Biotransformation of \([\text{H}]\text{serotonin}\) by cultured hamster skin to \([\text{H}]\text{-metabolites}\) corresponding to \(\text{N-acetylserotonin} (\text{NAS}), \text{melatonin, and 5-methoxytryptamine (5-MT)}\) was demonstrated. This process was time-dependent, with the highest production of radioactive NAS and melatonin metabolites after 3 and 5 h of incubation followed by a decrease in the rate of metabolite release into the media. Conversely, the formation of radioactive metabolite corresponding to 5-MT increased gradually during skin culture, reaching the highest level after 24 h of incubation. The production of \([\text{H}]\text{-metabolites, corresponding to NAS, melatonin, and 5-MT,}\) was stimulated by forskolin with a maximum effect of forskolin at 10 \(\mu M\) concentration. The gas chromatographic/mass spectroscopy analysis of the fraction eluting at the retention time of NAS standard material showed that it contained NAS, further confirming production and release of NAS into the media by hamster skin. Therefore, we conclude that mammalian skin can acetylate serotonin to NAS and postulate that the NAS is further metabolized by the skin to form melatonin which is subsequently transformed to 5-MT.

Melatonin serves as the main signal molecule which links the photoperiod to metabolic, endocrine, and immunological changes and which is mainly synthesized in the pineal gland, retina, brain, and Harderian gland (1). Depending on the site of production and target organ it can act as a hormone, neurotransmitter, cytokine, and biological modifier (2).

Melatonin is a product of a two-step conversion of serotonin, which involves the acetylation of serotonin (3) and subsequent methylation by hydroxyindole-O-methyltransferase (4). The majority of melatonin released into the bloodstream is metabolized in the liver and kidneys (5, 6), mainly by 6-hydroxylation and conjugation to glucuronate or sulfate (5, 6), and to a minor degree by deacetylation to 5-methoxytryptamine (5-MT),\(^1\) which is further deaminated (5-7). In contrast, melatonin bioconversion at the organ site of synthesis appears to be different from the metabolism of circulating melatonin (8-10). For example, in the retina melatonin is first deacetylated to 5-MT, which can then be deaminated, producing 5-methoxyindoleacetic acid and 5-methoxytryptophol (8, 9). Melatonin deacetylation to 5-MT was also detected in retinal pigment epithelium and non-mammalian skin that are target sites for melatonin bioregulation (8-10). Extracranial sites of both synthesis and metabolism of melatonin have also been demonstrated in the peripheral blood mononuclear leukocytes (11), and according to some authors in the gastrointestinal tract (12).

Melatonin production has not previously been demonstrated in skin, which is the largest body organ that can react to external and internal stimuli via the skin immune system (13), the pigmented system (14), and the skin endocrine system (15, 16). In lower vertebrates skin is a recognized target for melatonin action, e.g. melatonin has lightening activity on the skin (17). In mammals, it has been reported that melatonin can regulate hair growth (18), inhibit follicular melanogenesis (19, 20), and affect proliferation of epidermal keratinocytes (20) and malignant melanocytes (21). Specific binding sites for melatonin were also detected in the mammalian skin (20). In addition, we have identified two isozymic forms of aryamine N-acetyltransferase, NAT-1 and NAT-2, in hamster skin of which NAT-2 catalyzed the acetylation of dopamine to N-acetyldopamine and serotonin to NAS, a direct precursor of melatonin (22). This information has formed the basis for the present studies on the synthesis and degradation of melatonin by mammalian skin.

MATERIALS AND METHODS

Animals and Skin Culture—Three-month-old male Syrian golden hamsters (Mesocricetus auratus) were purchased from Charles River Laboratory, Wilmington, MA, housed in community cages with 12-h light periods, and fed ad libitum with water and rat/mouse chow. The animals were sacrificed, hair shafts were removed with an electrical animal clipper, the skin was dissected at the level of subcutis, and punch biopsies of skin were used for short-term skin culture as described previously (20). The incubations were started between 6 and 7 p.m. Three to five punch biopsies of skin per group were incubated together at the air-liquid interface in 300 \(\mu l\) of medium (Dulbecco’s modified Eagle’s medium + 10% fetal bovine serum and 1% antibiotic/antimycotic mixture, all from Life Technologies, Inc.) in 24-well plates at 37 °C, 5% CO\(_2\) in air and 100% humidity for 3.5, 6, 12, 14, and 24 h. Four-mm punch biopsies were used throughout the study in order to standardize the tissue volume and thereby the cell mass in each fragment. In control experiments we used primary cell culture of hamster melanotic melanoma cells (23).

\[^{[\text{H}]}\text{Hthyminde Incorporation—To measure DNA synthesis (20) skin cultures were pulsed with 1 \(\muCi/ml\) \([\text{H}]\text{thyminde and, after defined periods of incubation, washed with phosphate-buffered saline and incubated 3 times for 30 min with continuous shaking in 2 m NaBr to remove nonincorporated thyminde (20). The amount of } [\text{H}]\text{thyminde incorporated into each fragment was measured separately by liquid scintillation spectrometry (20) and expressed as the mean cpm (± S.E.) per skin biopsy.}

Melatonin Synthesis—To study melatonin synthesis, 1, 5, or 10 \(\muCi\) of \([\text{H}]\text{serotonin/ml (DuPont) was added at 7 p.m. to the media and skin cultures were incubated in the presence or absence of forskolin at the concentrations listed. The media and skin were collected and processed separately. The media was extracted with chloroform (Sigma). Chloroform and aqueous phases were further separated by centrifugation in

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\(^2\) The abbreviations used are: 5-MT, 5-methoxytryptamine; NAS, N-acetylserotonin; NAT, N-acetyltransferase; RP-HPLC, reverse-phase-high performance liquid chromatography; GC/MS, gas chromatographic/mass spectroscopy; EI, electron impact; CI, chemical ionization.
Microfuge at 16,000 \times g. Both fractions were collected separately, centrifuged again to remove any impurities, and subjected to analysis by reverse-phase high-performance liquid chromatography (RP-HPLC) analyses. Skin fragments from each culture were homogenized together in 0.1 M sodium phosphate buffer, pH 6.8, plus 1% Triton X-100, followed by centrifugation at 10,000 \times g for 15 min at 4°C. Supernatants were then extracted in chloroform and processed as described above for media samples.

RP-HPLC Analyses—Approximately a 100-\mu l sample of the tested fraction was injected onto the RP-HPLC column (Versapack C18 10U, Altitech, Deerfield, IL), which was equilibrated using 0.1 n acetic acid (pH 4.0, flow rate = 1 ml/min). The elution was 0–10 min with acetic acid 10–50 min with increased methanol from 0 to 100% and 50–60 min with a decreased methanol from 100 to 0% and was monitored at 280 nm using a UV detector. Starting at 20 min, elution fractions of 0.3-ml volume were collected. Each fraction was analyzed by liquid scintillation spectroscopy. During each separation via RP-HPLC of radiolabeled samples nonradioactive standards of serotonin, N-acetylserotonin (NAS), melatonin, and 5-MT (all from Sigma) were included and their retention was determined with ultraviolet detection at 280 nm.

Table 1. Time-dependent DNA synthesis in hamster skin cultured in vitro.

| Incubation Time (hours) | DNA SYNTHESIS (cpm/mll) |
|-------------------------|-------------------------|
|                         |                        |
| 0                       | 0                      |
| 2                       | 500                    |
| 4                       | 1000                   |
| 6                       | 1500                   |
| 8                       | 2000                   |
| 10                      | 2500                   |
| 12                      | 3000                   |
| 14                      | 3500                   |
| 16                      | 4000                   |
| 18                      | 4500                   |
| 20                      | 5000                   |

**RESULTS**

RP-HPLC Identification of Tritiated NAS, Melatonin, and 5-MT—To study the possible transformation of serotonin to melatonin by mammalian skin and further metabolism to 5-MT we used short-term skin culture system (20). Under the conditions tested, the hamster skin remained viable and metabolically active for at least 24 h of incubation as evidenced by progressive increase in DNA synthesis throughout the incubation period (Fig. 1).

After metabolic labeling with [3H]serotonin, the media and skin biopsies were prepared separately using chloroform extraction. Both chloroform and aqueous fractions were analyzed by RP-HPLC in the presence of nonradioactive serotonin, NAT, 5-MT, and melatonin standards. Fig. 2 shows representative elution times of triitated serotonin metabolites released into the media after 5 h incubation in the presence of 5 \mu Ci of [3H]serotonin. The radioactive peaks with retention times corresponding to those of unlabeled standards of NAS (28 min), 5-MT (32 min), and melatonin (36 min) were identified in chloroform extracts (Fig. 2A). HPLC analysis of the aqueous fraction from the same experiment showed the presence of a major radioactive peak corresponding to serotonin, the presence of a radioactive peak corresponding to NAS and the absence of radioactive peaks at the elution time of 5-MT and melatonin (Fig. 2B). The specificity of these findings were fur-
ther confirmed by the absence of radioactive peaks of NAS, 5-MT, and melatonin in control media from primary cell culture of hamster amelanotic melanoma cells radiolabeled with 5 μCi of [3H]serotonin (not shown).

The nature of the other radioactive peaks, including peaks eluting between NAS and 5-MT (30–31 min) and the major hydrophobic peak eluting at 40–42 min has not been determined. The GC/MS characteristic of the peak eluting at 26 min between serotonin and NAS is provided below. We have also attempted to characterize the 40–42 min hydrophobic peak using media from the culture performed in the presence of 100 μM unlabeled serotonin. Preliminary IR-mass spectroscopy suggests a nonaromatic compound (data not shown). This peak was also present in control medium (not shown) despite an absence of detectable transformation of serotonin into melatonin. We suggest that this peak may be unrelated to synthesis and degradation of melatonin and, therefore, we have narrowed our HPLC analyses to elution times of the serotonin, NAS, 5-MT, and melatonin standards.

The spectrum of the RP-HPLC separation of skin extracts was similar to that obtained from culture media. The NAS, 5-MT, and melatonin peaks were present in the chloroform fraction, while the majority of the radioactivity corresponding to serotonin and NAS remained in the aqueous fraction (Fig. 3).

**Comparison of Figs. 2 and 3 shows that the serotonin metabolites accumulate predominantly in the culture media.**

**GC/MS identification of NAS—**GC/MS analysis of fractions obtained from the HPLC analysis of nonradioactive standard confirmed the identity and retention of serotonin, NAS, melatonin, and 5-MT (data not shown). To analyze culture media an incubation time of 12–14 h was chosen, when appreciable production of [3H]NAS was evident (Fig. 4). For GC/MS analyses, skin biopsies were incubated in the presence of 10 μCi of [3H]serotonin and 10 μM forskolin. The media were extracted and chloroform (A) and aqueous (B) phases were separated by RP-HPLC. The GC/MS results indicated that greater than 80% of NAS still remained in the aqueous phase and that less than 10% of the NAS is transformed into melatonin or 5-MT (Fig. 4). We, therefore, focused our GC/MS analysis on the aqueous fractions with retention times corresponding to serotonin and NAS.

For GC/MS identification of NAS in skin culture media, an analysis of ion fragments resulting from E1 analysis of purified NAS was initially performed. Fig. 5A shows a total ion count chromatograph of a standard preparation of NAS which was derivatized and chromatographed. The mass spectra insert shows ion fragments with m/z 73, 290 (base peak), 303, 362, and 435. A proposed structure of the ion fragments is shown, along with the mass spectra, in Fig. 5B. The fragmentation pattern indicates a molecular ion with trisilylated derivatization. The major ion fragment with m/z 290 results from ion impact fragmentation of the carbon-carbon bond in the alkyl...
side chain of the indole ring, leaving a disilylated fragment ion. The ion with m/z 303 is consistent with the loss of silylated N-acetyl group. A minor abundance of a mono-desilated ion fragment with m/z 362 is also observed with the corresponding trimethyl silyl ion fragment at m/z 73. Ions with m/z 434 and 435 are consistent with the trisilylated molecular ion and its protonated form, respectively.

Based on the mass spectrum analysis of NAS, ions with m/z 290, 303, and 362 were used in the GC/MS identification of NAS in the aqueous layer of the media from skin culture. Results of the GC/MS analysis of the RP-HPLC fraction eluting at 27–29 min are shown in Fig. 5C. The upper and middle panels show selective ion chromatograms for the m/z range of 290 to 303 and 290 to 362, respectively. Both chromatograms show a single peak eluting at a retention time consistent with NAS. In the lower panel is shown the relative abundance of ions with m/z 290, 303, and 362. Fig. 5D shows a parallel GC/MS analysis of pure NAS. The relative abundance of fragment ions with m/z 290, 303, and 362 shown in the lower panel of Fig. 5, C and D, evidence a close agreement of ion ratios for pure NAS and the compound isolated from skin culture media. Thus, the compound isolated from the skin culture media is identified as NAS based upon HPLC retention, gas chromatographic retention, and mass spectral fragmentation ions.

Furthermore, we analyzed from aqueous phase the RP-HPLC fraction eluting at 25–26 min, which corresponds to the second major radioactive peak eluting between serotonin and NAS (Figs. 2–4). The analysis was performed by EI and CI modes. The major compound in this fraction has a gas chromatographic retention time of 11 min 48 s. The EI mass spectrum resulted in ion fragments with m/z 73, 354, 410, and 426 (base peak). The ion with m/z 426 also predominated in the CI analysis. Although we do not yet have the structural identity of this compound, we propose that it is a product of serotonin metabolism with a molecular weight of 425 daltons for the derivatized compound. The mass spectrum of the unknown compound was not consistent with the structures of 5-hydroxyindole acetaldehyde, 5-hydroxytryptophol, 5-hydroxyindole acetic acid, 5-methoxytryptophol, and 5-methoxyindoleacetic acid.

**Time-dependent Metabolism of Serotonin and the Stimulatory Effect of Forskolin—**To study time-dependent changes in transformation of serotonin to melatonin and further to 5-MT as well as the effect of forskolin (10 μM) on this process, media, after different times of incubation, were chloroform fractionated and separated by RP-HPLC (Fig. 6). The representative HPLC elution characteristics after 3, 5, and 24 h of incubation are shown in Fig. 6 A-C, respectively, and panel D (summary panel) shows the levels of tritiated metabolites fractionated into the chloroform phase. The highest production of [³H]NAS and [³H]melatonin occurred between 3 and 5 h of incubation, which corresponded to 10 and 12 p.m., and then decreased with
the lowest level observed at the 24 h of incubation (Fig. 6D). Conversely, the production of [3H]5-MT increased progressively during the time of incubation reaching the highest values at 24 h (Fig. 6, C and D).

The data from representative RP-HPLC separations performed on the chloroform phase obtained after 6 h of incubation in the absence or presence of 1, 10, and 100 μM forskolin are presented in Fig. 7, A-E. The addition of forskolin stimulated production of tritiated NAS, melatonin, and 5-MT in a dose-dependent manner with a maximal stimulation at 10 μM concentration (Fig. 7C). In a separate experiment we analyzed the aqueous fraction by a RP-HPLC. It appeared that the NAS concentration in the aqueous fraction was higher in the presence of 10 μM forskolin than in control (not shown), which is consistent with the data presented in Fig. 7.

DISCUSSION

In previous studies with hamster skin, we have identified and characterized the NAT-2 isozymic form of arylamine N-acetyltransferase that catalyzed the acetylation of serotonin to NAS, a direct precursor of melatonin, thus suggesting a non-rhythmic formation of N-acetylserotonin (22). We now have direct experimental evidence showing that mammalian skin can transform [3H] serotonin to radioactive metabolites eluting at the same time as norradioactive NAS, melatonin, and 5-MT standards. The GC/MS confirmed that coeluting nonradioactive standards separated by RP-HPLC were serotonin, NAS, 5-MT, and melatonin (MEL). The results are the summary of two skin cultures. A, 3 h of incubation; B, 5 h of incubation; C, 24 h of incubation; D, summary panel.

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**Fig. 6.** Time-dependent production of [3H]N-acetylserotonin, [3H]melatonin, and [3H]5-methoxytryptamine by hamster skin. Skin biopsies were incubated in the medium containing 10 μCi of [3H] serotonin. After defined time periods cultures were terminated, media were extracted, and chloroform phases were separated by RP-HPLC in the presence of nonradioactive standards of serotonin (SER), NAS, 5-MT, and melatonin (MEL). The results are the summary of two skin cultures. A, 3 h of incubation; B, 5 h of incubation; C, 24 h of incubation; D, summary panel.
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thesis in the gut. This last finding, however, was disputed by others (26). Presented here are data supporting the concept that there are extracranial and peripherally located sites of melatonin synthesis (11). Since the integumentum has the same embryonal origin as the central nervous system (27), it is not surprising that skin is capable of producing neurohormones and expressing corresponding receptors (15, 28). Skin is composed of many unrelated cells of neuroectodermal and mesenchymal origin including melanocytes, Merkel cells, keratinocytes, resident, and circulating immune response associated cells, fibroblasts, endothelial cells, and fat cells (13, 16, 18, 27). Future experiments with in situ hybridization techniques using molecular probes from the recently cloned gene coding for arylalkylamine NAT (29, 30) may help identify cutaneous cells producing melatonin and may better define the role of melatonin in skin function.

In conclusion, we show that the mammalian skin can produce NAS, melatonin, and 5-MT and suggest that production of 5-MT may represent a local mechanism of melatonin inactivation. Thus, the skin appears to be both a target for melatonin bioregulation and a site of its synthesis and degradation.

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Fig. 7. The effect of forskolin on production of [3H]N-acetylsereotonin, [3H]melatonin, and [3H]5-methoxytryptamine by hamster skin. Skin biopsies were incubated for 6 h in the medium containing 10 μCi of [3H]serotonin and different concentrations of forskolin: none (A, control), 1 μM (B), 10 μM (C), 100 μM (D), summary panel (E). Chloroform phases of the culture media from 2 skin cultures were separated by RP-HPLC in the presence of nonradioactive standards of serotonin (SER), NAS, 5-MT, and melatonin (MEL).