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Separation of Pineal Extracts on Sephadex G-10[1]

III. Isolation and Comparison of Extracted and Synthetic Melatonin

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Lerner *et al.* (1–3) isolated from bovine pineal glands an indole derivative which is a potent agent in lightening (blanching) frog skin. This compound is called melatonin. Heinzelman *et al.* (4) have studied the lightening activity of a number of synthetic tryptamine derivatives. It has been shown that extremely high lightening activity is limited to compounds which have a methoxyl group in the 5-position of the indole nucleus and an acetyl or large acyl substituent on the side chain amino group. Quay and Bagnara (5) studied the specificity of the body lightening of the larvae of *Xenopus laevis*. These authors have tested 46 synthetic compounds, indoles, and related substances. Only five of the compounds caused significant body lightening. These results are in good agreement with those of Heinzelman and Szmuszkovicz (4). The body lightening of *Xenopus* tadpoles proved to be ten times as sensitive as the most sensitive spectrofluorimetric procedure.

Although 100 kg of bovine pineal glands was used by Lerner *et al.* (3), it was at no time feasible to have more than 100 μg of melatonin available for analysis. The evidence for the structure of bovine melatonin (*N*-acetyl-5-methoxytryptamine) proposed by Lerner *et al.* (3) is quite convincing, but due to its availability in microgram quantities only it has not been possible to purify natural melatonin to the point at which it gives an infrared spectrum identical with that of synthetic melatonin. Szmuszkovicz *et al.* (6) have synthesized *N*-acetyl-5-methoxytryptamine and report that the synthetic compound shows an identical activity to that of natural melatonin when tested on the frog skin. Some years later McIsaac *et al.* (7), starting from 50 kg of bovine pineal tissue, isolated

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a substance which on reaction with picric acid in chloroform gave a crystalline material with the same melting point as *N*-acetyl-5-methoxytryptamine picrate and matching infrared spectrum.

Recently a rather simple and mild method was published for separating aqueous cockerel and sheep pineal as well as cerebral cortex extracts on Sephadex G-10 columns into several distinct fractions that show excitation and fluorescence maxima resembling those of indoles. Cockerel and sheep pineal extracts produce three peaks which cannot be observed in cerebral cortex (8,9). Thin-layer chromatography of Sephadex G-10 fractions in five different solvent systems revealed in one of these fractions the presence of a fluorescent, Ehrlich-positive spot, identical with that of synthetic melatonin. The fractions of this peak induced the blanching reaction in tadpoles of *Xenopus laevis*.

The present study deals with the isolation of melatonin from 300 gm of sheep pineal bodies by filtration of the aqueous extract on Sephadex G-10. The resulting fractions have been tested in the *Xenopus laevis* bioassay. The structure of melatonin, as proposed by Lerner *et al.* (3), does not explain clearly the extraction of melatonin from pineals by water. The differences in infrared spectra, as already mentioned by Lerner *et al.* (3), and the differences in fluorescence spectra, between isolated and synthetic melatonin (8) brought us to this study. In order to establish whether the isolated substance was identical with synthetic melatonin the infrared and mass spectra of the two substances were compared. Proton magnetic resonance spectra of synthetic melatonin before and after thin-layer chromatography were recorded.

MATERIALS AND METHODS

Synthetic melatonin was obtained from EGA-Chemie KG., Keppler und Reif, 7924 Steinheim bei Heidenheim (Brenz), W.-Germany.

All solvents used in these experiments were pro analysi from E. Merck A. G., Darmstadt, Germany.

Pineals. Sheep pineals are collected by ERSCO, San Mateo, California. These organs are frozen 1 to 3 hr after slaughter, shipped in dry ice, and preserved at −20°C.

Bioassay. The use of the blanching reaction of tadpoles of *Xenopus laevis* as a bioassay has been described previously by van de Veerdonk (10,11). Tadpoles of stages 47–49 according to Nieuwkoop and Faber (12) are used. The larvae were adapted to an illuminated black background in copper-free tap water during one night. The next morning they were divided into groups of two animals which were left swimming in 3 ml of tap water for 1 hr on an illuminated black background. Subsequently 3 ml of a Sephadex G-10 fraction or 3 ml of distilled water was

added. After 20 min the larvae were fixed by the addition of 0.5 ml of 40% formaldehyde, examined microscopically, and photographed.

Column Chromatography. Sephadex G-10, lot 3411 (Pharmacia, Uppsala, Sweden), is pretreated as described before (8,9). Gel filtration is performed on a column of 60 × 4.5 cm with distilled water as the eluant (20 ml/15 min/fraction). 300 gm of sheep pineals is extracted in batches of 100 gm. Each batch is homogenized with 100 ml of distilled water. The extraction is carried out as described by Ebels *et al.* (9). The residue is reextracted twice with 75 ml of distilled water. The combined supernatants of one batch (290 ml) are applied to the Sephadex G-10 column. All experiments are carried out at 3°C in darkness. The localization of the excitation and fluorescence maxima of the fractions has been described in detail (8,9). A Zeiss spectrofluorometer ZFM 4c is used. The amplification factor of the instrument is kept constant and slits of 2 mm are applied. 3 ml from each third fraction (up to number 225) and subsequently from each fifth fraction are taken for the blanching reaction in *Xenopus* tadpoles. In addition 3 ml from the top of each fluorescent peak is tested. The biological activity is expressed as the Melanophore Index (MI) of Hogben and Gordon (13). The fractions of a fluorescent peak are combined and lyophilized.

Thin-Layer Chromatography. Thin-layer chromatography is carried out on plates of silica gel (DC-Fertigplatten, Kieselgel F 254 (20 × 5 cm) from E. Merck A. G. Darmstadt, Germany) with a layer thickness of 0.25 mm. The lyophilized fractions which induce the blanching reaction in *Xenopus laevis* larvae are redissolved into a few microliters of chloroform/methanol (90:10 v/v), chromatographed on a thin-layer plate in the same solvent for 2 hr in darkness at room temperature. After drying the plates are examined under a Camag UV-lamp at 254 nm and at 356 nm wavelength. The spot corresponding to the R_f value of synthetic melatonin is removed from the plate and the silica gel eluted with acetone. The solvent is evaporated and the residue rechromatographed in the same way. The solution obtained after the second elution of the melatonin spot with acetone is used for infrared spectrometry.

Infrared Spectrometry. Infrared spectra were recorded with a Perkin-Elmer 225 infrared spectrophotometer. The sample obtained by thin-layer chromatography was brought into KBr and a micropellet (diameter 1.5 mm) was prepared. Infrared spectra of both isolated and synthetic melatonin were recorded. Infrared spectra of chloroform solutions of synthetic melatonin before and after thin-layer chromatography were measured in a micro Barnes cavity cell, path length 0.1 mm, with a beam condenser.

Mass Spectrometry. Mass spectra were recorded on an AEI-MS 902

mass spectrometer. The micropellets of isolated and synthetic melatonin were ground and extracted with acetone. The resulting solutions were transferred to the direct insertion probe of the mass spectrometer. After evaporation of the solvent the samples were introduced into the ion source. Spectra of samples of synthetic melatonin dissolved in acetone were recorded in the same way.

Proton Magnetic Resonance Spectrometry. Proton magnetic resonance (H-NMR) spectra of synthetic melatonin were recorded on a JEOL C-60 HL spectrometer with deuterochloroform as solvent. Tetramethylsilane (TMS) was used as internal standard.

RESULTS AND DISCUSSIONS

(I) *Separation of Aqueous Sheep Pineal Extract on Sephadex G-10*

Filtration of an aqueous sheep pineal extract on Sephadex G-10 gave rise to several fractions (Fig. 1). The bioassay (blanching reaction of tadpoles of *Xenopus laevis*) of the fractions is also presented in Fig. 1. The fractions of the last peak, eluted from the column between 7.894

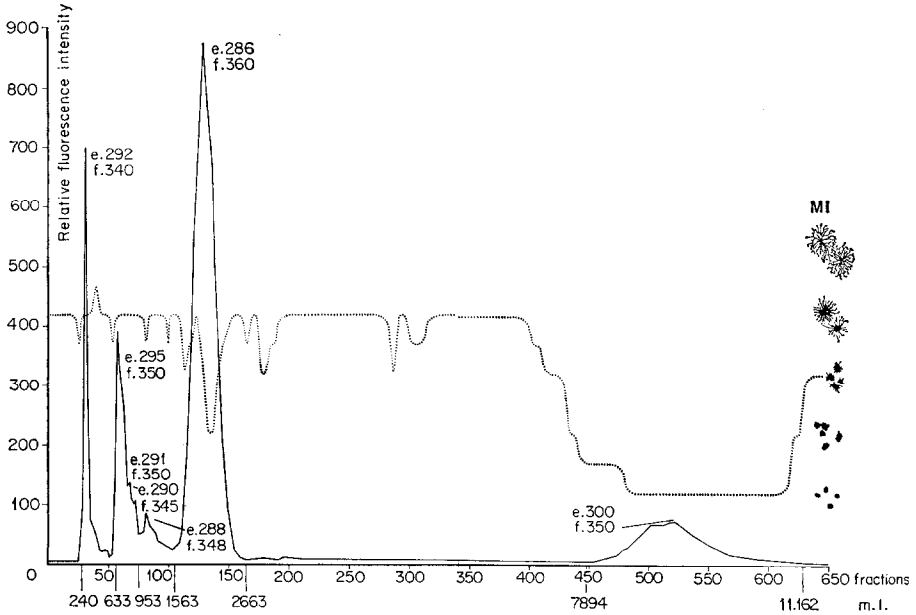


FIG. 1. Separation of sheep pineal extract on Sephadex G-10 column (60 × 4.5 cm) pretreated with pyridine acetate buffer, washed with 0.01 *M* NaOH, and subsequently equilibrated and eluted with distilled water: (···) Melanophore Index (MI)(13); (—) relative fluorescence intensity; e, excitation; f, fluorescence; ml, milliliter.

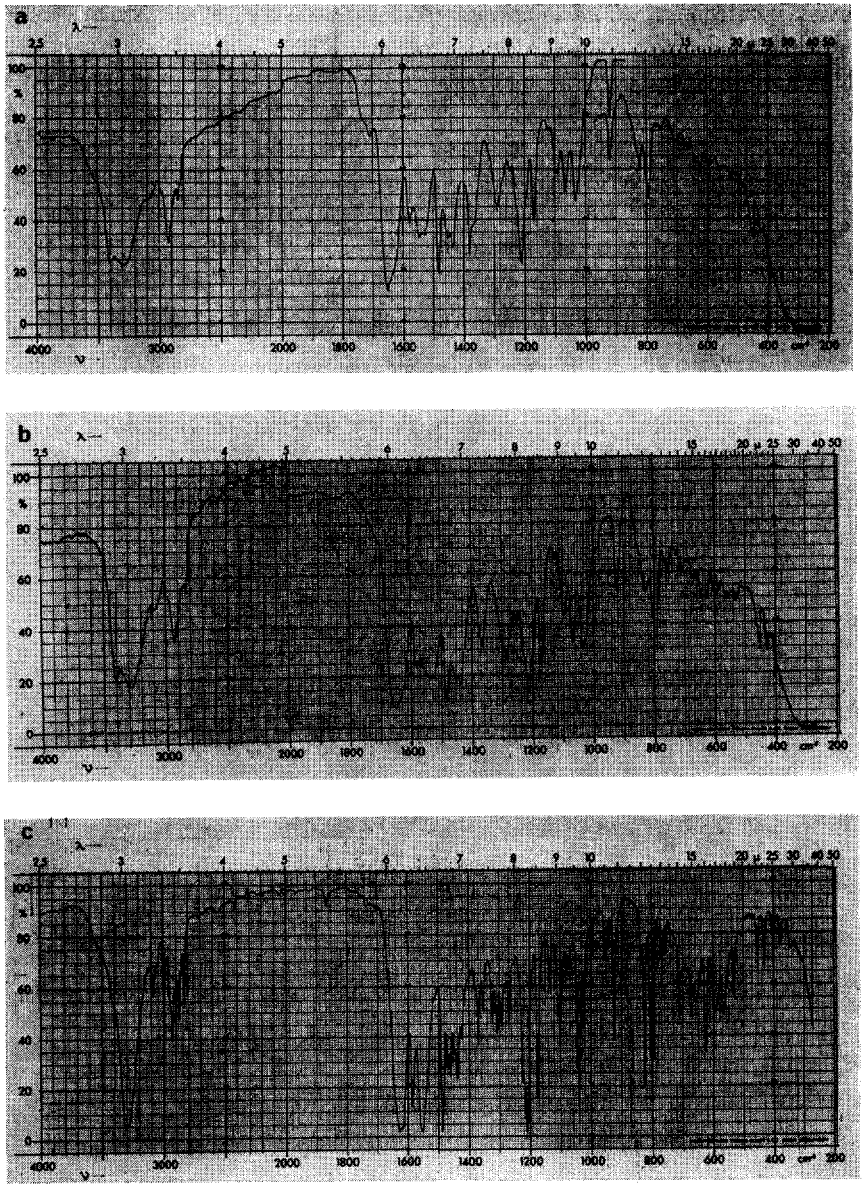


FIG. 2. Infrared spectra: (a) isolated melatonin (KBr pellet); (b) synthetic melatonin, treated in same way (KBr pellet); (c) synthetic melatonin without treatment (KBr pellet); (d and e) synthetic melatonin before (d) and after (e) thin-layer chromatography (in chloroform); (f) isolated melatonin (KBr pellet) after heating the KBr pellet 5 min at 110°C.

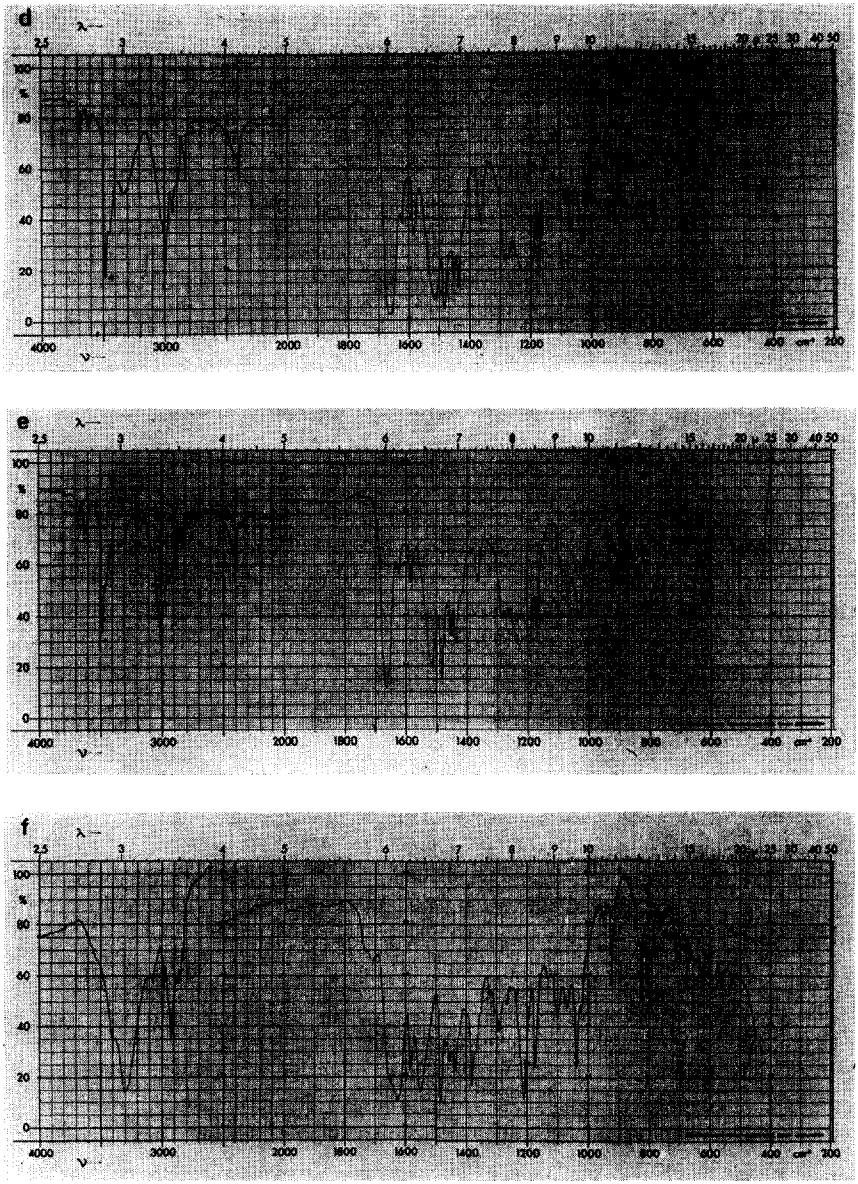


FIG. 2 (*Continued*).

ml and 11.162 ml, were used for further experiments as they developed a strong melanophore aggregating activity. The relative fluorescence intensities of isolated and synthetic melatonin were compared. At a rough estimate approximately 20 μg melatonin was obtained from 100 grams of sheep pineals.

(II) *Thin-Layer Chromatography*

After thin-layer chromatography of the lyophilized fractions of the last peak, three spots were generally observed in UV light. Besides the melatonin spot one spot is located just above the spot with the R_f value of synthetic melatonin and another could be detected at the front line. Rechromatography of the melatonin spot gave a spot with the R_f value of melatonin, and only one other spot—that at the front line.

(III) *Infrared Spectrometry*

The infrared spectrum of isolated melatonin (Fig. 2a) was compared with that of synthetic melatonin (Fig. 2b) treated in the same way as the isolated sample. These spectra are identical. They differ, however, from the spectrum of synthetic melatonin, which has not undergone any previous treatment (Fig. 2c). Spectrum 2c is in good agreement with the Sadtler spectrum of synthetic melatonin (14). Spectra 2a and 2c resemble each other to a high degree, but there are also some striking differences, particularly in the ranges 3500–3200 cm^{-1} and 1700–1500 cm^{-1}. The spectrum of synthetic melatonin shows peaks at 3300 (sharp), 3285 (shoulder), 1628 (amide I, sharp), 1618 (shoulder), and 1351 cm^{-1} (amide II, sharp). In the spectrum of the isolated sample, absorbances are located at 3400, 3300 + 3285 (broad), 1650 (amide I, broad), 1620 (shoulder), and 1535 + 1520 cm^{-1} (amide II, broad). These differences will be discussed later.

(IV) *Mass Spectrometry of Isolated and Synthetic Melatonin*

The mass spectra of the solutions obtained upon grinding the KBr pellets from which the IR spectra of Fig. 2a–c were recorded were identical. The mass spectrum of isolated melatonin from sheep pineals is shown in Fig. 3. This spectrum is identical with that obtained from synthetic melatonin dissolved in acetone and brought into the direct insertion probe of the mass spectrometer. All spectra are in accordance with the spectrum of synthetic melatonin published by Jamieson and Hutzinger (15). An analysis of the mass spectra, together with the fragmentation schemes of melatonin and two other indolic compounds isolated from sheep pineal bodies will be published elsewhere.

Note: In an attempt to explain the differences between the infrared

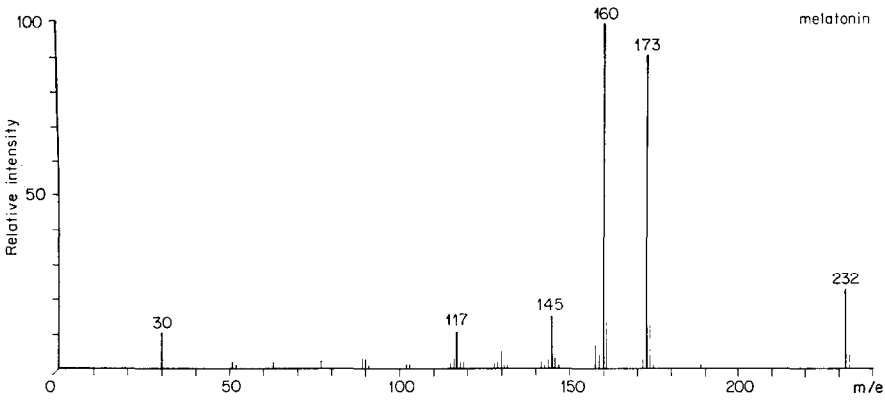
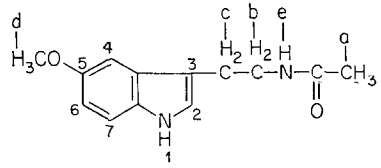


FIG. 3. Mass spectrum of melatonin isolated from sheep pineals (MW 232).

spectra of melatonin, before and after the isolation procedure, the following experiments were performed.

1. *Proton magnetic resonance spectra of synthetic melatonin before and after thin-layer chromatography.* The H-NMR spectrum of synthetic melatonin is given in Fig. 4a. The chemical shifts and coupling

TABLE 1
Chemical Shifts and Coupling Constants of Melatonin[a]



Proton	Chem. shifts (δ)	Multiplicity[b]	Relative intensity	Coupling constants (Hz)
a	1.86	broad	3H	
b	2.87	triplet	2H	$J_{b,e} = J_{b,c} = 6.0$
c	3.54	quartet	2H	
d	3.79	singulet	3H	
e	5.82	broad	1H	—
6	6.83	d.d.		
4	6.95	d.d.	3H	$J_{6,7} = 9.0$
2	7.03	d.		$J_{6,4} = 2.7$
7	7.24	d.d.	1H	$J_{4,7} = 0.6$
1	8.60	broad	1H	$J_{1,2} = 2.25$

[a] 1, 2, 4, 6, and 7 indicate the protons of the atoms of the indole nucleus; the other protons are indicated by a, b, c, d, and e.

[b] d. = doublet, d.d. = doublet of doublet.

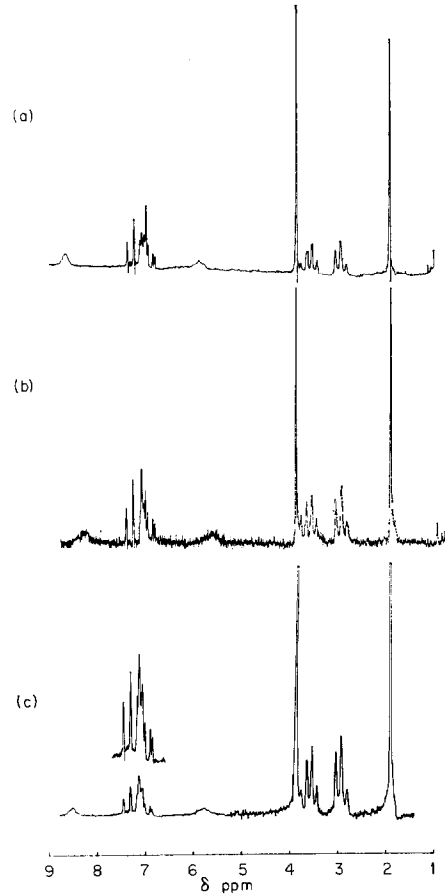


FIG. 4. Proton magnetic resonance spectra in chloroform of synthetic melatonin before (a) and after (b) thin-layer chromatography. (c) Spectrum of synthetic melatonin with added H_2O.

constants are reported in Table 1. The H-NMR spectrum of the same sample after thin-layer chromatography (Fig. 4b) differed only in the region between 6.8 and 7.1 ppm. From Fig. 4b it is concluded that proton H_2 did not couple with the ring >NH proton. This may be caused by a small amount of H_2O in the chromatographed sample. We found evidence for this supposition by adding some water to the original sample. The spectrum obtained (Fig. 4c) was identical with the spectrum of the chromatographed sample.

2. *Infrared spectra of synthetic melatonin in solution.* The infrared spectra of synthetic melatonin in chloroform, before and after thin-layer chromatography, proved to be identical (Fig. 2d–e).

3. *Influence of temperature on infrared spectra of KBr pellets of melatonin.* To investigate whether the presence of water causes the differences between the IR spectra of Fig. 2a (or 2b) and 2c we studied the effect of heating the KBr pellets from which the spectra were obtained. When a KBr pellet of synthetic melatonin was heated for 10 min at 120°C, the resulting infrared spectrum altered from that shown in Fig. 2b into one nearly identical with that of Fig. 2c. When the KBr pellet of the isolated sample of melatonin was heated for 5 min at 110°, its spectrum changed from that in Fig. 2a into spectrum 2f, which is also nearly identical with spectrum 2c.

From the experiments described above it was concluded, that the differences in infrared spectra are not caused by oxidation of melatonin, but may be due to a small amount of H_2O in the chromatographed sample.

GENERAL DISCUSSION

It is possible to isolate melatonin from sheep pineal bodies by rather simple and mild extraction followed by gel filtration of the aqueous extract on a Sephadex G-10 column which is eluted with distilled water. Although it seems that the melatonin isolated after thin-layer chromatography cannot be distinguished from synthetic melatonin as regards its elution volume (from the Sephadex G-10 column), as regards its excitation maximum, its blanching reaction with tadpoles of *Xenopus laevis*, its R_f value in thin-layer chromatography, and its mass spectrum. Minor differences in the infrared spectra of isolated and synthetic melatonin could be attributed to the presence of water in the isolated sample. The present data may give evidence that melatonin occurs in the cell in a hydrated form.

SUMMARY

Melatonin has been isolated from 300 gm of sheep pineal bodies by filtration of an aqueous extract on Sephadex G-10. Excitation and fluorescence maxima of the fractions of the column were measured and the fractions were tested for their blanching activity in tadpoles of *Xenopus laevis*. The microgram quantities of melatonin obtained after thin-layer chromatography were used for identification. The infrared spectrum of isolated melatonin in a KBr pellet is identical with that of synthetic melatonin treated in the same way as the isolated sample. It differs from the spectrum of synthetic melatonin, which has undergone no previous treatment. Heating of the KBr pellet of the isolated sample of melatonin for 5 min at 110°C, however, causes the spectrum to change into one which is nearly identical with that of untreated synthetic melatonin.

The mass spectra of isolated and synthetic melatonin are identical. Proton magnetic resonance spectra of synthetic melatonin before and after thin-layer chromatography are presented.

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