cording to Ledeen[8]. Soybeans *(Glycine max* L. cv Wayne) were grown for 4 days in darkness as described[9]. Intact hypocotyls (ca 500 g) from which the cotyledons were removed were homogenized in chloroform:methanol (2:1 v/v) using a Polytron 20ST homogenizer (Kinematica, Lucerne, Switzerland) and diluted to 2000 ml with the same chloroform:methanol mixture. The resulting homogenate was stirred for 3 h at 10 °C and filtered. The filtrate was extracted with an additional 2000 ml chloroform:methanol (1:1 v/v), stirred overnight, and filtered. The combined extracts were concentrated to a thick paste by rotary evaporation (50 °C), resuspended in 100 ml chloroform:-methanol:water (60:30:8 v/v) (solvent A), and applied (1-2 ml/min) to a DEAE sephadex column[8]; the column was washed with an additional 600 ml of solvent A (3-5 ml/min), and the acidic lipids eluted with 2000 ml of methanol:chloroform:0.8 M sodium acetate (60:30:8 v/v) and evaporated to dryness. The residue was resuspended in 100 ml 0.1 N methanolic sodium hydroxide, with stirring, saponified at 40 °C for 2 h, and lyophylized. This residue was resuspended in chloroform:methanol (4:1 v/v), filtered to remove insoluble material, and applied to a Unisil column[8]. The column was eluted first with 1000 ml chloroform:methanol (4:1 v/v) to remove contaminating lipids and then with 1000 ml chloroform:methanol (1:1). This eluate was concentrated to 10 ml and stored at −20 °C. Sialic acid was analyzed spectrophotometrically by the thiobarbituric acid method of Warren[10]. Additionally, the samples were chromatographed along with known amounts of authentic ganglioside G_{M3}[11] on silica gel G thin layer plates (Analtech, Inc., Newark, Del.) using chloroform:methanol:2.5 N ammonium hydroxide (60:40:9 v/v) as solvent. Lipids were visualized with iodine vapors and sialic acid was visualized using a resorcinol reagent[12].

Results and discussion. Analysis of the presumptive ganglioside fraction using the thiobarbituric acid assay[10] failed to detect sialic acid in each of 3 replicate experiments. Under ideal conditions, the assay will detect sialic acid amounts as low as 5 nmoles. Values for the maximum amounts of ganglioside sialic acid that might be present and still not be detected by this assay method would be on the order of 0.085 nmoles/g fresh wt of hypocotyl tissue. Normal values for rat liver are 2,600 nmoles/g fresh wt. Similarly, analyses of the soybean concentrate by TLC showed no compounds migrating with authentic G_{M3}, the simplest of the monosialogangliosides, and no compounds giving a positive reaction with resorcinol reagent[12] for sialic acid. Using this latter method, we were able to detect as little as 0.04 nmoles G_{M3} sialic acid. Based on this value, the maximum amounts of ganglioside sialic acid in the soybean hypocotyl tissue would be less than 0.02 nmoles/g fresh wt or less than 1/100,000 the amounts present in rat liver. Thus, we conclude that gangliosides, for all practical purposes, are absent from etiolated hypocotyls of the soybean.

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Estimation of the methylating capacity in the pineal gland of the rat with special reference to the methylation of N-acetylserotonin and 5-hydroxytryptophol separately

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Summary. In the present paper, an extension is presented of an earlier described method, by which the methylating capacity of the pineal gland can be determined. Supplementary to the earlier method, the synthesis of melatonin and 5-methoxytryptophol can now be qualified and quantified separately.

Recently Balemans et al.[2] described a method by which the methylating capacity of the pineal gland for several 5-hydroxyindoles and norepinephrine can be determined. However, in this method melatonin and 5-methoxytryptophol could not be determined separately. As melatonin and 5-methoxytryptophol are probably the most important indoles in the pineal-gonadal correlation[3-6], it remained necessary to determine the methylation of both 5-methoxyindoles separately. Therefore an additional method was developed.

The procedure was as follows: 1 rat pineal was slightly disrupted and incubated in 20 µl 0.1 M of phosphate buffer pH 8.0 and 10 µl of (^{3}H)-S-adenosyl methionine (containing 0.7 µCi in H_2SO_4 pH 2.5) for 60 min at 37 °C. The 5-hydroxyindoles present in the pineal were used as a substrate which implies that no substrate was added. After stopping the reaction with 10 µl H_2SO_4 pH 1.0, the pineal tissue was homogenized and synthetic 5-methoxyindoles were added for reference purposes. The pineal tissue together with the incubation medium was then chromatographed by TLC (Merck DC-Silicagel plates 60F 254, 025 mm No. 5729 were used). To minimize decomposition, chromatograms were developed in darkness. A bidimensional TLC-technique was applied. As a solvent system for the 1st direction, chloroform:methanol:acetic acid (93:4:3) was used. The plates were dried under nitrogen and developed in the 2nd direction in chloroform:methanol:ammonia 25% (60:35:5). After chromatography in the 1st direction S-adenosyl methionine, 5-methoxytryptophan, 5-methoxytryptamine and normetanephrine remain on the start. The R_f values of melatonin, 5-methoxytryptophol and 5-methoxyindole-3-acetic acid are respectively 34, 51 and 50. Thus in this solvent system 5-methoxytryptophol could not be separated from 5-methoxyindole-3-acetic acid. After turning the thin layer plate 90° and application of the 2nd solvent system, 5-methoxytryptophol and 5-methoxyindole-

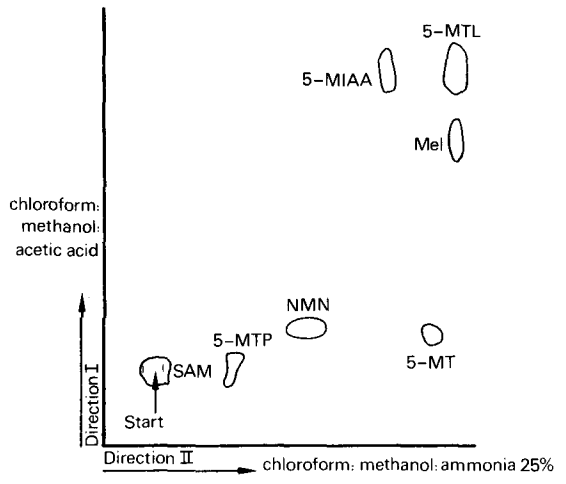

Fig. 1. Bidimensional chromatography of rat pineal tissue. The 5-methoxy-components are indicated. 5-MIAA, 5-methoxyindole-3-acetic acid; 5-MTL, 5-methoxytryptophol; Mel, melatonin; 5-MT, 5-methoxytryptamine; 5-MTP, 5-methoxytryptophan; NMN, normetanephrine; SAM, S-adenosyl methionine.

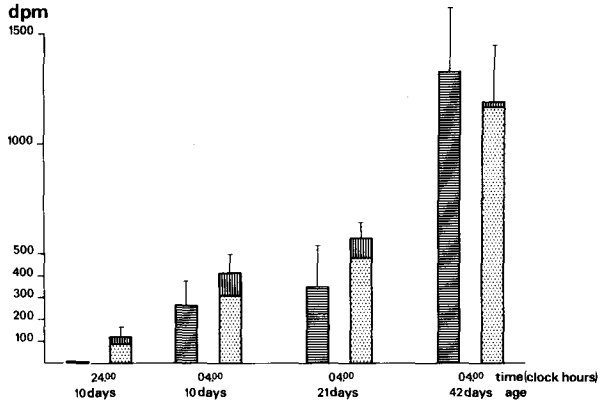

Fig. 2. A comparison between the result of an earlier described method in which the synthesized tritiated melatonin/5-methoxytryptophol was determined together, and the present method in which melatonin and 5-methoxytryptophol are determined separately. The HIOMT activity was determined at 24.00 and 04.00 h for the 10-day-old rats and at 04.00 h in the pineals of the 21- and 42-day-old rats in the month of October. Each vertical bar represents the mean HIOMT activity of 5 pineals±SEM. ▤, Melatonin/5-methoxytryptophol determined together with an earlier described method[2]. ▨, Melatonin determined with the present method. ▥, 5-Methoxytryptophol determined with the present method.

3-acetic acid are separated (figure 1), thus resulting in the separation of melatonin and 5-methoxytryptophol.

The spots were then scraped and counted in 75 µl of ethanol and 10 ml of a scintillation liquid (toluene 1000 ml; POPOP 0.1 g; PPO 5 g; Cabosil 40 g) with a liquid scintillation counter (Mark I of Nuclear Chicago). The whole procedure was carried out simultaneously without pineal tissue in order to identify the tritiated decomposition products of S-adenosyl-methionine. The small activity of the tritiated decomposition products (35–85 dpm) localized in the spots of melatonin and 5-methoxytryptophol are subtracted from the tritiated activity of the melatonin and 5-methoxytryptophol spots of the examined pineal tissue.

A comparison between the activity of the melatonin/5-methoxytryptophol spot of the earlier described method[2] and the sum of the activities of the separate

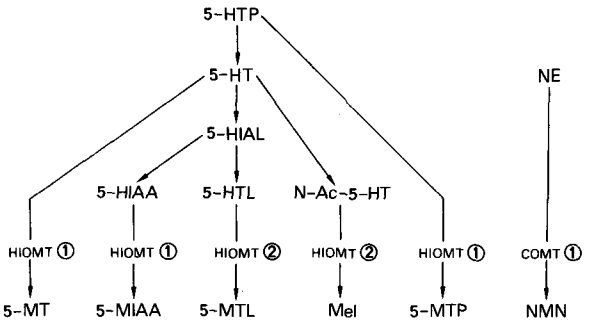

Fig. 3. Scheme of the 5-hydroxyindole metabolism in the pineal gland of a rat. 1. The HIOMT and COMT activity measured with the earlier described method[2]. 2. The HIOMT activity measured with the present method. 5-HTP, 5-hydroxytryptophan; 5-HT, 5-hydroxytryptamine; 5-HIAL, 5-hydroxyindole acetaldehyde; 5-HTL, 5-hydroxytryptophol; 5-HIAA, 5-hydroxyindole-3-acetic acid; N-Ac-5-HT, N-acetyl 5-hydroxytryptamine; 5-MTP, 5-methoxytryptophan; 5-MT, 5-methoxytryptamine; 5-MTL, 5-methoxytryptophol; MEL, melatonin; 5-MIAA, 5-methoxyindole-3-acetic acid; NE, norepinephrine; NMN, normetanephrine.

melatonin and 5-methoxytryptophol spots of the present method is demonstrated in figure 2. For this comparison, the HIOMT activity was determined with both methods in the pineals of 10-, 21- and 42-day-old Wistar rats, at 24.00 and 04.00 h for the 10-day-old rats and at 04.00 h for the 21- and 42-day-old rats. From the results represented in figure 2, it may be concluded that no significant difference in activity for melatonin/5-methoxytryptophol together[2] and the sum of the separate melatonin and 5-methoxytryptophol activity (present method) exists. Only for minimal values (about 100 dpm) significantly better results (24.00 h in the 10-day-old rats) were obtained using the present method.

The activities of the 5-methoxytryptophan, the 5-methoxytryptamine and 5-methoxyindole-3-acetic acid spots, however, do not give reproducible results. It is not known if this is possibly due to the influence of the solvent systems.

It may be concluded that both methods are supplementary in order to get detailed information about the total HIOMT activity in the pineal gland. The earlier described method should be applied for the methylation of 5-hydroxytryptophan, 5-hydroxyindole-3-acetic acid, 5-hydroxytryptamine, norepinephrine and N-acetylserotonin/5-hydroxytryptophol (together). The present method should be used to determine the methylation of N-acetyl-serotonin and 5-hydroxytryptophol separately.

Since no substrate was added to the incubation medium, each methylated product points to the presence in the pineal organ of a corresponding hydroxy-component as well as the presence of a methyltransferase capable of methylating this component. Thus with the combination of both methods, the hydroxyindole metabolism (figure 3) can be analyzed.

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