that when monocytes leave the blood and become tissue macrophages, they retain the ability to generate and release CSF. The wide distribution of these cells throughout the body would fit with the finding of CSF in multiple organs. Variation in activity in different organs may reflect the concentration of monocytes and macrophages in these tissues.

The finding that the monocyte stimulates the in vitro production of granulocytes is of considerable interest. However, this finding might not be totally unexpected since there are a number of physiological and biochemical events (*23*) as well as the growth pattern of these cells in vitro (*24*) which indicate a close relation between these cell lines. This finding may also be true in vivo since it is known that recovery from marrow aplasia or hypoplasia is nearly always preceded by the appearance of monocytes followed by the return of granulocytes. It may well be that the monocyte-macrophage system which is capable of performing many specialized functions (*25*) is also involved with the control of granulocyte proliferation and maturation. However, as yet, there is no convincing evidence that CSF is a physiological regulator of in vivo cell production.

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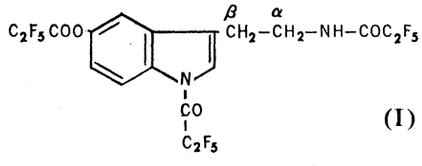
Gas Chromatographic–Mass Spectrometric Assay of Four Indole Alkylamines of Rat Pineal

Abstract. *Gas chromatography–mass spectrometry was used to quantitate serotonin, N-acetylserotonin, 5-methoxytryptamine, and melatonin in single rat pineal glands. After gas chromatographic separation, the ion density of specific fragments of each indole was measured with mass spectrometry. Sensitivity of this indole assay is of the order of 10^{-12} to 10^{-13} mole. Routinely, specificity is based on gas chromatographic retention time and the recording of the ion density generated by specific fragments. Absolute identification of the extracted indoles was based on multiple ion detection.*

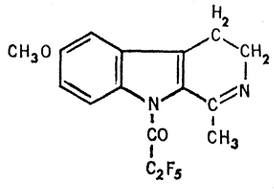
The technique of quantitative gas chromatography–mass spectrometry (*1*) is applied to measure the following indole alkylamines: serotonin, *N*-acetylserotonin (NAS), 5-methoxytryptamine (5MT), and melatonin. Serotonin, a putative neurotransmitter, is ubiquitous in the central nervous system, and existing methods are not absolutely specific (*2*). Until recently (*3*), it was believed that NAS, 5MT, and melatonin were uniquely located in the pineal gland. The specificity of the methods used is questionable since they involve either bioassay in the case of melatonin (*4*) or elaborate organic solvent extraction and reaction with *o*-phthalaldehyde to form fluorophores with the same emission and activation spectra (*5*).

To form indoles with the appropriate vapor pressure for gas chromatography, they are reacted with pentafluoropropionic anhydride (PFPA) to obtain acylation of hydroxyl and primary and secondary amine groups. When serotonin reacts with PFPA, the product (I) contains three pentafluoropropionyl (PFP) groups (molecular weight, 614). The product obtained with 5MT contains two PFP groups (molecular weight, 482),



(I)

and the internal standard α-methylserotonin (α-MS) contains three PFP groups (molecular weight, 628) (*6*). Those indoles with an *N*-acetyl side chain react with PFPA, forming a compound structurally similar to a β-carboline. The reaction product (II) of melatonin contains one PFP group (molecular weight, 360).



(II)

N-Acetylserotonin adds on two PFP groups (molecular weight, 492) and the internal standard *N*-acetyltryptamine (NAT), one PFP group (molecular weight, 330).

The structures and fragmentation patterns of these acylated derivatives have been determined with a LKB 9000 gas chromatograph–mass spectrometer. Gas chromatograph conditions were: 9-foot (1 foot = 0.3 m) glass column (inside diameter, 2 mm) packed with OV-17, 3 percent on Gas Chrom Q, 100 to 120 mesh; flash heater, 290°C; oven, 210°C; and helium flow, 15 ml/min. All the compounds have similar gas chromatographic properties and are completely resolved from each other (Table 1). Mass spectrometry conditions were: molecular separator, 250°C; ion source, 290°C; electron energy, 80 ev; trap current, 60 μa; and electron multiplier, 3.7 kv.

Mass spectral analysis of the indole-PFP derivatives revealed that their fragmentation pattern depends on the presence or absence of the *N*-acetyl group. Indoles with an *N*-acetyl group

undergo structural reorganization, forming a β-carboline (II). Upon electron impact, the ionized β-carbolines (M+, parent ion) are the most abundant ions (base peak) (Table 1). The base peak from the PFP derivatives of serotonin, 5MT, and α-MS resulted from cleavage between the bond between α-carbon and β-carbon and the bond between α-carbon and nitrogen (Table 1). Internal standards with similar gas chromatographic–mass spectrometric characteristics were used (NAT for NAS and melatonin; α-MS for serotonin and 5MT), and thus correct for any fluctuations in experimental conditions, that is, a small change in flow velocity through the gas chromatograph column.

Acylation of the six indoles by PFPA reached a steady state after 3 hours at 60°C. The derivatives were stable for at least 24 hours. This was tested by keeping the concentration of the internal standards NAT and α-MS constant (20 pmole) and varying the concentration of serotonin, NAS, 5MT, and melatonin (1 to 600 pmole). The indoles, together with a constant concentration of the internal standards, were dried under nitrogen and reacted with PFPA (100 μl) in ethyl acetate (20 μl) at 60°C for 3 hours. Prior to analysis, the excess PFPA was evaporated under a stream of nitrogen and the residue dissolved in ethyl acetate (10 μl). Usually 2 μl of this solution was injected into the gas chromatograph port. Under these conditions, the reaction yield for serotonin-PFP was 60 percent, and 100 percent for NAS-PFP, 5MT-PFP, and melatonin-PFP.

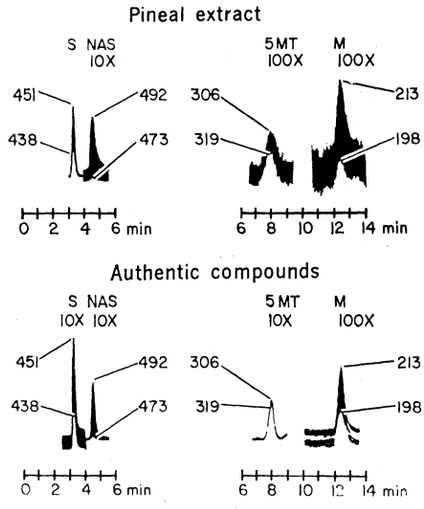


Fig. 1. Composite photograph of the ultraviolet tracings of multiple ion detection of pineal extract (upper panel) and authentic compounds (lower panel) of serotonin-PFP (*S*), *N*-acetylserotonin-PFP (*NAS*), 5-methoxytryptamine-PFP (*5MT*), and melatonin-PFP (*M*). Gas chromatographic–mass spectrometric conditions are reported in the text. Multiple ion detection was obtained by using the accelerating voltage alternator. Initially the magnetic field was set so the lower fragment (*m/e*) to be recorded was focused. By decreasing the accelerating voltage the fragment with the higher *m/e* is focused. The accelerating voltage alternator automatically alternates between the original voltage setting and the lower voltage, thereby allowing for the consecutive recording of the ion density of the two fragments.

For quantitation the ion density of the base peak of each indole was recorded as it was eluted from the gas chromatograph column (Table 1). The peak height ratio between the indoles and their respective internal standards (α-MS-PFP, NAT-PFP) was plotted (ordinate) against the absolute concentration of each indole (abscissa). In this way a linear relation was obtained for each of the indoles over a range of 1 to 600 pmole. This linearity supports the contention that the ion density of the recorded fragment is proportional to the original concentration of indole. These graphs also serve as calibration curves for analysis of unknown concentrations of endogenous indoles. The slope of calibration curves made on separate days is completely reproducible with all points falling on a single line.

With authentic standards we have shown that it is possible to measure less than 1 pmole of the four indoles and that α-MS and NAT are suitable as internal standards. When compared with the four indoles under study the internal standards have (i) similar gas chromatographic properties, (ii) similar rates of acylation and breakdown, and (iii) similar cracking patterns. In addition, the tissue extract does not have "background" activity at the gas chromatographic retention time and fragments [mass to charge (*m/e*)] used to measure the internal standards.

This assay was applied to the measurement of the endogenous indole content contained in a single pineal gland of the rat. The pineal was homogenized in 50 μl of ice-cold 0.1*M* $ZnSO_4$. After neutralization with 50 μl of 0.1*M* $Ba(OH)_2$ and centrifugation, a portion

Table 1. Parameters used for quantitation and multiple ion detection of the 5-substituted indole alkylamine pentafluoropropionyl (PFP) derivatives. For gas chromatographic–mass spectrometric conditions, see text; *m/e*, mass to charge; GC, gas chromatographic; percent relative intensity refers to relative intensity of the fragment compared to the most abundant (base) peak which is 100 percent. Origin is obtained from normal mass spectral analysis of each compound: 1, α-Carbon-nitrogen bond cleavage. The charge is retained on the indole moiety with transfer of a hydrogen atom to the neutral fragment. 2, Parent ion. 3, α-β-Carbon atom cleavage, charge retained on the indole moiety. 4, Parent ion (M) minus fluorine atom (M − 19). 5, Parent ion minus PFP (M − 147). 6, Parent ion minus PFP (147) and a methyl atom (15) (M − 162). Multiple ion detection was accomplished by holding the magnetic field constant and automatically alternating the accelerating voltage, thereby consecutively recording the ion density of the two fragments (Fig. 1). The fragment ratios are computed from the peak heights of each fragment. Multiple ion detection was not done on the internal standards.

Indole PFP derivatives	GC retention time (min)	Fragments used for quantitation			Multiple ion detection			
					Fragments assayed			Fragment ratio
		m/e	Relative intensity (%)	Origin	*m/e*	Relative intensity (%)	Origin	
α-Methylserotonin	2.7	465	36	1				
Serotonin	3.4	451	100	1	438	44	3	2.3
					451	100	1	
N-Acetylserotonin	4.5	492	100	2	473	7	4	14.3
					492	100	2	
N-Acetyltryptamine	5.8	330	100	2				
5-Methoxytryptamine	8.0	306	100	3	319	96	1	1.0
					306	100	3	
Melatonin	12.3	360	100	2	213	40	5	2.1
					198	19	6	

of the supernatant was processed with the internal standards and analyzed by mass fragmentography (Table 1). Extraction recoveries for each of the indoles were (mean of six determinations): serotonin, 68 percent; 5MT, 53 percent; NAS, 74 percent; and melatonin, 69 percent. As measured by mass fragmentography, rat pineal contains (in micrograms per gram; mean of four assays ± standard error of the mean): serotonin, 54 ± 4.4; 5MT, 4.2 ± 0.38; melatonin, 3.9 ± 0.10; and NAS, 0.13 ± 0.022. Values for melatonin and NAS do not agree with those of Miller and Maickel (*5*), but our melatonin value does agree with melatonin values reported for bioassay (*7*). Since the relative fluorescence of melatonin is three times greater than that of NAS (*8*), it is possible that with the extraction procedures used for the *o*-phthalaldehyde method, there is contamination of melatonin in the NAS sample, thereby giving a higher value for NAS (7.8 μg/g) and a lower value for melatonin (0.48 μg/g) (*7*). The pineal 5MT concentrations are comparable to those obtained by the *o*-phthalaldehyde method (*7*), and serotonin values are the same as those measured by *o*-phthalaldehyde or ninhydrin fluorescence (*9*).

Absolute identification of the analyzed pineal indoles was by multiple ion detection (Fig. 1) (*10*). Multiple ion detection of the assayed indoles was done with the fragments listed in Table 1, and the fragment ratio obtained for both the authentic compounds and the pineal extract (Fig. 1) is the same as that listed in Table 1, thereby confirming the specificity of the assay. The pineal extracts processed without internal standards do not have any "biological background."

In summary, we have described a gas chromatographic–mass spectrometric assay for the simultaneous measurement of serotonin, NAS, 5MT, and melatonin with a sensitivity greater than 1 pmole. Routinely, specificity is based on the gas chromatographic retention time of the compound and measurement of the ion density of a specific fragment (*m/e*) at this time. Absolute identification of compounds extracted from pineal gland is obtainable by multiple ion detection.

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2. With the routine analytical methods presently used there is no differentiation between serotonin and *N*-methylserotonin, whereas with the method presented here, these two compounds are differentiated.
3. Using this method, we have detected significant concentrations of some of these indoles in various brain nuclei. Enzymes for the synthesis of these compounds have also been reported to be present in the rat retina [D. P. Cardinali and J. M. Rosner, *J. Neurochem.* **18**, 1769 (1971)].
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6. Those indole derivatives lacking the *N*-acetyl group also form PFP derivatives in which all the available primary and secondary amine groups or hydroxy groups are not acylated. These derivatives have different gas chromatographic retention times and fragmentation patterns from the fully acylated compound described. Since the amount of each derivative formed is proportional, only the sensitivity of the method is affected without changing the precision or accuracy.
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Water-Soluble Insulin Receptors from Human Lymphocytes

Abstract. *Specific insulin receptors from human lymphocytes in culture have been prepared in aqueous solution without use of detergents or related compounds. Receptors prepared in this fashion exhibit characteristics identical to those reported in intact cells.*

The solubilization of polypeptide hormone receptors from broken cell preparations (*1*) has contributed to our knowledge of hormone-receptor interactions. In all of these procedures, detergents or vigorous mechanical techniques are used (*1, 2*). However, the study of physical and chemical properties of detergent-solubilized protein is subject to uncertainties because of the presence of residual detergent molecules bound to the protein (*3*). Similarly, studies on the "solubilized" hormone receptors prepared by mechanical extraction are limited by the presence of microfine particles (*4*).

Circulating human lymphocytes have specific membrane insulin receptors that are similar to those in the fat cell and liver cell membranes of rats (*5*). Further, specific insulin receptors found in cultured human lymphocytes are identical to those in the circulating cells (*5*). The accessibility of the cultured lym-

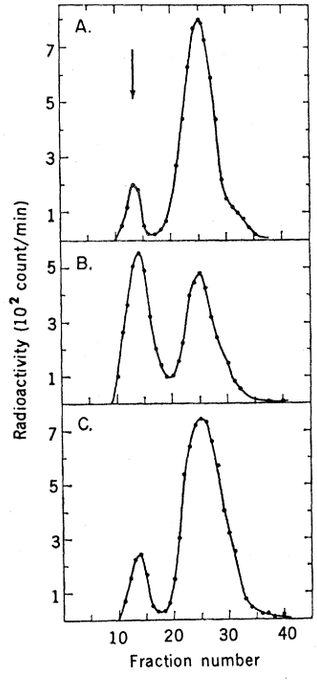

Fig. 1. Gel filtration of soluble receptor from lymphocytes. [^{125}I]Insulin ($2 \times 10^{-11}M$) was incubated with receptor (approximately 0.3 mg of protein) in the presence and absence of $10^{-5}M$ unlabeled insulin for 75 minutes at 23°C in a total volume of 0.25 ml. The mixture was made 3 percent in sucrose and chromatographed on a 1 by 30 cm column of Sephadex G-50 previously equilibrated with PBS (*p*H 7.6) containing 0.1 percent serum albumin. Flow rate was about 5 ml per hour. Results are shown for (A) [^{125}I]insulin alone; (B) [^{125}I]insulin plus soluble receptor; and (C) [^{125}I]insulin plus receptor in the presence of $10^{-5}M$ unlabeled insulin. When material in the unretarded peak (fractions 12 to 16) from (B) was rechromatographed, approximately 85 percent of the radioactivity appeared in the void volume, and the remainder eluted in the position corresponding to [^{125}I]insulin. The arrow marks the position of the void volume, as determined by chromatography of Blue Dextran 2000 (Pharmacia).