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The influence of GABA on the synthesis of N-acetylserotonin, melatonin, O-acetyl-5-hydroxytryptophol and O-acetyl-5-methoxytryptophol in the pineal gland of the male Wistar rat.

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Summary.

The influence of GABA on the synthesis of N-acetylserotonin, melatonin, O-acetyl-5-hydroxytryptophol and O-acetyl-5-methoxytryptophol has been investigated using different experimental procedures. It was demonstrated that when GABA and an acetyl donor were added to the incubation medium together, a significant increase in synthesis of the N-acetylated products occurred during the night. Moreover there was a large increase in N-acetylserotonin synthesis at 15^{00} hrs although none was observed in the control experiments.

However, when GABA was added 20 min before the acetyl donor, synthesis of the N-acetylated products was significantly less. The opposite effect was observed for the O-acetylated indoles. These results confirm the proposal by Ebadi *et al.* (1982) that GABA, like norepinephrine, may be a regulator of melatonin synthesis. As melatonin is implicated in the regulation of reproduction it may be that GABA is equally significant in this regulatory effect.

Introduction.

GABA has been identified recently as a compound which occurs abundantly in the central nervous system (Enna, 1981 ; Defeudis and Orensanz-Munoz, 1980). Both GABA (Schon *et al.*, 1975 ; Labella *et al.*, 1968 ; Waniewski and Suria, 1977) and GAD (Kanazawa *et al.*, 1976) are also present in the pineal

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The following abbreviations have been used (Smith, 1982) : Serotonin, HT ; N-acetylserotonin, aHT ; melatonin, aMT ; O-acetyl-5-hydroxytryptophol, aHL ; O-acetyl-5-methoxytryptophol, aML ; N-acetyltransferase, NAT ; hydroxyindole-O-methyltransferase, HIOMT ; γ-aminobutyric acid, GABA ; glutamic acid decarboxylase, GAD (Glutamic acid → GABA) ; GABA transaminase, GABA-T (GABA → succinic semi-aldehyde) ; acetylcoenzyme A, ac-CoA.

gland and this strongly suggests that synthesis occurs. GABA is metabolized by GABA-T and this enzyme has also been demonstrated in the pineal gland (Schon *et al.,* 1975 ; Waniewski and Suria, 1977). Furthermore GABA can be taken up from the circulation (Schon *et al.,* 1975 ; Waniewski and Suria, 1977).

GABA in the central nervous system occurs in both neurons and glial cells. In neurons the function of GABA resembles that of an inhibitory neurotransmitter causing a change in ion conductance. However, the role of GABA in glial cells is still obscure and it is not even known if there is any relationship between neurons and glial cells in this respect. As it has been shown by Schon *et al.* (1975) that GABA is taken up exclusively in glial cells of the pineal gland, a possible correlation with functional compounds of the pineal cannot be excluded. The pineal contains a number of types of biologically active compounds (peptides, Benson, 1977 ; pteridines, Ebels, 1981 ; and indoles, Birau and Schloot, 1981) but it is the indoles and their metabolism which have been most extensively studied. Most investigations have been done on melatonin and its action on the reproductive system (Reiter, 1980), and on N-acetyltransferase, the rate-limiting enzyme in melatonin synthesis (Moore and Klein, 1974) which converts HT to aHT. The latter product is subsequently methylated to form aMT. It is generally accepted that norepinephrine is synthesized by the nervi conarii which projects from the superior cervical ganglia to the pineal and is released in darkness. This catecholamine activates as β-receptor which in turn stimulates adenylate cyclase to form cAMP which stimulates NAT activity (see review : Balemans, 1979, 1981a).

The influence of GABA on indole metabolism is not well known even though a direct action of GABA on NAT and of GABA on NAT stimulated by norepinephrine has been shown. In the rat neither experimental procedure had any effect (Mata *et al.,* 1976 ; Wheler and Klein, 1980). In bovine pineals, however, Ebadi and Chan (1980) and Chan and Ebadi (1980) demonstrated an inhibition of the norepinephrine-induced stimulation of NAT.

N-acetyltransferase, however, shows a day/night rhythmicity with a peak of activity at the beginning of the night (Klein and Weller, 1970). In the experiments with GABA, only one point in the 24 hour period was examined. Therefore it was decided to investigate the influence of GABA on the synthesis of N-acetylated (aHT, aMT) and O-acetylated (aHL, aML) indoles in the pineal of the male Wistar rat at 10 points in the 24-hour period.

Materials and Methods.

Male Wistar rats of 137 ± 3 g were used. They were kept at a constant temperature of 23 °C, under a 12 L : 12 D schedule (lights-on at 7 a.m. and off at 7 p.m.), and a relative humidity of 60 p. 100. Water and food were available *ad libitum.*

GABA, melatonin and N-acetylserotonin were obtained from Sigma Chemical Co., St. Louis, U.S.A. ; (^{3}H)-acetyl coenzyme A from Amersham International, England.

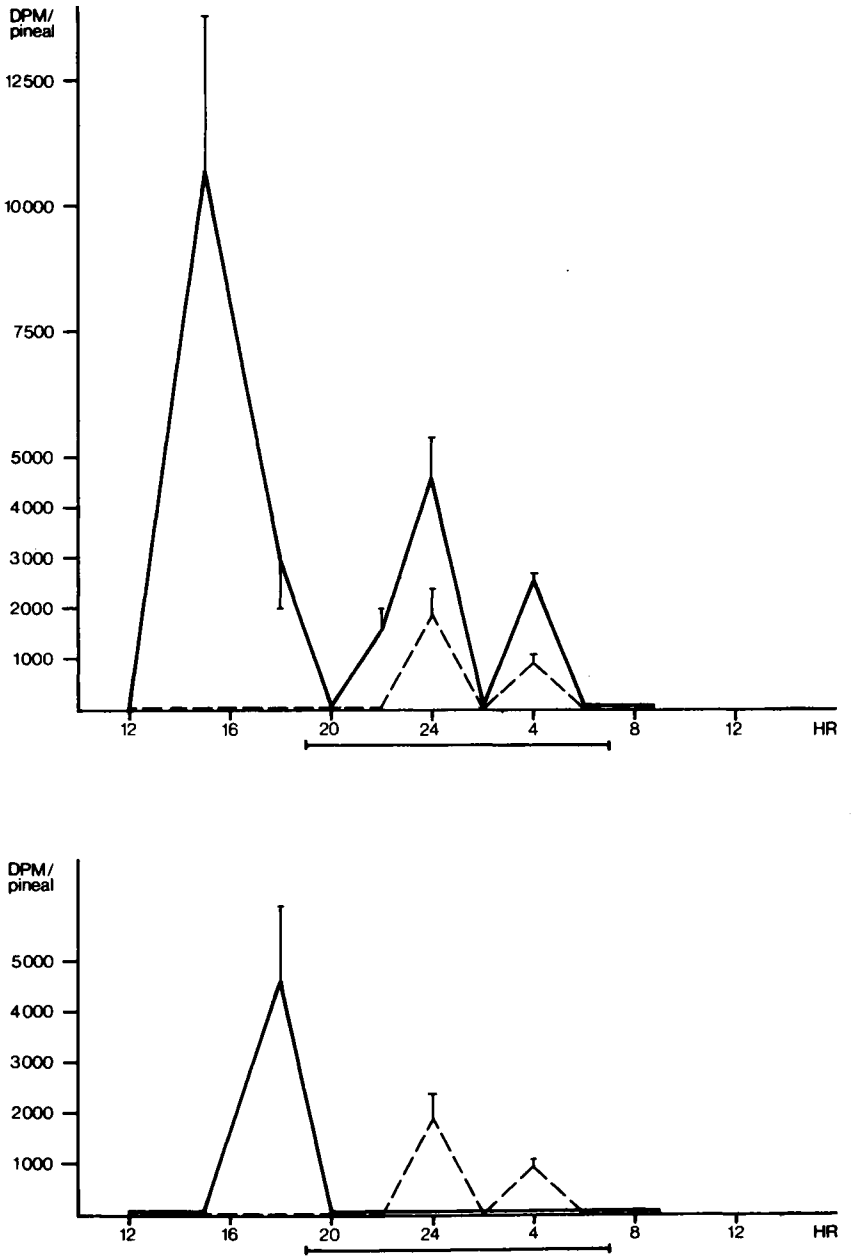

FIG. 1. — *Circadian rhythm of N-acethyltransferase capacity to synthesize N-acetylserotonin whether influenced* (————) *or not* (----------) *by GABA.*
A. (*above*) : acetyl CoA was administered simultaneously with GABA for 60 min.
B. (*below*) : GABA was first administered for 20 min, followed by the addition of acetyl CoA, and then left for the remaining 40 min.

The following solutions were used for the experiments : phosphate buffer pH 8.0, 0.1 M ; (^{3}H)-acetyl coenzyme A was dissolved at a concentration of 1.5 μC_i in 10 µl of HCl, pH 5.0 ; GABA at 2×10^{-3} M in the above phosphate buffer. Each incubation lasted one hour.

Thin-layer chromatography was carried out on Merck DC silica gel plates 60 F 254 using chloroform : methanol : acetic acid (93 : 4 : 3) for the first direction and chloroform : methanol : ammonia (60 : 35 : 5) for the second direction. The plates were dried under nitrogen between runs.

Nine animals, taken every 3 hrs during the light period and every 2 hrs during the dark period, were killed and their pineals excised. Three pineals were incubated separately in 20 µl of phosphate buffer plus 10 µl of acetyl-CoA and served as controls ; three pineals were separately incubated in 20 µl of buffer containing GABA, and 10 µl of acetyl-CoA. The last three were separately incubated in 20 µl of buffer containing GABA for twenty minutes after which time the 10 µl of acetyl-CoA was added and left for the remaining 40 min. Each incubation was stopped by adding 10 µl of H_2SO_4 pH 1.0. Synthetic, non-radioactive indoles were added for reference purposes, the pineal was homogenized in its incubation mixture and the whole was transferred to a thin-layer plate for bi-dimensional chromatography.

The standard indoles were visualized with the aid of a 254 nm UV light and marked. Using this technique, aHT, aMT, aHL and aML were separated from each other and from the various acetyltryptophans which all ran together near the origin. The indole spots were scraped off into vials, 75 µl of ethanol was added to dissolve them and 5 ml of scintillant was added (toluene : 1 000 ml ; popop : 0.1 g ; PPO : 5 g and Cabosil : 40 g). The vials were counted in a liquid scintillation counter (Balemans *et al.,* 1981c).

Results.

In the control pineals, aHT was synthesized only during the night. Two peaks of activity were observed, namely at 24^{00} and 04^{00} hr (fig. 1 A). When GABA and acetyl CoA were added simultaneously, these two peaks increased greatly and a third, very much larger, peak was observed at 15^{00} hr (fig. 1 A). However, when the GABA was added first and the acetyl-CoA some 20 min later the night-time peaks were not observed, and the day-time peak was diminished and shifted to 18^{00} hr (fig. 1 B).

Melatonin was also synthesized at night in the control groups with greatest activity between 02^{00} and 04^{00} hr. As with aHT, its synthesis was also increased when GABA and acetyl-CoA were added simultaneously (fig. 2 A). In both cases a second, smaller peak was observed at 09^{00} hr. However, when GABA was added first and followed by the acetyl-CoA later, the night-time peak diminished but the early morning peak was unaffected (fig. 2 B). aHL was synthesized in much smaller amounts than the aHT and aMT but it showed a broad peak between 06^{00} and 12^{00} hr in the controls (fig. 3 A). Adding GABA plus ac-CoA together resulted in a very similar pattern but with a very small peak at 02^{00} hr also. However, the situation was quite different when GABA was followed by

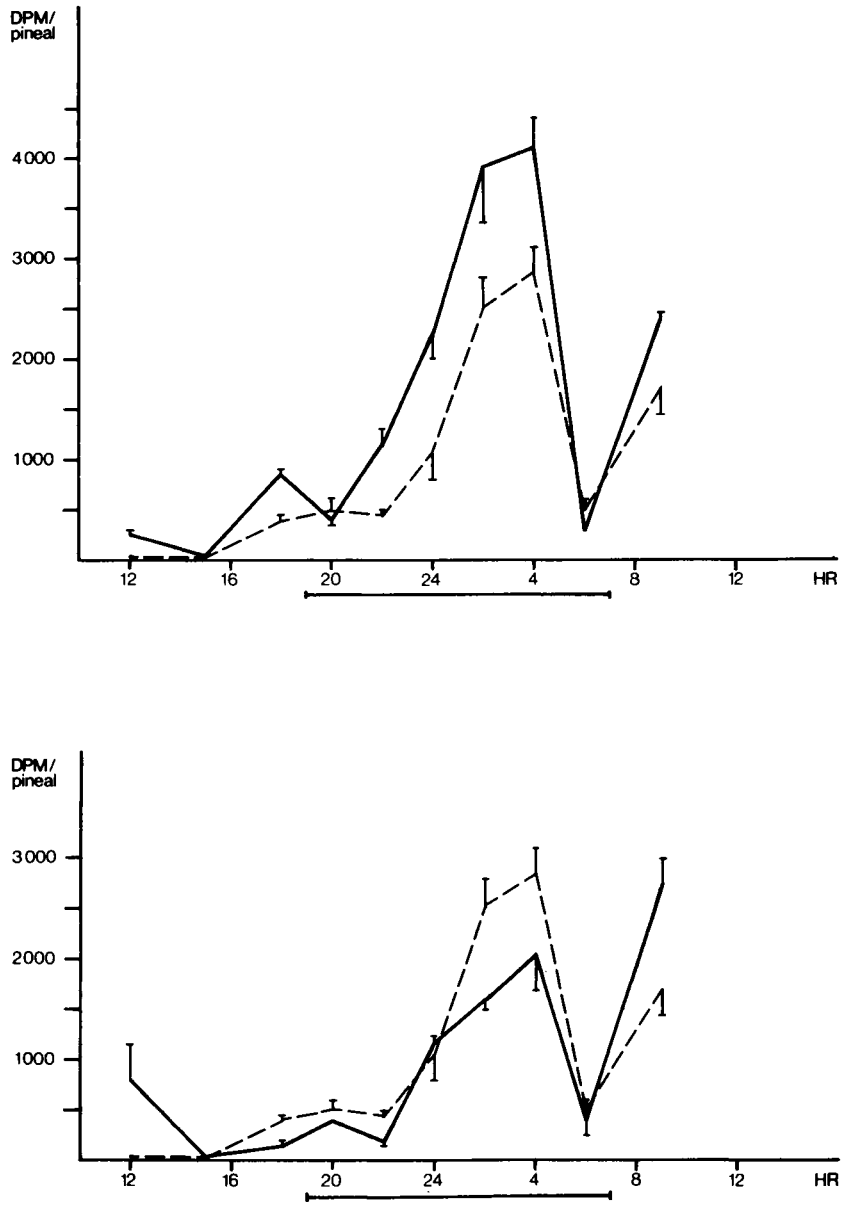

FIG. 2. — *Circadian rhythm of N-acetyltransferase capacity to synthesize melatonin whether influenced* (————) *or not* (----------) *by GABA.*
A. (*above*) : acetyl CoA was administered simultaneously with GABA for 60 min.
B. (*below*) : GABA was first administered for 20 min, followed by the addition of acetyl CoA, and then left for the remaining 40 min.

ac-CoA later as both the previous peaks observed then showed substantial increase and a third peak was evidenced at 20^{00} hr (fig. 3 B).

The situation with aML was still different. The amounts formed were intermediate between those of aHL on the one hand and aHT and aMT on the other. The controls seemed to show one peak of activity through the night, starting at about 20^{00} hr and continuing to 09^{00} hr (fig. 4 A), and a second peak

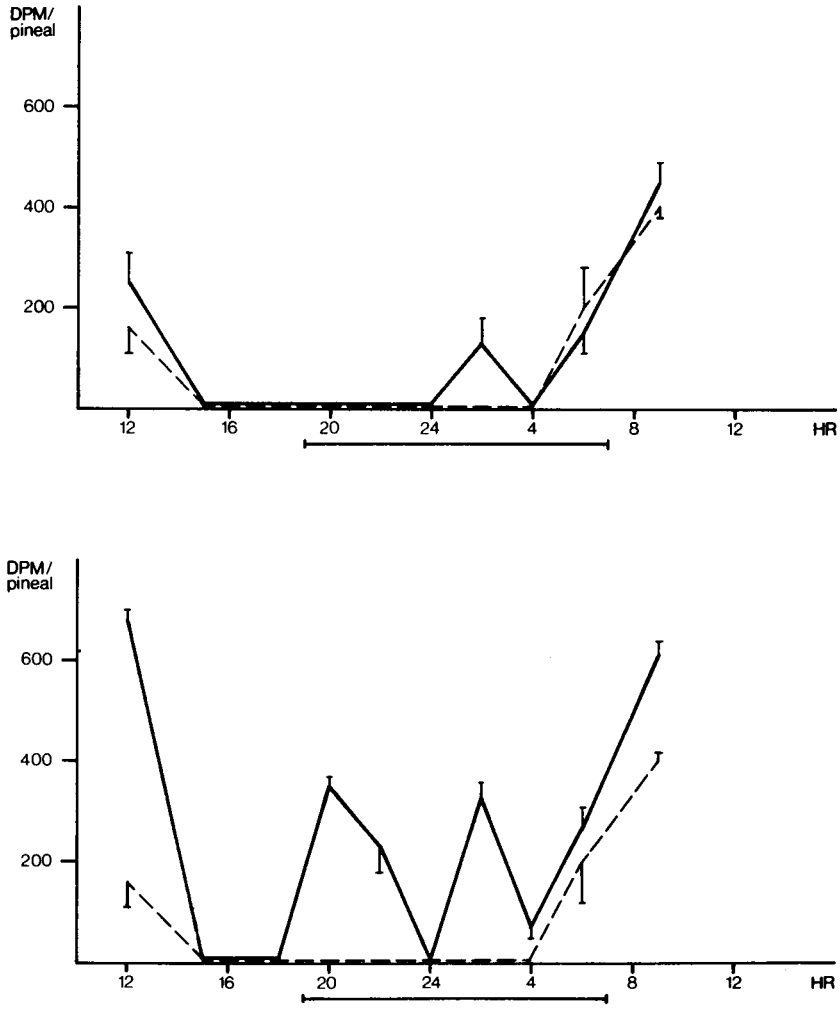

FIG. 3. — *Circadian rhythm of O-acetyltransferase capacity to synthesize O-acetyl-5-hydroxytryptophol whether influenced* (————) *or not* (----------) *by GABA.*
A. (*above*) : acetyl CoA was administered simultaneously with GABA for 60 min.
B. (*below*) : GABA was first administered for 20 min, followed by the addition of acetyl CoA, and then left for the remaining 40 min.

at about 12^{00} hr. With GABA plus ac-CoA synthesis diminished (fig. 4 A) but with GABA added before ac-CoA the reverse was again seen with substantial increases during the whole night, although the peak began and ended earlier and the second peak then occurred at about 09^{00} hr (fig. 4 B).

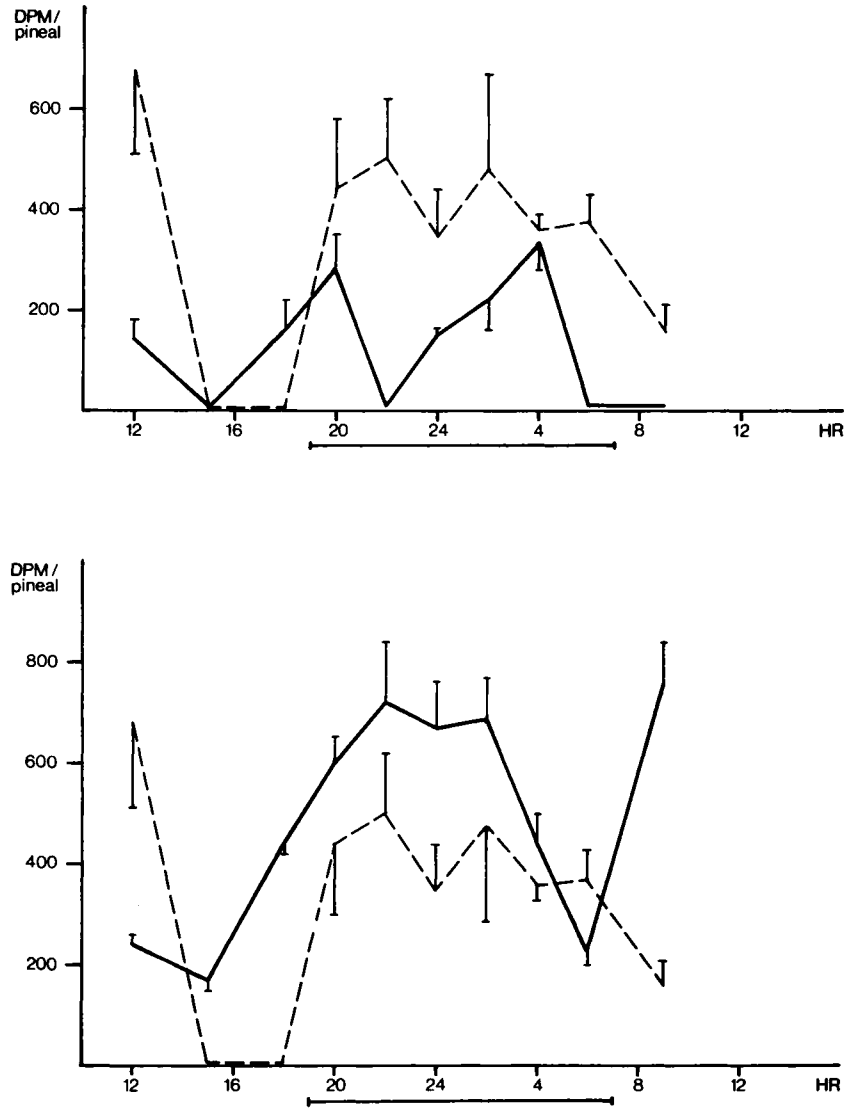

FIG. 4. — *Circadian rhythm of O-acetyltransferase capacity to synthesize O-acetyl-5-methoxy-tryptophol whether influenced* (————) *or not* (----------) *by GABA.*
A. (above) : acetyl CoA was administered simultaneously with GABA for 60 min.
B. *(below)* : GABA was first administered for 20 min, followed by the addition of acetyl-CoA, and then left for the remaining 40 min.

Discussion.

The data presented here demonstrate that the two different experimental conditions produced results which were different and completely opposite to each other when compared with the controls.

The finding that N-acetylserotonin and melatonin syntheses were increased after simultaneous treatment with both GABA and the acetyl donor contradicts the finding that GABA has an inhibitory effect on N-acetyltransferase activity in the bovine pineal (Ebadi and Chan, 1980 ; Chan and Ebadi, 1980) and no effect on N-acetyltransferase activity in the rat pineal (Mata *et al.,* 1976). However, our data showing that the synthesis of N-acetylserotonin and melatonin occurs when GABA is added first and the acetyl donor 20 min later, agree with those reported in the bovine pineal gland but not with data on the rat pineal. These different results may be explained by variation in melatonin synthesis in different animal species (Ebadi *et al.,* 1982), the age of the animals, the time of day they are killed, and the different technical procedures used. Ebadi and Chan (1980), using pineal explants in culture and an enzyme assay with tryptamine or serotonin as a substrate, aimed to influence the norepinephrine-induced stimulation of N-acetyltransferase activity. In the present work the effect of GABA on the synthesis of two N-acetylated and two O-acetylated indoles has been investigated during a light/dark rhythm in the juvenile rat using endogenous substrates.

The question arises as to which of the two experimental conditions in our work can be thought to be nearest to the natural conditions, and whether both conditions can occur. Several interpretations are possible.

1) The influence of GABA on N-acetylation took place in the first 20 min. Hence, when the acetyl donor was given together with GABA the tritiated acetylated products were synthesized and could be investigated. When the tritiated acetyl donor was given 20 min later, then (on the basis of this hypothesis) the reaction would already have taken place with the endogenous acetyl donor and little or no synthesizing activity would be observed.

2) It has been shown already that N-acetylserotonin (Balemans *et al.,* in preparation) and melatonin syntheses (Balemans *et al.,* 1980, 1981b) show light/dark rhythmicity which differs during the course of the year due to shifting of the peaks of maximal synthesis. It therefore might be that in one season the highest GABA synthesis or content would coincide with the highest N-acetyltransferase activity (or sensitivity), causing an increase in N-acetyltransferase activity. When GABA did not coincide with N-acetyltransferase activity (or sensitivity) no influence would be found.

It is striking that the synthesis of the O-acetylated indoles after GABA treatment shows the opposite effect when compared to the synthesis of N-acetylated indoles. In this case, one might wonder whether there was an inverse relationship between N- and O-acetylation. If there was no HT or MT precursor available at that time for N-acetylation, then O-acetylation would take place. This hypothesis not only explains the increased synthesis when ^{3}H-acetyl donor is added 20 min after GABA administration, but it also accounts for the small amount of synthesis.

Earlier investigations on the influence of white and green light on N- and O-acetyltransferase capacity of the pineal have suggested a relationship between O-acetylmethoxytryptophol and melatonin. The green light/dark rhythmicity of O-acetylmethoxytryptophol is identical to the light/dark rhythmicity of melatonin (Balemans *et al.,* 1981c). No correlation between the present results and data obtained after white and green light application can be made yet. Therefore, in order to understand the influence of light/dark on GABA synthesis it is necessary to investigate these different light conditions. Since, besides norepinephrine, cAMP, taurine and indole derivatives, acetyl CoA is also implicated in N-acetyltransferase activity (Balemans, 1981a ; Chan and Ebadi, 1981, 1982), it seems that the regulation of melatonin synthesis is more complicated than believed.

Concerning the role of GABA, it can be concluded that one has to be very cautious in explaining the results on indole metabolism obtained with GABA treatment, especially when suggesting that GABA may be an inhibitory or stimulatory compound.

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Résumé. *Influence de l'acide amino butyrique (GABA) sur la synthèse de la N-acétylsérotonine, de la mélatonine, de l'O-acétyl-5-hydroxytryptophol et de l'O-acétyl-5-méthoxytryptophol dans la glande pinéale du rat mâle Wistar.*

L'influence de GABA sur la synthèse de la N-acétylsérotonine, de la mélatonine, de l'O-acétyl-5-hydroxytryptophol et de l'O-acétyl-5-méthoxytryptophol a été étudiée grâce à divers procédés expérimentaux.

Lorsque du GABA et un donneur du groupement acétyle sont introduits simultanément dans le milieu d'incubation, un accroissement significatif de la synthèse des produits N-acétylés a lieu pendant la nuit. De plus, la synthèse de la sérotonine est fortement augmentée à 15 h 00, ce qui n'est pas observé dans les expériences de contrôle.

Lorsque du GABA est ajouté 20 min avant le donneur d'acétyle, la synthèse des produits N-acétylés est significativement réduite. Un effet inverse est observé pour les indoles O-acétylés.

Ces résultats confirment la suggestion faite par Ebadi *et al.* (1982) selon laquelle le GABA, comme la norepinephrine, peut participer à la régulation de la synthèse de la mélatonine, donc indirectement à la régulation de la reproduction.

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