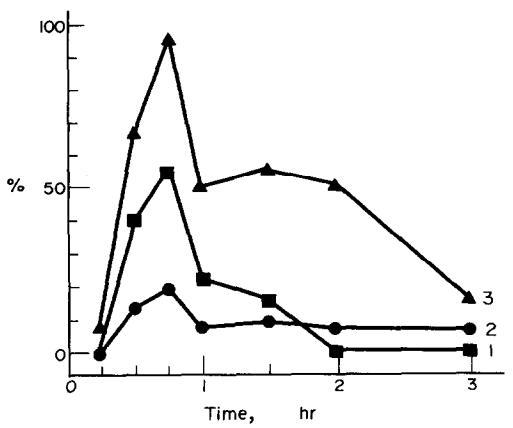

FIG. 1. Kinetics of the total protection against the audiogenic seizure following i.m. injection of: 1, adenosine (130 mg/kg) ; 2, nicotinamide (400 mg/kg) ; 3, adenosine (130 mg/kg) + nicotinamide (400 mg/kg). Population size : 15–20.

Sedative effect of adenosine. In all the animals we have studied, a striking change in behaviour was observed within 2 min after injection of adenosine. The animals could no longer stand on their feet and rested their bellies and heads on the ground, their eyes half closed. After a variable lapse of time, varying with the dose injected (Fig. 3) their activity returned progressively to normal. Figure 2 represents the integration of spontaneous activity after injection of physiological serum and various amounts of adenosine (25 mg/kg, 75 mg/kg). This figure indicates the latent period and the duration of sedation. As can be seen in Fig. 3, where each point represents a group of 10 mice, there is a linear relationship between the dose of adenosine

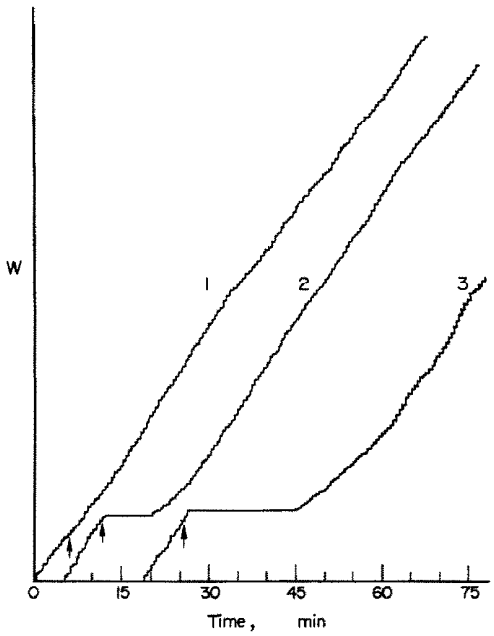

FIG. 2. Sedative effect of adenosine: cumulative spontaneous activity (arbitrary units) $W = \Sigma\, dw$ following an i.m. injection in mice. (1) Control; (2) adenosine (25 mg/kg); (3) adenosine 75 mg/kg. The arrows mark the injection. The large horizontal lines characterize the sedation period.

and the length of sedation. Nicotinamide injected alone did not produce sedation and when injected with adenosine did not alter the sedation time of adenosine alone. It is notable that there was no parallel between the sedative phase (Fig. 3) during which the animals always reacted to tactile stimuli, and the onset of resistance to auditory stimuli (Fig. 1). Indeed, even 15 min after injection of adenosine + nicotinamide, when the sedative effect was well established, there was still no protection

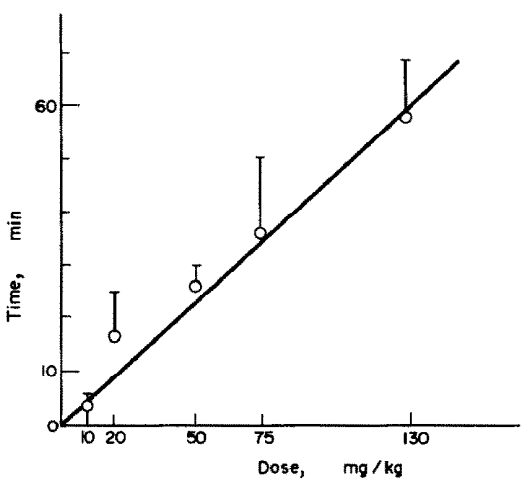

FIG. 3. Sedation time following i.m. injection of adenosine. Population size: 10. The vertical bars represent the standard deviation.

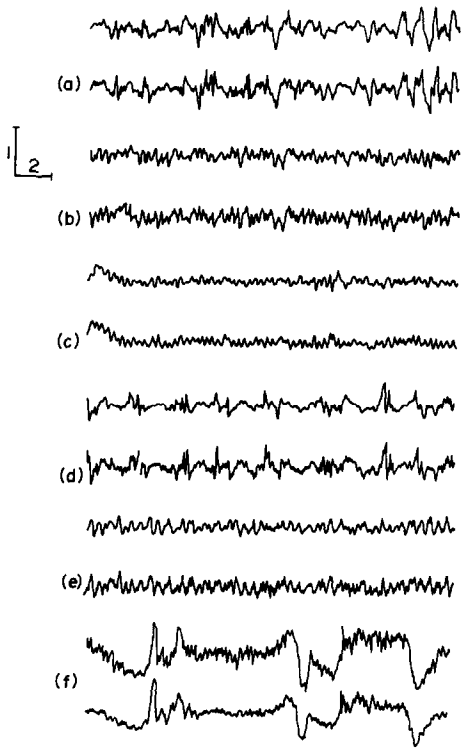

FIG. 4. EEG left and right fronto-occipital derivations. (a) Control; (b) injection of 400 mg/kg nicotinamide + 130 mg/kg adenosine; (c) 90 sec later; (d) 15 min later; (e) acoustic stimulus on a protected mouse 45 min after the injection (100 per cent of mice protected); (f) acoustic stimulus on an unprotected sensitive mouse. Scale 1, 400 μV, scale 2, 1 sec.

against seizures. An hour after the same injection, when the sedative effect was over, 50 per cent of the animals were immune to the auditory stimulus.

Electroencephalography. Figure 4 represents the EEG trace after injection of adenosine + nicotinamide (130 mg/kg, 400 mg/kg). Almost immediately (20 sec), the EEG trace was modified; passing from a slow rhythm with a high voltage to a faster rhythm with a weak voltage characteristic of a waking rhythm. One minute after the injection, and for about the next 10 min, this rhythm was pure, without any other activity. Then the trace returned to its initial pattern. The EEG trace registered during an acoustic stimulus, 45 min after the injection, when 100 per cent of mice are protected, was identical to that recorded from a protected animal receiving no stimulus.

Measurement of arterial pressure. In the mouse, injection of adenosine (130 mg/kg) caused a lowering in pressure, the minimum value (fall of about 25 per cent) being reached after 20 sec. The recovery time was greater than 90 min. Injection of nicotinamide induced a fall in pressure, the minimum being 30 min after injection (Fig. 5). This fall was about 40 per cent, and the recovery time was greater than 90 min. Adenosine and nicotinamide together caused a fall in pressure, the lowest value being 10 min after administration (fall of 45 per cent). It should be noted that the lowest

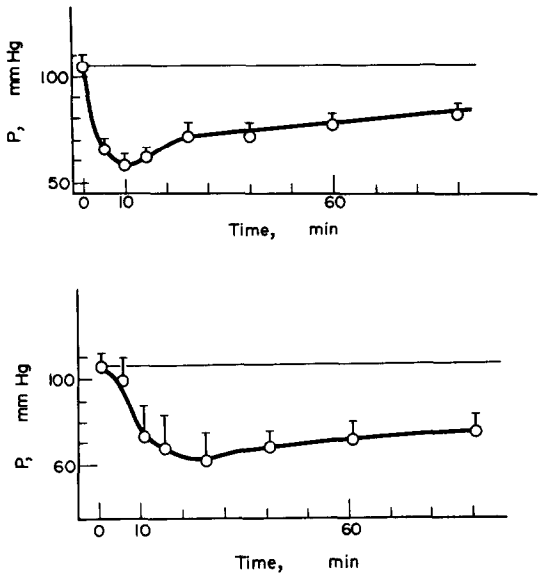

FIG. 5. Mean blood pressure following injections in unanaesthetized mice. Vertical bars represent standard deviations. Top: adenosine 130 mg/kg + nicotinamide 400 mg/kg; bottom: nicotinamide 400 mg/kg.

pressure was reached at a time when there was no protection against auditory stimuli, and that the pressure was still decreased by 20 per cent at the time of maximum protection.

Biochemical studies

Levels of ATP and phosphocreatine. After injection of adenosine + nicotinamide (130 mg/kg, 400 mg/kg) there was a significant increase in the cerebral levels of ATP and phosphocreatine. They reached their maximum values 45 min after injection. This increase of about 30 per cent in energy rich compounds coincided with the time of maximum protection against auditory stimuli (Fig. 6). During this same period,

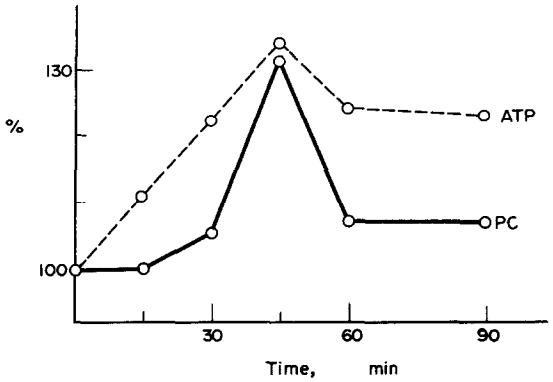

FIG. 6. Creatine phosphate and adenosine triphosphate after an i.m. injection of nicotinamide (400 mg/kg) + adenosine (130 mg/kg). Standard deviation < 7 per cent.

there were no significant variations in the cerebral levels of NAD and GABA (0·19 ± 0·025 μmole/g and 1·70 ± 0·20 μmole/g wet wt respectively).

Incorporation of ^{32}P into ATP, phosphocreatine, and NAD. These studies were undertaken with the aim of clarifying the cause of an increased rate of synthesis of ATP and phosphocreatine or possibly the decrease in their rates of utilization. The results are shown on Table 1. There was no significant difference in the relative specific activities of the control mice and the treated mice with regard to ATP, the γ-phosphate of ATP, phosphocreatine and NAD.

TABLE 1. RELATIVE SPECIFIC ACTIVITIES OF BRAIN CREATINE PHOSPHATE, ADENOSINE TRIPHOSPHATE AND γP-ADENOSINE TRIPHOSPHATE

	Creatine phosphate	ATP	γP-ATP
Control	1·45 ± 0·36 (6)	4·35 ± 0·50 (5)	2·04 ± 0·22 (4)
Adenosine + nicotinamide (130 mg/kg; 400 mg/kg)	1·22 ± 0·26 (4)	3·75 ± 0·20 (4)	1·92 ± 0·15 (4)

Relative specific activities are expressed as the mean ± S.E.M. Mice were killed 6 hr after i.p. injection of ^{32}P and 45 min after i.p. injection of adenosine + nicotinamide. Number of experiments is given between brackets.

Utilization of noradrenaline. After blocking noradrenaline synthesis by the dopamine β-hydroxylase inhibitor, Fla 63, we noticed that following an injection of caffeine, there was a significant fall in the cerebral level of noradrenaline (17 per cent), whereas, after injection of adenosine, the fall was 27 per cent (Table 2).

TABLE 2. LEVEL OF CEREBRAL NORADRENALINE AFTER INHIBITION OF DOPAMINE-β-HYDROXYLASE AND CAFFEINE OR ADENOSINE INJECTION

	Noradrenaline (ng/g wet wt)	No. of animals	P
Control	500 ± 46	8	<0·005
Fla 63, 50 mg/kg	345 ± 28	8	
Fla 63, 50 mg/kg + caffeine 10 mg/kg	288 ± 26	8	<0·0025
Fla 63, 50 mg/kg + adenosine 130 mg/kg	252 ± 44	8	<0·0005

DISCUSSION

The feeble protective effect afforded by nicotinamide injected alone is in accord with the results of Lehmann *et al.*[6] with regard to 2·5 month–old sensitive mice, undergoing a single stimulus after injection. The 100 per cent protection afforded against audiogenic convulsions when adenosine and nicotinamide are injected together is of short duration and takes place about 45 min after injection. However, the development of this protection does not parallel that of the sedative nor the hypotensive effect of adenosine. We noticed that 50 per cent of the animals were still protected 1–2 hr after injection. After injection of adenosine and nicotinamide, we did not observe a significant increase in the cerebral NAD level.

Our kinetic studies on the sedative effects and the lowering of arterial pressure, show that protection reaches its maximum towards the end of the sedative effect, whereas the arterial pressure reaches its minimum value after 30 min, then increases towards normal. Indeed, 50 per cent of the protection remains when the arterial pressure is almost back to normal, and when there is no longer a sedative effect.

There was no sedation after injection of nicotinamide alone, whereas this compound induced a 40 per cent fall in blood pressure, thus, there is no direct relationship between the fall in pressure and the sedative phase. It is worth noting that the maximum protective effect coincides with the maximum cerebral levels of ATP and phosphocreatine. This elevation is most probably the result of a diminution in the rate of utilization of these energy rich compounds, as suggested by our studies on ^{32}P incorporation. This increase in ATP level is not specific for adenosine. It also occurs after administration of numerous antiepileptic agents, in particular *n*-dipropyl acetic acid, which causes an increase of about 15 per cent in the cerebral levels of ATP and phosphocreatine.[19]

The sedative effect of adenosine could be compared with the inhibitory effect of adenosine derivatives, particularly ATP, on the smooth muscle of guinea pig intestine. From these results a non-adrenergic inhibitory system, using ATP as a mediator, has been postulated.[20]

After injection of adenosine, the reduction in the cerebral noradrenaline level, as far as we could tell from our experiment, can be compared with the effect of caffeine, as described by Waldeck.[21] There is a structural analogy between these two compounds, which however produce opposite effects with regard to motility, arterial pressure, basal metabolic rate and body temperature.

The effect of adenosine on the noradrenaline level can be compared with that of caffeine, the inhibitory action of which on phosphodiesterase activity, causes an increase in the cerebral level of cyclic AMP. At the cerebral level, adenosine potentiates the action of noradrenaline in increasing the cAMP level.[22] Liberation of ATP accompanies the release of catecholamines in numerous nervous tissues.[23,24] In both cases, on injection of caffeine or adenosine, an increase in the cAMP level was noticed.[25]

At the same time, a lowering of the cerebral level of noradrenaline is seen. This lowering cannot be caused by sedation, for a similar effect on the cerebral level of noradrenaline is seen after administration of either caffeine or adenosine, which cause opposite behavioural effects.

The pharmacological effects of ATP described by Mathieu-Levy[26] are most probably, at the cerebral level, those of adenosine. The effects of the two compounds are identical, yet it is known that ATP does not cross the blood–brain barrier, whereas adenosine does so easily.[27] Thus, ATP must undergo degradation in the body before producing the described effects. Adenosine is incorporated in the brain tissues of the guinea-pig, and released by electrical stimulation. At low concentrations it is utilized in ATP synthesis.[28]

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