

also to an increase of carboxyl-residues close to or at the protein's active site.

Zusammenfassung. Anhand von Modellversuchen konnte gezeigt werden, dass SH-gebundenes N-Äthylmaleinimid bei pH-Werten über dem Neutralpunkt allmählich

zur entsprechenden N-Äthylsuccinamidsäure hydrolysiert. Dadurch verändert sich das chromatographische Verhalten NÄM-markierter Peptide und möglicherweise auch die biologische Aktivität NÄM-blockierter Proteine.

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17 March 1972.

⁷ This study was carried out with the support of the Deutsche Forschungsgemeinschaft.

The Amine and Amino Acid Composition in the Retzius Cells of the Leech *Hirudo medicinalis*

It has become clear in recent years that precise biochemical information on the functioning of nervous systems is best obtained from experiments on individual neurons. The so-called 'colossal' or RETZIUS cells¹ in each abdominal ganglion of the leech *Hirudo medicinalis* are particularly suitable for biochemical studies because they can be dissected from their surrounding tissues.

These neurons are globe-shaped with a diameter of up to 80 μ m and are known to contain serotonin^{2,3}. Although the Retzius cells can vary in size, their constant position and superficial location allow them to be easily distinguished from the surrounding cells in living preparations. We applied the microbiobiochemical technique developed by NEUHOFF et al.^{4,5} to investigate the amine and amino acid content of the Retzius cells and also the abdominal nerve chain of the leech. The content of the Retzius cells is compared with that of the serotonin-containing neuron in the metacerebral ganglion of the snail which has been described elsewhere^{6,7}.

Materials and method. Active leeches were obtained from a local dealer and groups of about 7 ganglia were isolated as described by WALKER⁸. The ganglia were pinned, ventral side up onto the base of a small dissecting dish containing a sheet of plastic, filled with cold saline⁹, and were placed on a specially cooled dissecting microscope stage. Single Retzius cells (there are two in each abdominal ganglion of the nerve chain) were carefully freed from the surrounding nerve tissues using thin tungsten needles and sharpened forceps. Cells were then individually lifted free by suction from the ultra thin tip (15 μ m in diameter) of a glass pipette, attached to the mouth by rubber tubing. 40 neurons were transferred to a 10 μ l capillary (Drummond microcap) which had been heat-sealed at one end and contained 3 μ l of 0.05 M sodium bicarbonate pH 10. The cells were then homogenized using a special nerve canal drill, 3 μ l acetone were added to precipitate the proteins and the capillary was then centrifuged⁶. The supernatant was next transferred to a clean microglass tube and reacted with an equal volume of 5 μ M/ml ¹⁴C-dansyl-chloride (Schwarz/Mann, Orangeburg, New York) specific activity 49 mC/mM). A number of trial experiments were initially carried out to determine the concentration of ¹⁴C-dansyl-chloride required, since it is known that this is of importance in order to obtain optimal reaction conditions¹⁰. After incubating the micro-glass tube and its contents for 30 min at 37°C, the mixture was evaporated to dryness under reduced pressure and the dansylated substances resuspended in 2 μ l acetone/acetic acid (3:2 v/v). Aliquots of 0.5 μ l were spotted onto a corner of a 3 \times 3 cm polyamide layer and developed by two dimensional chromatography (Figures 1a and 2a). A third chromatography in the second dimension was required to separate alanine from

dansyl-NH₂, aspartic acid from glutamic acid and serine from threonine, glutamine and asparagine (Figures 1b and 2b), although this resulted in a poor definition of some other substances. The chromatographic procedure, a method for obtaining autoradiograms and for the quantitative measurement of each substance on a single microchromatogram is described elsewhere⁴⁻⁶. In addition, after the Retzius cells had been dissected, the abdominal nerve chain was homogenized (1 mg tissue per 10 μ l sodium bicarbonate) and centrifuged and 3 μ l of the supernatant was then reacted with the same quantity of 5 μ M/ml ¹⁴C-dansyl-chloride and incubated for 30 min at 37°C. The mixture was then evaporated to dryness, redissolved in 5 μ l acetone/acetic acid and applied in aliquots of 0.5 μ l to microchromatograms for analysis.

Results and discussion. Autoradiograms showing the occurrence of substances which react with dansyl chloride are shown in Figures 1 and 2. Each substance can be identified by comparing the spot numbers on the maps with those shown in the Table. The radioactivity associated with each substance from chromatograms of 2 different experiments for both Retzius cells and abdominal nerve chain are also shown in the Table. Comparison of the percentage composition of each dansyl derivative in the different experiments demonstrates that reproducible results are obtained. Since the method depends on the reaction of dansyl-chloride with aliphatic amino or hydroxy groups to form fluorescing compounds at alkaline pH, a single substance which contains an aliphatic amino and hydroxy group, as does serotonin, can provide 3 possible fluorescing compounds, i.e. N-, OH- and Bis-form. In practice, however, the number of compounds produced depends on the experimental conditions^{4,11}. One of the most striking features of these

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Composition of ^{14}C -dansylated compounds separated by microchromatography, isolated and counted in a scintillation spectrometer

Spot No.	Substance	Abdominal nerve cells (free of Retzius cells)				Retzius cells			
		Experiment 1		Experiment 2		Experiment 1		Experiment 2	
		Counts (cpm)	Composition (%)	Counts (cpm)	Composition (%)	Counts (cpm)	Composition (%)	Counts (cpm)	Composition (%)
1	Starting point	—	—	—	—	—	—	—	—
2	Dans-OH (not counted)	—	—	—	—	—	—	—	—
3	N-serotonin	246	1.78	252	1.83	3033	11.08	2008	10.65
16	Bis-serotonin	192.5	1.39	191	1.39	1339	4.89	379	4.66
4	Tryptophan	180.5	1.31	161	1.17	1590	5.81	1025.5	5.44
32	N-5-HTP	129	0.93	126	0.91	1250	4.57	850.5	4.51
5	Bis-5-HTP	absent	absent	absent	absent	474	1.73	331	1.75
6	Bis-Lysine	424.5	3.08	468	3.40	790	2.88	534	2.83
7	Bis-ornithine	895	6.50	922	6.71	1184.3	4.33	760.5	4.03
8	Methionine	73	0.53	72	0.52	98	0.35	100	0.53
9	Phenylalanine	205	1.48	203	1.47	363	1.32	298.5	1.58
10	Bis-histidine	228	1.65	231	1.68	255	0.93	191.5	1.01
11	Leucine	439	3.18	416	3.03	757	2.76	542	2.87
12	Isoleucine	294	2.16	256	1.86	340	1.24	221	1.17
13	Unknown	184	1.33	167	1.21	313	1.14	298.5	1.58
14	Unknown	97	0.70	102	0.75	300	1.09	258	1.36
33	N-tyrosine	129	0.93	129	0.93	251	0.91	149	0.78
15	Bis-tyrosine	248.5	1.80	265	1.93	366	1.33	251	1.33
17	5-Hydroxyindole	251.5	1.82	244.5	1.78	291	1.06	173.5	0.92
18	Proline	273	1.98	287	2.09	266	0.97	194	1.02
19	Valine	429	3.11	419	3.05	488	1.78	414	2.19
20	GABA	60	0.43	61	0.44	61	0.22	65	0.34
21	Alanine	1498	10.88	1450	10.56	1471	5.37	1100.5	5.84
22	Ethanolamine	282	2.04	274	1.99	370	1.35	216	1.14
23	Unknown	absent	absent	absent	absent	337	1.23	223	1.18
24	Unknown	absent	absent	absent	absent	362.9	1.32	291.6	1.54
25	Glycine	1667	12.10	1628	11.86	1803	6.59	1266.5	6.72
26	Glutamic acid	1321	9.59	1316	9.58	3510	12.83	2327.5	12.35
27	Aspartic acid	383	2.78	369	2.69	1119	4.09	792	4.20
28	Threonine + Glutamine + Asparagine	303	2.20	310	2.25	281	1.02	208.5	1.10
29	Serine	1092	7.93	1040	7.57	763	2.78	648	3.43
30	Arginine, α -amino-histidine + ϵ -lysine	514	3.73	580	4.22	1580	5.77	935	4.96
31	Unknown + taurine	absent	absent	absent	absent	1381	5.04	839	4.45
34	Unknown	497.5	3.60	525	3.82	absent	absent	absent	absent
35	Unknown	869	6.31	880	6.41	592	2.16	448	2.27
36	Unknown	127	0.92	131	0.95	absent	absent	absent	absent
37	Unknown	114	0.82	122	0.88	absent	absent	absent	absent
38	Unknown	122	0.88	128	0.93	absent	absent	absent	absent

results is the occurrence of high levels of 5-HTP (5-hydroxytryptophan) and serotonin (both substances occur as 2 dansyl forms) in the Retzius cells. The existence of serotonin in these neurons had previously been proved by fluorescence microscopy^{2,3}, microspectrometry³ and chromatography³. This, however, is the first report describing the presence of 5-HTP, although it has been shown earlier that the Retzius cells can take-up and convert 5-HTP to serotonin¹². It is worth while noting that the abdominal nerve chain also has a fairly high serotonin content, which is not surprising since each ganglion contains at least 4 large serotonin-containing neurons¹³.

Generally the distribution of amino acids in the Retzius cells and abdominal nerve chain of the leech is similar. Both have approximately the same amounts of tyrosine, lysine, ornithine, isoleucine, leucine, phenylalanine, methionine, γ -amino-butyric acid (GABA) and arginine (plus α -amino-histidine and ϵ -lysine) although the abdominal nerve chain contains greater quantities of histidine, serine, proline, valine, threonine, glutamine and asparagine, alanine, glycine and lesser amounts of aspartic acid and glutamic acid. Of special interest is the occur-

rence of very much more tryptophan and less serine in the Retzius cells. The significance of the high level of serine in the abdominal nerve chain is not yet understood.

The presence of 5-hydroxyindole in leech nerve tissue is worthy of special note. This substance has already been detected in cat spinal cord¹¹ and mollusc nerve tissue⁵ where serotonin is known to exist. Furthermore its concentration increases in the snail brain after electrical stimulation¹⁴. Because of its close similarity in structure to 5-hydroxyindoleacetic acid (a known breakdown product of serotonin) it seems likely that 5-hydroxyindole is also a metabolite of serotonin synthesis. Also interesting are the absence of 3 unknown substances (spot 36, 37, 38), and the presence of 2 unknown substances (spot 23 and 24) in the Retzius cells, whereas the opposite is true of the abdominal nerve chain of the leech.

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There are some difficulties in making a comparison between the contents of the Retzius cell and the serotonin-containing neuron which exists in the snail^{6,7}, because the amine and amino acid content of snail nervous tissue⁵, is different from that of the leech. It does appear, however, that the Retzius cell, like the serotonin-containing neuron in the snail, contains greater amounts of serotonin, 5-HTP and tryptophan than its surrounding nerve tissues. In addition the Retzius cell, like the snail neuron, contains large amounts of glycine and alanine, but differs from the snail in having lower amounts than its surrounding nerve tissue. It also contains more glutamic and aspartic acid

(the latter to a lesser extent) than the snail serotonin-containing neuron. So that although the amine and amino acid content of the serotonin-containing neurons in the leech and snail are similar, there is some variation in the quantities of individual substances. The significance of this variation is complicated by the fact that not only does each animal species vary in the amino acid composition of its nervous system, but that even individual animals can differ within the one species. However, it must be emphasized that studies like these on individual neurons are required to obtain precise biochemical information on the functioning of nervous

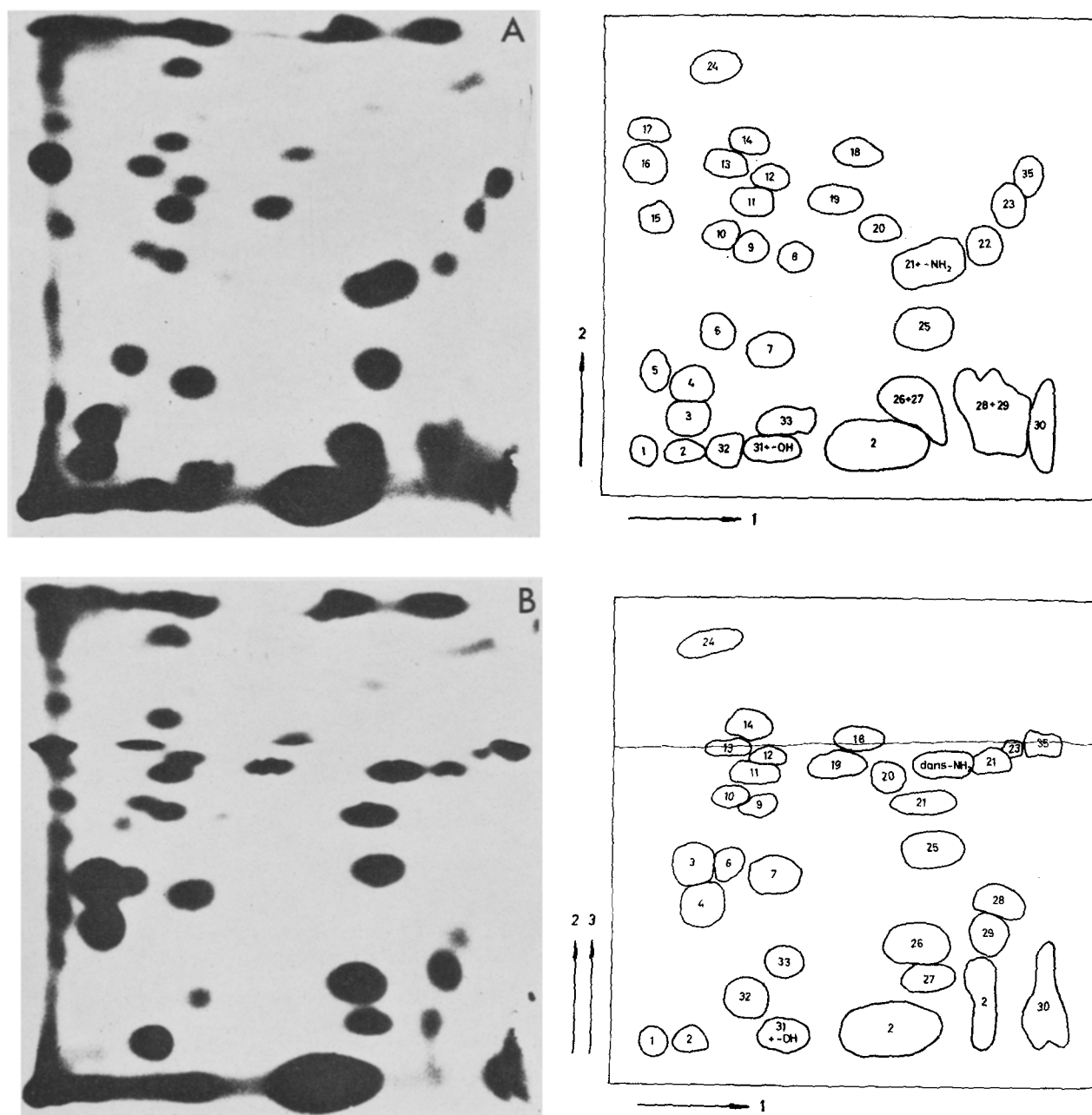


Fig. 1. Autoradiograms from extracts of Retzius neurons after having been reacted with ¹⁴C-dansyl-chloride. A single chromatogram originally measured 3 × 3 cm and was developed in either 2(a) or 3(b) solvent systems. The solutions used were: 1st direction, water/formic acid (100:3 by volume); 2nd direction, benzene/acetate acid (9:1 by volume); 3rd direction, ethyl acetate/methanol/acetic acid (20:1:1 by volume). By comparing the spot numbers on each accompanying map with those shown in the Table, each substance can be identified. The unmarked spots on chromatograms belong either to impurities of ¹⁴C-dansyl-chloride or to other unknown compounds.

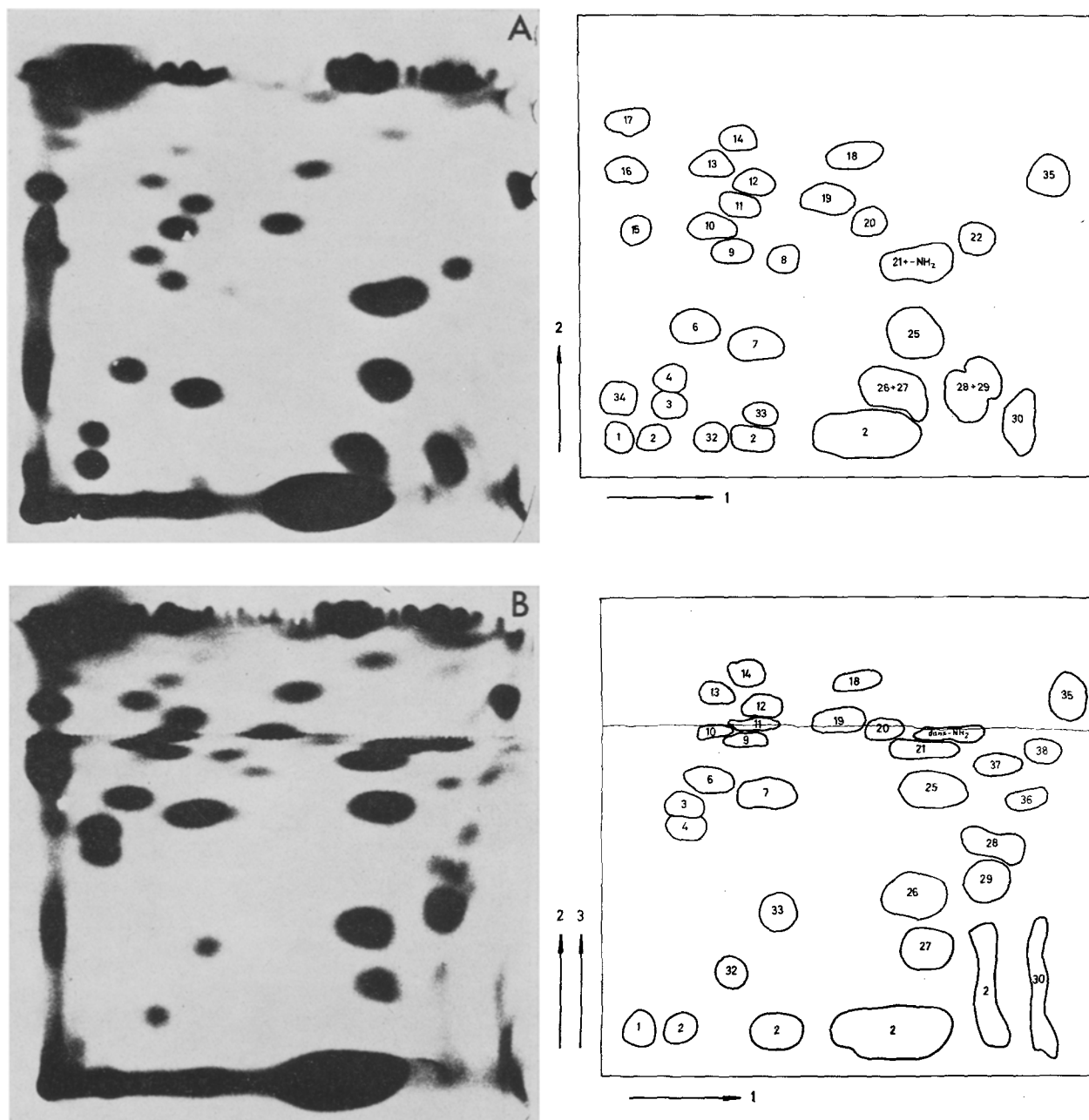


Fig. 2. Autoradiograms and corresponding maps of ^{14}C -dansyl chloride compounds in abdominal nerve chain (excluding Retzius neurons) separated by microchromatography in either 2 (a) or 3 (b) solvent systems.

systems. The comparatively high levels of the serotonin precursors, 5-HTP and tryptophan for example, in the serotonin-containing neurons of the leech and snail^{6, 7, 15} may be a characteristic of all such neurons.

Zusammenfassung. Die Mikrochromatographie von dansylierten Verbindungen wurde angewandt, um die Verteilung von Aminen und Aminosäuren in den Retzius-Zellen und der Bauchganglienkeite des Blutegels *Hirudo medicinalis* zu untersuchen. Die Retzius-Zellen weisen sehr grosse Mengen an Serotonin, 5-HTP und Tryptophan auf. Ihr Gehalt an Asparagin- und Glutaminsäure ist ebenfalls, allerdings in geringerem Masse, grösser als im umgebenden Nervengewebe, während Histidin, Prolin,

Valin, Alanin, Glycin sowie Threonin, Glutamin und Asparagin in geringeren Mengen vorkommen.

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¹⁶ We thank Miss MARIANNE MAIER for her technical assistance.

¹⁷ Acknowledgment supported by a long term fellowship from the European Molecular Biological Organisation.