Discussion. The question is, how the evacuation of the wings takes place when they contain an excess of haemolymph after expansion (8 times normal in *Bombyx*[17], 5 times in *Attacus*) and how an efficient exchange of haemolymph with that from the abdomen is guaranteed in species with relatively large wings. The generally accepted model of circulation in the wings with afferent and efferent sinuses, joined to a longitudinal body circulation[8,16,18], would imply a short circuit wing supply: the haemolymph once sucked out of the wings by the PO's must be taken to the head by the aorta. It leaves the frontal sac and enters the thoracic cavities, whence it can enter the anterior wing veins again, only a part of it being exchanged. The delay in the start of transport activity of the PO's seems to be a key for the understanding of heartbeat reversal and the mechanism of wing supply. As the head and thorax become drained shortly after backward peristalsis has begun, there must be a haemolymph deficiency when the PO's start their activity. Thus the anterior aorta and the lateral thoracic passages to the PO's[16] do not compete with those from the wings, so that haemolymph can be sucked mainly from the latter; additionally no haemolymph can be available to enter the wing veins during backward periods. The wings must also become drained. One function of the haemolymph oscillations, instead of a pure circulation, would be to accomplish an effective exchange in the wings containing relatively large amounts of haemolymph. The correlation of backward pulsations to the quantity of wing haemolymph becomes obvious in *Caligo*, where the duration of backward periods is increased after wing expansion[15]. The to and fro movements of blood cells observed in the wings, especially the pupal wings of *Ephestia*[19], show that similar conditions may exist in Microlepidoptera.

When the wing haemolymph becomes reduced after wing expansion, the soft cuticular surfaces can give way and approach one another, while the veins in fully developed adults are rather sclerotized. Changes in haemolymph pressure are thus presumed to be compensated by the large tracheae in the wing sinuses and by the airsacs of the thorax and head, which must expand by haemolymph evacuation during the backward periods. The wing tracheae must contract under their own elasticity as soon as haemolymph flows in from the anterior body at the beginning of the forward period. The activity of the PO's on the one hand, and the elasticity of the tracheae on the other, would thus be antagonistic forces for air- and haemolymph ventilation in the wings, supported by coordinated directional changes of heart peristalsis and abdominal movements.

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Immunohistological Investigations of N-Acetylserotonin in the Rat Cerebellum after Parachlorophenylalanine Treatment

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Summary. The amount of N-acetylserotonin (NAS) in the granule layer of the rat cerebellum was investigated using immunohistologic double antibody technique. After 5 days of treatment with parachlorophenylalanine (PCPA) an increase of NAS was observed. The possibility of a differential effect of PCPA on serotonin synthesis in the neurons and the nerve terminals is discussed.

N-acetylindolealkylamines (NAI) are derivatives of serotonin. The two best known NAIs, melatonin and its precursor N-acetylserotonin (NAS) were first identified in the pineal gland[2], however, subsequently were localized in our laboratory in the cerebellum, retina[3], hypothalamus, Harderian gland and the lower brain stem[4] using immunohistological double antibody techniques. The first antibody developed by Grota and Brown[5] did not distinguish between NAS and melatonin. However, crossreactivity to other derivatives of serotonin was only minimal (to serotonin only 0.02%) and to other hormones and neurotransmitters essentially no crossreactivity was found[3]. Recently a new type of antibody was developed which is almost exclusively specific to melatonin (Table I). We have now used this antibody to reexamine tissues in which NAIs were previously seen. The new antibody did not induce any specific staining in the granule layer of cerebellum (Figure 1). On the other hand in tissues where synthesis of melatonin in vitro has been shown[6] and in the Harderian gland where melatonin has been identified by gas chromatography-mass spectrometry (J. Warsh, personal communication) positive staining was seen[4]. On the basis of these findings and the known crossreactivity of the NAI antibody to NAS and melatonin we now conclude that the previously identified NAI in the granule layer of cerebellum[3] is NAS. In order to check the relationship of NAI positive substance to serotonin, we have chosen to treat experimental rats with parachlorophenylalanine (PCPA). PCPA is a blocker of tryptophan-5-hydroxylase (TR-5-OHase), an enzyme essential in the synthesis of serotonin from tryptophan (Table II). The brain of animals treated for 3–4 days with PCPA exhibits only 15–20% of the original amount of serotonin[7],

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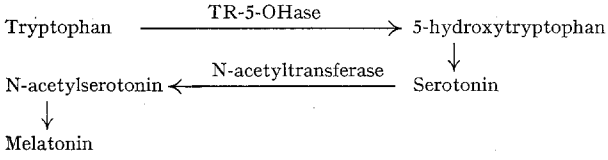
Table I. Crossreactivity of antiserum (calculated on a weight basis as a equivalence at 50% displacement of ^{3}H-melatonin)

Antigen	Melatonin-M-BSA
Melatonin	100.0
N-Acetylserotonin	1.3
Serotonin creatinine sulphate	< 0.1
5-Methoxytryptamine	< 0.1
5-Methoxytryptophol	< 0.1

Table II. Biosynthesis of melatonin from tryptophan



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which is regarded as the only known precursor of NAIs[8]. The results of our study can be characterized as paradoxical to that we have expected.

Materials and methods. 3 adult male rats were treated with 2-daily doses of 160 mg of PCPA/kg injected i.p. Another 3 adult male rats were treated with 320 mg of PCPA/kg injected once a day also i.p. Both groups were sacrified after 5 days of treatment, 12 h after last injection of PCPA. After decapitation the brains were quickly removed and immediately frozen in a cryostat to −20 °C. 10 μm sections of cerebellar cortex were cut and then stained using the double antibody technique described previously[3].

Results. The distribution of NAS positive staining was identical to that observed in untreated animals (Figure 2). However in all sections of all animals treated with PCPA, the amount of diaminobenzidine granules indicating the presence of NAS appeared increased regardless of treatment schedule (Figure 3). There were no differences between animals treated once or twice a day.

Discussion. NAS was described as one of many derivatives of serotonin present in the pineal gland[9] without obtaining recognition as a physiologically significant substance. Our discovery of NAI in the granule layer of cerebellum[3] is supported with the observation of HSU et al.[10,11]. They have investigated 15 regions of CNS on the content of N-acetyltransferase, the enzyme participating in the synthesis of NAS (Table II) and reported that the highest concentration of the enzyme was found in cerebellum. Our findings of NAI positive substance in the cerebellum

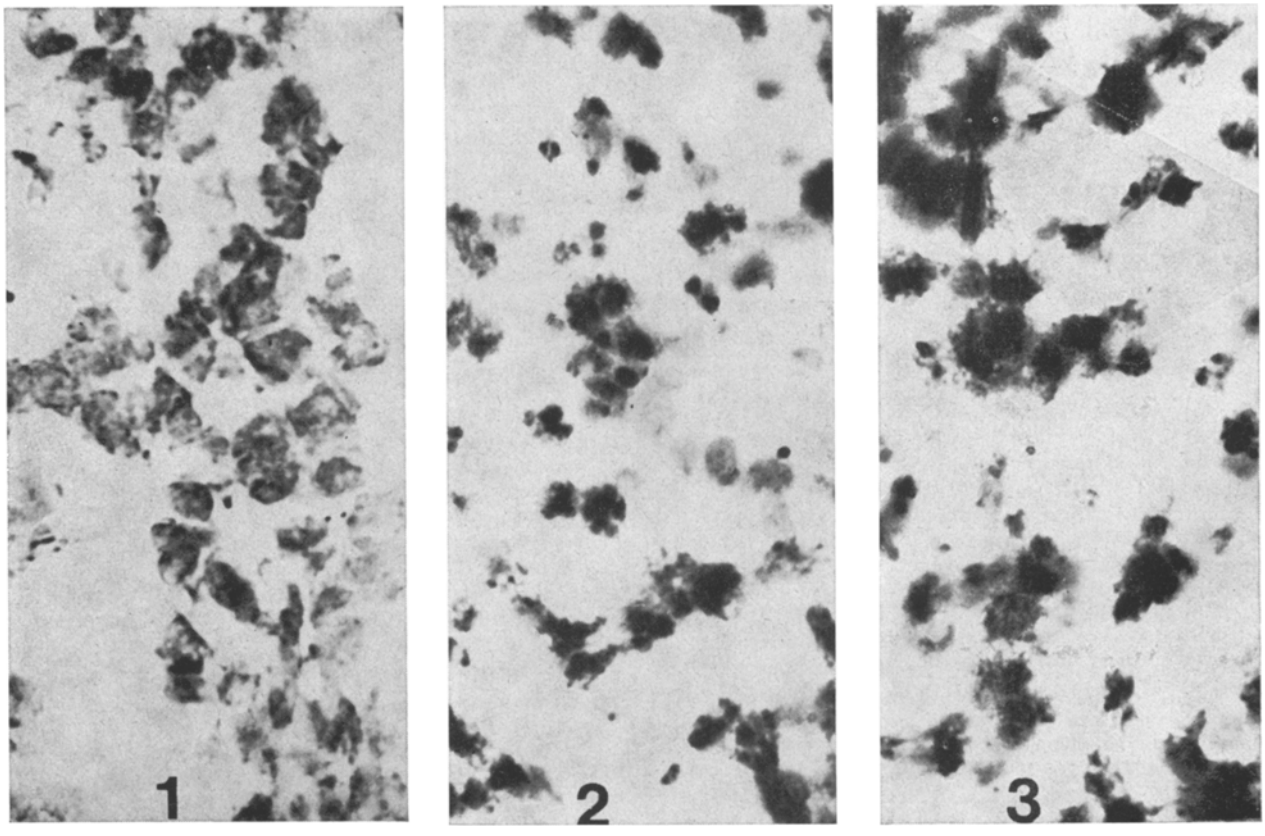

Fig. 1. Granule layer of cerebellum. Peroxidase-labelled antibody technique; using anti-melatonin antibody in the normal rat. No specific staining present. × 1,000.

Fig. 2. Granule layer of cerebellum. Peroxidase technique, using N-acetylindolealkylamine antiserum in the normal rat. Note the dark granules of diaminobenzidine indicating NAI. × 1,000.

Fig. 3. Granule layer of cerebellum. Peroxidase technique, using N-acetylindolealkylamine antiserum in the rat treated with PCPA. Note an increase of diaminobenzidine granules compared to Figure 2. × 1,000.

even 6 weeks after pinealectomy also indicated the independent synthesis of NAS in this tissue[3]. Using PCPA treatment we have expected a decrease of NAI positive substance in the granule layer of cerebellum. The opposite findings seems to contradict the established effect of PCPA on serotonin synthesis. However this paradoxical observation is not unique. In 7 brain areas PCPA was reported to decrease the content of serotonin (range 31–46%). On the other hand in cerebellum the serotonin was increased by 44%[12]. AGHAJANIAN et al.[13] discovered that PCPA was not able to prevent the L-tryptophan induced increase of serotonin fluorescence of the neurons of the raphe system, even though the region of terminals in the forebrain was substantially depleted. In 1974 HARVEY and GAL[14] observed no blockade of TR-5-OHase in the septal region after PCPA. The authors of these two findings offer two different explanations of our unexpected results. AGHAJANIAN et al. hypothesized that synthesis of serotonin by PCPA is blocked in the nerve terminals while in the perikaryon of the neurons the serotonin production continues due to new synthesis of TR-5-OHase. The same explanation may hold for granule cells. Together with a feedback response to depletion of serotonin synthesis in the terminals of the granule cells it could even explain an increase of serotonin synthesis in the granule cells, supplying the substrate for increased NAS synthesis. Another possible explanation is the speculation of HARVEY and GAL[14] that at least 2 TR-5-OHase exist, one which is blocked by PCPA and the other which is not. This explanation does not provide any reason for increase in NAS.

The third possibility is an as yet unknown metabolic pathway for NAS synthesis which does not involve TR-5-OHase. Such a pathway might involve the metabolism of tryptophan to tryptamine, then to N-acetyltryptamine and finally hydroxylation to NAS by an unidentified reaction. However such a pathway seems less probable in view of recent findings of L. HSU et al. (personal communication). They observed an increase of N-acetyltransferase in the rat cerebellum after PCA treatment. Although this report correlated with our findings it does not help to explain the mechanism by which NAS is synthesized in the granule layer of cerebellum after PCPA. We hope that further investigations will contribute to the solution of this problem.

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Is Glutamic Acid the Pyramidal Tract Neurotransmitter?

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Summary. Applied by microiontophoresis, 1-hydroxy-3-amino-pyrrolidone-2 (HA-966) antagonized excitation by glutamic acid but not by acetylcholine of neurones in the rat cuneate nucleus. HA-966 blocked the short latency excitation of cuneate neurones following stimulation of the pyramidal tract on 28 of 40 cells (70%). Thus, glutamate or a related amino-acid may be the neurotransmitter released by pyramidal tract neurones.

The pyramidal tracts have at least two important physiological functions. They are involved in the control of fine movements of distal limb muscles[2], and they modulate the amount of sensory information reaching higher centres such as the cerebral cortex[3]. Yet little attention has been paid to the possible neurotransmitter released by the pyramidal tract (PT) neurones. From neurochemical analyses the transmitter is unlikely to be acetylcholine[4,5] or a monoamine such as noradrenaline[6,7].

Studies have therefore been carried out using the amino-acid antagonist 1-hydroxy-3-amino-pyrrolidone-2 (HA-966) to determine whether an excitatory amino-acid could be involved.

Materials and methods. Adult male rats were anaesthetised with urethane, 1.25 g/kg. i.p. and placed on a heating pad to maintain the rectal temperature at 37–38 °C. The left cerebral cortex and right cuneate nucleus were exposed and then covered with 5% agar in saline to reduce pulsatory movements. Pyramidal tract fibres were excited by single anodal pulses of 0.1 msec duration applied to the motor or sensory areas of the cortex by a silver ball electrode. Evoked spike activity in the cuneate was considered to be monosynaptically induced, and therefore directly due to PT activity a) if the spike had a minimum latency not exceeding 5.0 msec[8]; b) if the minimum spike latency did not vary by more than $\pm$ 0.2 msec with just threshold and 5 $\times$ threshold stimuli, and c) if the spike would follow 25–100 Hz stimulation but not more than 100 Hz. These criteria were intended to eliminate from study any polysynaptically induced spikes.

Drugs were applied to single cells in the cuneate nucleus by microiontophoresis using 5-barrelled micropipettes as described elsewhere[9,10]. One barrel always contained 200 m*M* NaCl solution for current balancing and current testing[9]. The remaining 4 barrels were filled with a selection of: sodium L-glutamate 200 m*M*, pH 8.0; acetylcholine chloride 200 m*M*, pH 5.0; atropine sulphate 100 m*M*, pH 5.0; 1-hydroxy-3-amino-pyrrolidone-2 (HA-966) 100 m*M*, pH 4.5 in 0.2 *N* HCl.

Extracellular unit activity was recorded by a single electrode fixed alongside the multibarrel assembly[10]. Spikes were amplified in a Fenlow AD55 preamplifier,

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