The Distribution of Protein-Bound *N*-Acetylneuraminic Acid in Subcellular Fractions of Rat Brain

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Protein-bound *N*-acetylneuraminic acid and hexosamine, including the sialomucopolysaccharides, occur mainly in the least dense particles sedimented in the microsomal fraction from rat whole brain. Particles rich in protein-bound *N*-acetylneuraminic acid and hexosamine are also found in the subcellular fraction separated as a layer between 0.8M- and 1.2M-sucrose after centrifuging the crude mitochondrial preparation in a density gradient. This distribution is similar to that of the gangliosides and suggests an association of all of these substances in the same subcellular structures. It is postulated that the sialomucopolysaccharides, as well as the gangliosides, are components of cell membranes. Evidence is presented that indicates that there are quantitative differences between distribution of the gangliosides on the one hand, and protein-bound *N*-acetylneuraminic acid and hexosamine on the other. The ratio of protein-bound *N*-acetylneuraminic acid (and hexosamine) to gangliosidic *N*-acetylneuraminic acid (and hexosamine) present in individual subcellular fractions obtained by density-gradient centrifugation tends to increase with increasing particle density. Exposure of the crude mitochondrial fraction to osmotic 'shock' before density-gradient centrifugation causes a shift of the protein-bound *N*-acetylneuraminic acid and gangliosides to the less dense fractions. In some experiments, a selective shift of the protein-bound *N*-acetylneuraminic acid was observed.

The present work is a continuation of investigations designed to ascertain the subcellular localization of the insoluble glycoproteins associated with particulate fractions obtained from rat whole brain. Brunngraber & Brown (1964, 1967) reported that most of the protein-bound NANA* of the isolated particulate matter of rat brain after the gangliosides are extracted is present in a protein-linked sialomucopolysaccharide. The sialomucopolysaccharides contain NANA, hexose, hexosamine and fucose; the sialomucopolysaccharide molecule consists of a repeating unit containing hexosamine and hexose to which NANA and fucose are attached. Individual sialomucopolysaccharide molecules differ in the relative proportions of fucose and NANA attached to this repeating unit. By-products obtained in the isolation of the sialomucopolysaccharides include a fraction that was resistant to a 24hr. incubation period in the presence of papain ('papain-resistant' fraction), as well as diffusible fragments containing some NANA and much hexosamine liberated by the proteolytic procedure.

The distribution of all of these substances in the

crude mitochondrial, microsomal and nuclear pellets (Brunngraber & Brown, 1964) was similar to that of the gangliosides. To draw appropriate conclusions with regard to their subcellular localization, it was necessary to prepare subcellular fractions of a higher degree of purity than those previously studied. In the present work, the crude mitochondrial fraction was separated into myelin-, synaptosome- and mitochondria-enriched fractions by utilizing the density-gradient centrifugation technique introduced by Whittaker and co-workers (Whittaker, 1959; Gray & Whittaker, 1962) and utilized by Wolfe (1961) in his investigations designed to localize the subcellular distribution of the gangliosides. The same procedure was also applied to the crude microsomal pellet.

MATERIALS AND METHODS

Preparation of subcellular fractions. The crude mitochondrial and microsomal pellets from three rat whole brains were obtained as described by Brunngraber & Brown (1964), except that the microsomal pellet was sedimented by centrifuging for 90 min. at 105400g in the no. 40 rotor of the Spinco model L preparative ultracentrifuge. The mitochondrial and microsomal pellets were

* Abbreviation: NANA, *N*-acetylneuraminic acid.

suspended in 10 ml. of 0.32M-sucrose. Each was layered over a density gradient consisting of 10 ml. of 0.8M-sucrose and 10 ml. of 1.2M-sucrose, and centrifuged in the SW25.1 rotor of the Spinco ultracentrifuge at 53500g for 140 min. The procedure is that reported by Wolfe (1961). The layers were separated by use of a Pasteur pipette. The mitochondrial preparation (P_2) yielded a layer (P_2A) of white material between 0.32M- and 0.8M-sucrose. The material of intermediate density (P_2B) formed a layer between 0.8M- and 1.2M-sucrose. A tan-coloured residue (P_2C) was beneath the 1.2M-sucrose layer. Most of the microsomal pellet sedimented in the layer between 0.32M- and 0.8M-sucrose (P_3A). The remaining material sedimented between 0.8M- and 1.2M-sucrose (P_3B) with only a very small amount of material found at the bottom of the tube (P_3C).

The layers were put into centrifuge tubes and made up to 13 ml. by filling the tube with water and stirring gently. These were then centrifuged in the no. 40 rotor for 45 min. at 105400g. The process was repeated once. The purpose of these washings was to remove most of the sucrose, which would interfere with the analytical methods. The pellets corresponding to the six layers were then frozen and stored.

The procedure was repeated with three rats at a time, until material from 15 rat brains had been accumulated. The corresponding fractions obtained from each experiment were combined to give a 15 rat preparation of layers P_3A , P_3B , P_3C , P_2A , P_2B and P_2C .

A crude mitochondrial pellet from cat cerebellum was prepared and subfractionated into five fractions (P_2A' , P_2B' , P_2C' , P_2D' and P_2E') by the procedure described by De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnaiz & Salganicoff (1962).

Osmotic-'shock' treatment of the crude mitochondrial pellet from whole rat brain consisted of successively suspending the mitochondrial pellet obtained by procedures described by Brunngraber & Brown (1964) in 0.02M-tris-chloride buffer, pH 8.0, centrifuging at 105000g for 30 min., resuspending in water and recentrifuging. Suspension of the particulate fraction was performed by homogenizing by hand in a TenBroeck homogenizer. This procedure solubilizes approx. 25% of the protein content of the pellet (Brunngraber & Aguilar, 1962), and additional treatment solubilizes very little more. The 'shocked' material was separated on a density gradient consisting of equal volumes of 0.8M- and 1.2M-sucrose to give three fractions P_2A'' , P_2B'' and P_2C'' analogous to fractions P_2A , P_2B and P_2C .

Isolation of NANA- and hexosamine-containing substances. The subcellular fractions were extracted with chloroform-methanol (2:1, v/v) to remove the gangliosides. The defatted protein residue was subjected to proteolytic digestion with papain. The papain-resistant material was removed by centrifugation. The NANA- and hexosamine-containing materials solubilized by papain were dialysed to recover the diffusible fragments. The high-molecular-weight constituents were treated with cetylpyridinium chloride to precipitate the nucleic acids and glucuronic acid-containing mucopolysaccharides. Details of this procedure have been published (Brunngraber & Brown, 1964, 1967).

Analytical methods. Hexosamine was determined by the Elson-Morgan method as modified by Boas (1953) after hydrolysis in 1.5N-HCl for 6 hr. NANA was determined by the thiobarbituric acid method of Warren (1959) after mild

hydrolysis of the sample at 80° for 1 hr. in 0.1N-H₂SO₄. Heating was extended to 3 hr. to determine gangliosidic NANA. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Materials. NANA and papain (twice recrystallized, sodium acetate suspension) were both obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. The rats were of the Wistar strain obtained from Abrams Small Stock Breeder Inc., Chicago, Ill., U.S.A.

RESULTS

NANA- and hexosamine-containing constituents of subcellular fractions from rat whole brain. The gangliosides were recovered mainly in the fraction of intermediate density (P_2B) derived from the crude mitochondrial pellet (Tables 1 and 2). This fraction was also enriched in gangliosides when their concentration was expressed in terms of μ g. of gangliosidic NANA/mg. of protein. The microsomal pellet (P_3) was also rich in gangliosides. These findings agree with those reported by Wolfe (1961). Gangliosides present in the microsomal pellet are especially enriched in the least dense fraction (P_3A) derived from it.

The distribution of the sialomucopolysaccharides, diffusible fragments and papain-resistant material is similar to that of the gangliosides in all these respects. It appears that all of the hexosamine- and NANA-containing substances are closely associated in brain tissue and may be present in the same subcellular structures. Fraction P_2B is known to be enriched in nerve endings and membranes (Whittaker, 1959; Gray & Whittaker, 1962); fraction P_3A is reported to contain membranous fragments (Toschi, 1959).

The major NANA-containing materials in rat brain are the gangliosides, the 'papain-resistant' fraction, and the sialomucopolysaccharides. Diffusible NANA-containing fragments released by the action of papain represent only about 4-10% of the total brain content of NANA, and of the NANA content of subcellular fractions prepared from brain. The 'papain-resistant' material contains largely sialomucopolysaccharides that have not been removed by papain by the 24 hr. digestion procedure, as well as some gangliosides not removed by extraction with chloroform-methanol (2:1, v/v) (Suzuki, 1965).

The major hexosamine-containing constituents in brain are the sialomucopolysaccharides, although large percentages are also present in each of the other fractions. The NANA/hexosamine ratio for the gangliosides, 'papain-resistant' material, diffusible fragments and sialomucopolysaccharides were 1.6, 1.7, 0.2 and 0.5 respectively. Papain is relatively more efficient in putting the hexosamine (80-85%) of the defatted brain residue into

Table 1. *NANA- and hexosamine-containing substances in subcellular fractions*

Amounts of NANA and hexosamine present in the ganglioside, 'papain-resistant', diffusible and sialomucopolysaccharide fractions isolated from subcellular fractions (P_2A , P_2B , P_2C ; P_3A , P_3B , P_3C), obtained by subjecting crude mitochondrial (P_2) and microsomal (P_3) pellets respectively to sucrose-density-gradient centrifuging, were determined. The numbers in parentheses are the percentages of the total amount of each class of NANA-containing material occurring in the subcellular fraction indicated. The average weight of a rat brain was 1.9g. For further details see the text.

NANA-containing fraction	Subcellular fraction ...	NANA (μ moles/brain)				Hexosamine (μ moles/brain)			
		P_2A	P_2B	P_2C	Total	P_2A	P_2B	P_2C	Total
Ganglioside		0.13 (28)	0.31 (68)	0.015 (4)	0.46	0.11 (37)	0.17 (59)	0.018 (4)	0.30
'Papain-resistant'		0.11 (17)	0.50 (79)	0.023 (4)	0.63	0.07 (19)	0.24 (72)	0.031 (9)	0.34
Diffusible		0.02 (29)	0.05 (64)	0.006 (7)	0.08	0.09 (25)	0.25 (67)	0.032 (8)	0.37
Sialomucopolysaccharide		0.09 (24)	0.24 (70)	0.020 (6)	0.35	0.17 (22)	0.52 (68)	0.077 (10)	0.77
Total		0.35	1.10	0.064	1.51	0.44	1.18	0.16	1.78

NANA-containing fraction	Subcellular fraction ...	P_3A	P_3B	P_3C	Total	P_3A	P_3B	P_3C	Total
Ganglioside		0.41 (81)	0.089 (18)	0.005 (2)	0.50	0.23 (74)	0.08 (24)	0.006 (2)	0.31
'Papain-resistant'		0.12 (59)	0.087 (41)	0 (0)	0.21	0.07 (44)	0.07 (47)	0.015 (9)	0.16
Diffusible		0.03 (60)	0.020 (40)	0 (0)	0.05	0.20 (62)	0.12 (36)	0.009 (2)	0.32
Sialomucopolysaccharide		0.18 (64)	0.095 (34)	0.003 (2)	0.28	0.33 (77)	0.08 (20)	0.014 (3)	0.42
Total		0.74	0.29	0.008	1.04	0.83	0.35	0.044	1.21

Table 2. *Distribution of protein, gangliosides and protein-bound NANA in subcellular fractions of rat whole brain*

For nomenclature of subcellular fractions see the legend to Table 1 and the text.

Subcellular fraction	Protein (mg./brain)	Gangliosidic NANA (μ g./mg. of protein)	Protein-bound NANA (μ g./mg. of protein)
P_2A	14.7	2.73	4.62
P_2B	27.2	3.50	8.94
P_2C	15.9	0.29	0.95
P_3A	10.7	11.84	9.53
P_3B	7.7	3.57	8.10
P_3C	1.2	1.29	0.81

solution than it is in putting NANA into solution (60–65%).

Possibility of a difference in subcellular localization of the gangliosides and protein-bound NANA. The ratio between the gangliosidic NANA and protein-bound NANA reveals a trend that may be indicative of a quantitative difference between individual subcellular fractions. The proportion of each of the protein-bound constituents ('papain-resistant' material, diffusible fragments and sialomucopolysaccharides) usually increases relative to ganglioside with increasing density of the subcellular fraction (Table 3). This trend is evident when the ratio is computed on the basis of NANA and hexosamine. The protein-bound NANA/ganglio-

sidic NANA ratio is higher for the denser particles obtained by fractionating the crude mitochondrial and microsomal pellets (Table 4). It is also higher in the mitochondrial preparation (more dense) than in the microsomal preparation (less dense).

Change in distribution of gangliosides and protein-bound NANA by osmotic 'shock'. Subjection of the crude mitochondrial pellet to osmotic 'shock' treatment before density-gradient centrifugation in several experiments led to a shift of the protein-bound NANA to the less dense P_2A fraction, leaving the gangliosides predominantly in the P_2B fraction (Brunngraber, Ziboh & Occomy, 1965). However, in other experiments both gangliosides and the protein-bound NANA have shifted to the

Table 3. *Ratios of protein-bound NANA and hexosamine to gangliosidic NANA and hexosamine in subcellular fractions*

For nomenclature of fractions see the legend to Table 1 and the text.

Protein-bound NANA fraction	Subcellular fraction ...	NANA			Hexosamine		
		P_2A	P_2B	P_2C	P_2A	P_2B	P_2C
'Papain-resistant'		0.84	1.6	1.5	0.63	1.4	1.7
Diffusible		0.15	0.16	0.40	0.81	1.5	1.7
Sialomucopoly- saccharides		0.69	0.77	1.30	1.5	3.0	4.2
	Subcellular fraction ...						
		P_3A	P_3B	P_3C	P_3A	P_3B	P_3C
'Papain-resistant'		0.29	0.97	—	0.30	0.87	2.5
Diffusible		0.07	0.22	—	0.86	1.5	1.5
Sialomucopoly- saccharides		0.43	1.06	0.60	1.4	1.0	2.3

Table 4. *Ratio of protein-bound NANA to gangliosidic NANA in subcellular fractions*

Results of Expt. 1 (average of five experiments) are taken from Table 1. Results of Expt. 2 (average of two experiments) are taken from previously published material (Brunngraber & Brown, 1964). Expt. 3 is an average of two experiments in which cat cerebelli were used to prepare the crude mitochondrial (P_2) and microsomal (P_3) fractions. Fraction P_2 was subsequently fractionated to yield fractions P_2A' , P_2B' , P_2C' , P_2D' and P_2E' by the method of De Robertis *et al.* (1962).

Expt. no.	Subcellular fraction	Protein-bound NANA/ gangliosidic NANA	Protein-bound hexosamine/ gangliosidic hexosamine
1	P_2A	1.69	3.00
	P_2B	2.54	5.94
	P_2C	3.26	7.77
	P_3A	0.80	2.60
	P_3B	2.26	3.37
	P_3C	—	6.33
2	P_2	2.15	—
	P_3	1.23	—
3	P_2	1.14	—
	P_3	0.78	—
	P_2A'	0.54	—
	P_2B'	0.65	—
	P_2C'	1.00	—
	P_2D'	1.14	—
	P_2E'	1.75	—

less dense fraction (Table 5). The failure consistently to reproduce the selective shift of the protein-bound NANA probably reflects our failure to standardize this procedure. Selective results may be dependent on strict control of the clearance of the homogenizer and the number of strokes used to disperse the particulate material. Osmotic-'shock' treatment caused a loss of about 9% of the ganglioside content of the crude mitochondrial fraction and a 31% loss of the protein-bound NANA. The work of Quamina & Bogoch (1965) suggests that extensive treatment with aqueous solvents of low ionic strength will liberate appre-

ciable amounts of glycoproteins. The detergent, Triton X-100, solubilizes about 70% of the protein-bound NANA and only 30% of the gangliosides (Brunngraber & Bejnarowicz, 1965).

DISCUSSION

The nerve endings and associated membranes are largely present in the P_2B fraction isolated from the crude mitochondrial pellet from brain (Whittaker, 1959). This fraction also contains most of the gangliosides, sialomucopolysaccharides and other NANA- and hexosamine-containing sub-

Table 5. *Distribution of ganglioside NANA and protein-bound NANA in subcellular fractions from a crude mitochondrial preparation from rat whole brain after exposure to osmotic 'shock'*

The crude mitochondrial fraction was subjected to osmotic 'shock' by successive extractions with 0.02M-tris-chloride buffer, pH 8.0, and water. After centrifugation, the pellet was suspended in 0.32M-sucrose and layered over a density gradient consisting of 0.8M- and 1.2M-sucrose. High-speed centrifugation yielded subfractions P_2A'' , P_2B'' and P_2C'' . The results relate to one brain. Values in parentheses in columns 3 and 4 are the amounts of NANA expressed as percentages of the total NANA of each class of compound.

Expt. no.	Subcellular fraction	Protein (mg.)	Ganglioside NANA (μ g.)	Protein-bound NANA (μ g.)	Ganglioside NANA (μ g./mg. of protein)	Protein-bound NANA (μ g./mg. of protein)
1	P_2A''	11.0	110 (34)	91 (57)	10.0	8.3
	P_2B''	10.0	193 (60)	55 (35)	19.3	5.5
	P_2C''	13.4	18 (6)	12 (8)	1.3	0.9
2	P_2A''	11.5	182 (74)	92 (67)	15.8	8.0
	P_2B''	10.1	54 (22)	38 (26)	5.3	3.7
	P_2C''	11.0	9 (4)	10 (7)	0.8	0.9

stances present in the original crude mitochondrial pellet from which it is derived. The concentration of these substances, when expressed in terms of μ g. of NANA or hexosamine/mg. of protein, is also higher. The concentration of all of these substances is also greater in the microsomal fraction than it is in the mitochondrial fraction. Moreover, they are predominantly present in the less dense particles of the crude microsomal pellet. These particles are believed to consist of fragments of nerve-cell membranes (Wherrett & McIlwain, 1962; Toschi, 1959; Hanzon & Toschi, 1959, 1960).

The present work suggests that the mitochondria (P_2C), RNA granules (P_3C) and lysosomes (P_2C) do not contain appreciable amounts of NANA or hexosamine. The lysosomes have been demonstrated to be present in the most dense fractions of the crude mitochondrial preparation (Koenig, Gaines, McDonald, Gray & Scott, 1964). The nuclear pellet is relatively poor in these substances (Brunngraber & Brown, 1964), and recent experiments in this Laboratory have indicated that most of the NANA and hexosamine that appear in this preparation are present in the less dense fractions and not in the more dense, nuclei-enriched, fractions. The myelin fraction (P_2A) contains appreciable amounts of hexosamine and NANA, but purified myelin from ox-brain white matter (Autilio, Norton & Terry, 1964) contains less than 5% of proteins that are insoluble in chloroform-methanol (2:1, v/v).

Hess (1953) reported a positive periodic acid-Schiff reaction at the periphery of nerve cells that was attributed to the presence of mucopolysaccharides in the intercellular cement substance. In view of the low volume of extracellular space in brain, this positive reaction may be a reflection of the nerve-cell membrane itself.

Aboud, Kurahasi, Brunngraber & Koketsu (1966) have isolated sialomucopolysaccharides from frog sartorius-muscle membrane preparations by procedures identical with the ones used here. In the above paper, it was erroneously reported that the sialomucopolysaccharides of the preparation are precipitated by cetylpyridinium chloride. The sialomucopolysaccharides from muscle membrane are in fact similar to those of rat brain in that they are not precipitated by this reagent.

The cell membrane of the Ehrlich ascites-carcinoma cell (Cook, Laico & Eylar, 1965) and the red blood cell (Cook & Eylar, 1965) also appear to contain non-ganglioside, membrane-bound NANA and hexosamine; thus the protein-bound and hexosamine-containing constituents may have a membrane function as well as the gangliosides (McIlwain, 1963).

The present work has provided evidence that the protein-bound NANA/gangliosidic NANA ratio increases with particle density. This is not entirely unexpected, since it is known that this ratio varies considerably in different parts of the brain (Papadopoulos, 1960). There is no reason to believe that the entire neuronal membrane is of the same composition. One might expect chemical differences in membranes that are involved in the processes associated with the inhibitory post-synaptic potential, excitatory post-synaptic potential and the action potential (Eccles, 1964). The physiological attributes of differing regions of the nerve-cell membrane, such as the axon, axon hillock, perikaryon, pre-synaptic and post-synaptic membranes, suggest differences in chemical structure. Further differences may be associated with the glial membranes, which must be present in the subcellular particulates obtained after homogenizing nerve tissue.

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