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STUDIES ON THE MUCOLIPIDS AND THE CEREBROSIDES OF CHICKEN BRAIN DURING EMBRYONIC DEVELOPMENT*

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SUMMARY

The formation of mucolipids, cerebrosides, and other brain constituents has been examined in the developing chick embryo. Mucolipids were isolated from adult chicken brain and a corresponding fraction from 10 developmental stages (starting at 4.5 days) of the chick-embryo brain. These fractions are compared with regard to amount per cell, composition, and several physical properties. Included in the discussion are the relationship between mucolipid formation and cerebroside formation, the presence of "pre-myelin" cerebrosides, the unusual composition of the hyperphasic fractions of the early stages, and the limitations of a chemical inventory in growing and differentiating tissue.

INTRODUCTION

The most easily grasped, though only partially relevant, distinction between organs exerting specific functions is their chemical composition. The high content in lipids of brain and the nervous system—some of these occurring almost exclusively in these tissues—has rightly occupied the attention of biochemists for a long time; there can be little doubt that, when the correlation between chemical composition and physiological function is better understood, it will be possible to assign definite roles to many of the molecular species found among the brain lipids.

This laboratory has for some time been interested in a class of complex lipids, soluble in water and in organic solvents, for which the designation of mucolipids has been proposed[1]. This group, for different representatives of which various names, such as gangliosides, strandin, etc., have been used, combines constituents characteristic of both the cerebrosides and the mucoids. We may refer to a previous discussion of this problem[2]. The most striking mucolipid constituent, which together with the sugar components renders these lipids soluble in water, is, perhaps, sialic acid (*N*-acylneura-

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minic acid). Whereas our knowledge of the sialic acids is making satisfactory progress (compare ref. 3 for a recent review), many uncertainties still persist with respect to size, structure, homogeneity, and composition of the mucolipids themselves.

The remarkable solubility properties of the mucolipids make it likely that they may act as mediators at oil-water interfaces in facilitating transfers from one phase to the other; and we have previously referred to some interesting charge properties of these complex lipids[2]. The present study is, however, concerned with a different level of interest, namely, with the appearance of mucolipids in developing embryonic brain, principally in comparison with their monomeric relatives, the cerebrosides. In the extraction procedure used here, *viz.*, the method of partition dialysis developed by FOLCH *et al.*[4], the extract is separated into three phases: (a) *hypophasic* chloroform, containing the hydrophobic lipids; (b) *interphasic* proteolipids; (c) *hyperphasic* water, containing the mucolipids. In the present study of the lipids of developing chick-embryo brain, the content of hyperphasic sialic acid is taken as an indication of the presence of mucolipids, being compared with the accumulation of hypophasic cerebrosides. We shall also have occasion to mention the abnormal composition of the hyperphasic material in the early developmental stages and the appearance of pre-myelin cerebrosides. A very brief report on some of the findings has appeared[5].

MATERIALS AND METHODS

Materials

Fertilized hen's eggs of a White Leghorn strain* were incubated in a humidified atmosphere at 38°. Pooled brain specimens corresponding to a total of ten developmental stages were examined, extending from Stage I (4.5–5-day embryo) to Stage 10 (two days after hatching). The stage of development of each embryo was determined by morphological criteria[6]. The whole brain of the chick embryo was taken in each case, the spinal cord being severed just below the myelencephalon. For the 5-day embryo, in which the brain accounts for about one-third of total body weight, only an approximation could be used: the embryo was decapitated, the eyes and beak were removed, and the remainder was taken as brain. Adult-chicken heads of various strains were obtained at local markets immediately after decapitation and kept in ice for several hours before the brains were processed.

Removal of organs

The embryos were removed from the egg and freed from embryonic membranes and external fluid. The brains were dissected from the embryos, freed from adhering liquid on a glass plate and immediately placed in a covered flask kept at the temperature of a dry ice–acetone mixture. The hearts were similarly separated from the remainder of the embryos.

Hyperphasic material

The tissue was extracted without delay at 4° for 2 min in a high-speed mixer with a 10-fold amount (v/w) of a pre-cooled mixture of chloroform–methanol (2:1, v/v). The partition dialysis method[2,4] was applied (3 days at 4°) to the filtered extract, procedural details being kept constant for each preparation. The upper, aqueous

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phase then was lyophilized. This material will be referred to as "hyperphasic fraction" or, in the later developmental stages and in adult brain, as "crude mucolipid"

Hypophasic lipids

The chloroform phase recovered after dialysis was taken to dryness under nitrogen and the residue weighed as a measure of total hydrophobic lipids. The lipids were taken up again in chloroform and samples were taken for phosphorus and cerebroside determinations. Lipid phosphorus was determined colorimetrically[7]. Cerebrosides were isolated from the lipid mixture by column chromatography and determined by a modified anthrone reagent[8]. This method is much more specific than cerebroside determinations based on differences in reducing value of a total extract before and after acid hydrolysis; it gave quantitative recovery of a sample of phrenosine.

Estimation of cell number

The extracted tissue was dried, pulverized with mortar and pestle, and 20-mg samples were taken for the determination of deoxyribonucleic acid[9,10]. The DNA content was checked by the Dische method[11]. Cell number was calculated from the published value of DNA phosphorus per fowl nucleus[12].

Determination of mucolipid components

Sialic acid was determined routinely by the direct Ehrlich reaction[13]; the results were checked frequently by Bial's orcinol reaction[13], the diphenylamine reaction[13], and the method using 2-thiobarbituric acid[14]. As a standard for these procedures, sialic acid was prepared from ox-brain mucolipid by mild acid hydrolysis (pH 1.2 at 100° for 4 h in a sealed tube) and elution from a column of Dowex-50 resin with an increasing gradient of formic acid[15]. The crystalline sialic acid obtained was *N*-acetylneuraminic acid containing trace amounts of *N*-glycolylneuraminic acid.

Mucolipid hexose was estimated by means of anthrone[16], free hexosamine (after hydrolysis) by a modification of the Elson–Morgan procedure[17].

Sphingosine and fatty acids were usually determined on the same methanolysate. Samples of mucolipid (about 5 mg) were heated at 100° for 6 h in a sealed tube with 2 ml of 10% methanolic H_2SO_4 or with dry methanolic HCl (3 N). Methyl esters of fatty acids were extracted seven times with an equal volume of low boiling petroleum ether; the extract was washed with water, taken to dryness under nitrogen, and the esters were determined as the hydroxamic acids[2]. The methanol phase was made alkaline with 20% aqueous KOH and extracted exhaustively with diethyl ether. Sphingosine was determined by reaction with fluorodinitrobenzene[18] with the use of authentic sphingosine sulfate as the standard.

Amino acids were determined by the VAN SLYKE manometric method[19]. The conditions selected for amino acid release, after a time study, were 20 h at 110° in 6 N HCl in a sealed tube under nitrogen. The hydrolysates were evaporated to dryness *in vacuo* over NaOH pellets, and the filtered aqueous solutions of the residues diluted to volume. The pH was adjusted to 2.5 with citrate powder and the dissolved CO_2 driven off by boiling before the amino acids were made to react with ninhydrin. Individual amino acids were determined as the dinitrophenyl derivatives[20,21].

An appreciable part of the hyperphasic material was of unknown composition. In a search for the presence in these fractions of compounds other than those found

in purified preparations, analyses were performed for total phosphorus[7], nitrogen[22] and sulfur[23] content. Sulfur was also identified qualitatively by oxidative and reductive tests[24].

Inositol was identified in 42-h hydrolysates (6 N HCl in a sealed tube at 110°) of mucolipid by paper chromatography using isopropanol–acetic acid–water (3:1:1, v/v/v), with the spots visualized by a modified silver reagent[25,26]. Quantitative estimation of inositol was carried out through periodate reduction[27] on extracts of paper chromatograms obtained with the same solvent system.

RESULTS

Specimens of developing chick embryo

The development of the whole embryo (Table I), and of the chick-embryo brain, was found to be in approximate agreement at each stage during the 3-week incubation time with the results of many investigators of this classical material[28]. The present investigation is, perhaps, more reliable with regard to dating as it is based not merely on incubation time or embryo weight, but on the morphological changes described in the HAMBURGER–HAMILTON normal stages[8]. The earliest stage of the embryo selected for investigation was HAMBURGER–HAMILTON Stage 26, which is described as 4.5–5 days of incubation. Reference ordinarily will be made to the age of the embryo, which is most conveniently given as incubation time in days. The average hatching time was 20.5 days.

TABLE I

SPECIMENS OF DEVELOPING CHICK EMBRYO*

Stage	*H–H stage*	*Age (incubation time in days)*	*Number of individuals pooled*	*Fresh weight per embryo (g)*
I-1	26	4.5–5	36	0.123
I-2	26	4.5–5	212	0.131
II	34	8	72	1.02
III-1	35	8.5–9	198	1.52
III-2	35	8.5–9	201	1.48
IV	36	10	36	2.32
V-1	39	13	23	6.16
V-2	39	13	35	7.28
VI	41	15	24	13.7
VII	44	18	11	24.8
VIII	46	20	36	41.7
IX	46	20.5	10	37.8
X		22.5	9	34.7
Adult (1)			51	
Adult (2)			33	
Adult (3)			61	

* The stages distinguished here are indicated by roman numerals followed by arabic numerals when separate preparations of the same stage were used. H–H Stage, HAMBURGER–HAMILTON normal stage. Stage V-2 is more properly dated as 13.4 days.

Chick-embryo brain

Enough brain tissue was used to isolate 10–100 mg of the hyperphasic material at each stage (Table II). A progressive, but irregular, desiccation of the chick-embryo brain during development was observed. As can be seen in Table II, the dry weight

amounts to 6.4 % of the wet wt. in Stage II, but to 13.6 % in Stage IX. Duplicate preparations were made at Stages I, III, and V. Adult crude mucolipid (1.35 g total) was obtained from three separate preparations.

TABLE II

COMPOSITION OF EMBRYONIC BRAIN*

Amounts per organ.

Stage	*Age (days)*	*Wet weight (mg)*	*Dry weight (mg)*	*Total lipids in chloroform phase (mg)*	*Inter-phase (μg)*	*Lipid phosphorus (μg)*	*Cerebrosides (mg)*	*Crude mucolipid (mg)*	*Hyperphasic sialic acid (μg)*
I-1	5	38.6	2.03	0.58	8.3		0.031	0.016	0.65
I-2	5	48.5	2.00	0.48	10.4	11.7	0.045	0.033	1.32
II	8	136.8	8.72	2.31		62	0.132	0.189	16.28
III-2	9	246	15.59	3.78	100			0.316	22.5
IV	10	167.2	15.83	3.92	114	108	0.259	0.247	34.6
V-1	13	339	24.2	8.57		231	0.615	0.627	89.6
V-2	13	357	29.0	9.19	143	251	0.665	0.661	72.6
VI	15	557	55.3	12.9		352	1.032	1.125	165.5
VII	18	794	79.8	25.2		373	1.640	2.045	393.5
VIII	20	860	119.0	37.9	750	980	2.690	3.59	550
IX	20.5	803	109.4	34.5	520	933	3.84	1.86	417
X	22.5	814	135.0	40.1		1057	4.18	2.59	533

* Compare Table I for the description of stages.

The hyperphasic material from the early embryo brains is far from homogeneous, and it is not identical, as will be shown, with the corresponding material from the adult organ. It is, however, a fraction of unusual solubility properties. Extracted from the wet tissue along with the other lipids by chloroform–methanol, it passes into the water phase from which it is not dialyzable. The weight of the total hyperphasic material recovered at each stage is relatively constant in different experiments; and it increases in amount as the chick-embryo brain develops.

A hyperphasic fraction corresponding to crude mucolipid was isolated from one dozen unfertilized hen's eggs. 100 g of wet yolk tissue yielded 16.8 mg of hyperphasic material, containing 2 % chromogen; 100 g of wet egg white yielded less than 5 mg of hyperphasic material, containing about 1% chromogen. The chromogen found in the yolk is less than 1 % of the sialic acid found in an equal weight of adult-chicken brain.

The corresponding hyperphasic fractions of chick-embryo heart and chick-embryo carcass, in parallel experiments at each stage, showed no orderly increase in chromogen content comparable to the brain material.

Composition of the hyperphasic material

The components of the hyperphasic fractions that were determined quantitatively in the various stages are listed in Table III. Only in the later developmental stages and in adult brain can they be said to comprise the bulk of the material found in the aqueous phase after partition dialysis. For ease of comparison, we have also tabulated the principal components in form of integral molar ratios (Table IV). It will be seen that the hyperphasic material at term resembles in composition that of the adult organ, whereas the early stages are quite dissimilar.

The ordinary components of mucolipids (sialic acid, hexose, hexosamine, fatty acid, and sphingosine) account for the following approximate percentages of the hyperphasic material: 5-day, 30%; 13-day, 40%; 18-day, 55%, 22.5-day, 70%; adult, 70%. Further information had to be gained about the remaining components, especially in the early stages.

TABLE III

COMPOSITION OF HYPERPHASIC FRACTIONS*

Stage	I	II	III	IV	V	VI	VII	VIII	IX	X	*Adult*
Age (days)	*5*	*8*	*9*	*10*	*13*	*15*	*18*	*20*	*20.5*	*22.5*	*Adult*
Sialic acid	4	8.6	7.1	14	14.3	15	19	15.5	22	21	15
Hexosamine	3.1	2.5		2.3	3.3	3.0	3.5	2.9	3.2	3.2	3.0
Hexose	12	8	7	13	12	13	14	14	19	18.5	19.5
Sphingosine	3	3		3	4	3	5	6	8	9	8
Fatty acids**	6	4		6	7	6	9	12	14	17	15
Amino acids			40					26			17
Nitrogen	3.9		5.4		3.3		2.8			5.6	3.2
Phosphorus	1.8		1.6		0.8	0.8	0.7	0.5	0.6	0.8	0.7
Sulfur			1.4					0.8			0.7

* The components are given as percent of total crude mucolipid.
** Calculated as stearic acid.

TABLE IV

INTEGRAL MOLAR RATIOS OF SOME COMPONENTS OF THE HYPERPHASIC FRACTIONS

Stage	I	II	III	IV	V	VI	VII	VIII	IX	X	*Adult*	*Adult**
Age (days)	*5*	*8*	*9*	*10*	*13*	*15*	*18*	*20*	*20.5*	*22.5*	*(crude)*	*(purified)*
Sialic acid	1	2	2	3	3	3	4	4	5	5	4	7
Hexosamine	1	1		1	1	1	1	1	1	1	1	2
Hexose	5	4	3	5	5	5	5	5	7	7	7	11
Sphingosine	1	1		1	1	1	1	1	2	2	2	4
Fatty acid	1	1		2	2	2	2	3	3	4	4	4
Amino acid			20					15			10	1.6

* This preparation was purified, by a modification of previously reported procedures, to a degree corresponding to purification stage IV (ref. 2).

The amino acid content of the hyperphasic material was found to be: adult, 1.4 μmoles/mg; 20-day, 2.1 μmoles/mg; and 9-day, 3.2 μmoles/mg. Hence, amino acids in some non-dialyzable form are present in greatly increased amounts in the hyperphasic fraction of the early embryo brain.

It can be calculated that amino acids comprise about 40% of the weight of the hyperphasic material from the 9-day brain. The distribution of the individual amino acids in the hyperphasic material of the 9-day chick brain differs considerably from that of the adult-chicken brain. A comparison of two extreme cases, namely, the amino acid distribution in a highly purified specimen of mucolipid from adult-chicken brain and in the total hyperphasic material of the 9-day chick brain, appears in Table V.

The determination of total hyperphasic nitrogen (Table III) failed to show any overall trend. Calculated from the integral molar ratios, almost all the mucolipid

nitrogen in the hatching and adult stages can be related to identified components, but in the 9-day chick there are 6 g atoms of hyperphasic nitrogen per mole of hexosamine which are present in yet unidentified components. The total nitrogen content of purified ox brain mucolipid previously reported[2] was 2.7% (calculated) and 3.8% (Kjeldahl).

TABLE V

AMINO ACIDS IDENTIFIED IN LIPID PREPARATIONS FROM CHICKEN BRAIN

Amino acid	*Hyperphasic fraction from 9-day chick embryo**		*Purified mucolipid from adult-chicken brain***	
	Absolute amounts (as mμmoles/mg)	*Relative amounts (as percent of total amino acid residues)*	*Absolute amounts (as mμmoles/mg)*	*Relative amounts (as percent of total amino acid)*
Glycine	465	15	19	9
Alanine	133	4	26	12
Valine	199	6	13	6
Leucine	232	7	21	10
Threonine	166	5	13	6
Serine	133	4	17	8
Glutamic acid	647	20	26	12
Aspartic acid	232	7	17	8
Lysine	66	2	13	6
Arginine	66	2	8	4
Histidine	166	5	4	2
Phenylalanine	149	5	8	4
Tyrosine	16	1	2	1
Proline***	530	17	23	11
Total	3200		210	

* Stage III (Table III).
** As in Table IV.
*** Compare the reservations concerning the occurrence of this amino acid made in a previous paper[2].

Inositol-containing lipids were considered likely components of the hyperphasic fraction. Paper chromatography revealed traces of inositol in hydrolysates of the hyperphasic material from both the adult chicken and the 8-day embryo. By quantitative estimation, the concentration of inositol was, however, found to be lower in the embryo material (approx. 0.5%) than in the adult (approx. 1%). In neither case can the contribution of inositides have been important.

The hyperphasic fraction from the early embryo contained about twice the quantity of phosphorus and sulfur than that of the hatching chick (Table III). The phosphorus content dropped rather sharply between the 9th and 13th days of incubation. Insufficient material precluded a detailed study of the nature of the sulfur compounds in the hyperphasic material. Only traces of sulfur amino acids were found in any of the hyperphasic preparations. It may also be noted that there is not enough fatty acid (especially in the early stages) to allow for considerable amounts of phosphatides or sulfatides.

These studies of the composition of hyperphasic fractions account for about 70% of the material of the earlier stages and for about 90% of the later stage and adult material. It is, in any event, clear that the unusual components of the early-stage

hyperphasic material comprise increased quantities of polypeptides and of compounds containing phosphorus, sulfur, and some unidentified form of nitrogen.

Redistribution, solubility, electrophoretic behavior

The difference in composition of the hyperphasic fractions is reflected also in redistribution studies. Crude mucolipid from adult-chicken brains, when treated with chloroform–methanol (2:1, v/v) containing 10% $CaCl_2 \cdot 2H_2O$ and then dialyzed[2], can be considered as enhanced in purity, if sialic acid content is used as a criterion. That is, 50% of the starting material, but 90% of the sialic acid are found in the aqueous phase after dialysis. When this procedure was, however, applied to a sample (65 mg) of 9-day hyperphasic material, only 1% of the sialic acid was found to have gone into the aqueous phase. In fact, most of the hyperphasic material did not dissolve in the $CaCl_2$–chloroform–methanol mixture. The insoluble residue, suspended in water and dialyzed to remove the salt, yielded 60 mg and contained about 90% of the original sialic acid.

Also in their solubility in acetate buffer (pH 4.7), there was a considerable difference between the samples of hyperphasic material from early and late embryonic stages. About one-fifth of its weight and the same proportion of its sialic acid content of the material from Stage III (9-day embryo) were insoluble; virtually all of the corresponding fraction from adult brain remained in solution.

The electrophoresis of solutions in acetate buffer (pH 4.7, $I = 0.15$) and in veronal buffer (pH 8.7, $I = 0.15$) showed only one peak with respective mobilities of 7.7 and $9.7 \cdot 10^{-5}$ in the case of the preparation from the adult organ, although the fraction still was, by other criteria, quite inhomogeneous. The electrophoresis did, however, result in some purification: the sialic acid content of the starting material was 15.7%; this value had risen to 22.9% after electrophoresis at pH 4.7 and 8.7.

Hydrophobic lipids of chick-embryo brain

The total lipids (Table II) extracted from the tissue included the hydrophobic lipids of the chloroform phase, the "proteolipids" of the interphase, and the hyperphasic material. Cold chloroform–methanol is an efficient solvent mixture for the extraction of embryonic lipids. Exhaustive re-extraction of the tissue residues in a Soxhlet apparatus with petroleum ether and diethyl ether yielded less than 0.2% additional lipid material.

The lipid phosphorus in Table II is hydrophobic lipid phosphorus, determined on samples of the chloroform phase. It is a measure of total phosphatides, sphingomyelin, and phosphoinositides. The values reported here are generally intermediate between two sets of values reported previously[12,29]. The amount of phosphorus in the hyperphasic lipid material adds an average of 3% to the values of hydrophobic lipid phosphorus.

Cerebrosides were determined after separation from the other lipids of the chloroform phase. The values (Table II) are lower than those reported elsewhere[29] with the use of less specific analytical methods. There is, in any event, definite evidence for the presence of cerebrosides at the earliest stage (4.5 days).

Comparison of mucolipid formation and cerebroside formation

The determination of hyperphasic non-dialyzable sialic acid is probably a more refined measure of mucolipids than is the weight of the hyperphasic fraction. Some of the necessary reservations will be discussed later. Fig. 1 compares the accumulation of

cerebrosides in the developing chick brain with the accumulation of hyperphasic sialic acid. From such a comparison it appears that a sharp increase in the mucolipids of brain precedes the notable rise in cerebroside formation which accompanies hatching. The picture which these data give is a rather gross one. It is complicated, among other things, by the partial dehydration which occurs during the development of the brain (Table II). Another basis of comparison is, however, available, namely, the calculation of the average amounts per cell. The DNA content per cell nucleus may be taken to be a constant[30]; for this particular species it has been determined as 0.235 pg of DNA phosphorus per nucleus[12]. In each stage of the developing chick and in the adult, the dry lipid-free brain tissue remaining after the chloroform–methanol extraction was examined for DNA content. The number of cells ($\times 10^7$) per organ at each stage (age in days given in parentheses) was: 2.2 (4.5 days), 8.0 (8 days), 10.0 (10 days), 13.2 (13 days), 20.8 (15 days), 27.7 (18 days), 33.4 (20 days), 34.6 (22.5 days), and 73.3 (adult). The variation in average cell mass is illustrated in Fig. 2. Once again, the comparison of hyperphasic sialic acid with hypophasic cerebrosides, expressed as average amounts per cell (Fig. 3), indicates that mucolipid formation precedes cerebroside formation. Sphingosine, one of the constituents common to mucolipids and to cerebrosides, may be considered as another indicator of the presence of mucolipids. Fig. 4 compares hyper- and hypophasic sphingosine per cell, with the values expressed as percent of the amount of sphingosine per cell in the corresponding fractions

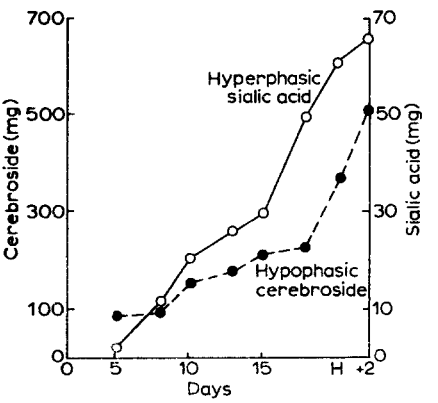

Fig. 1. Changes in the concentration of hyperphasic sialic acid (right ordinate) and hypophasic cerebroside (left ordinate) in chick-embryo brain during development. The figures refer to milligrams of component per 100 g of wet wt. The abscissa indicates the age of the embryos, with H denoting the time of hatching.

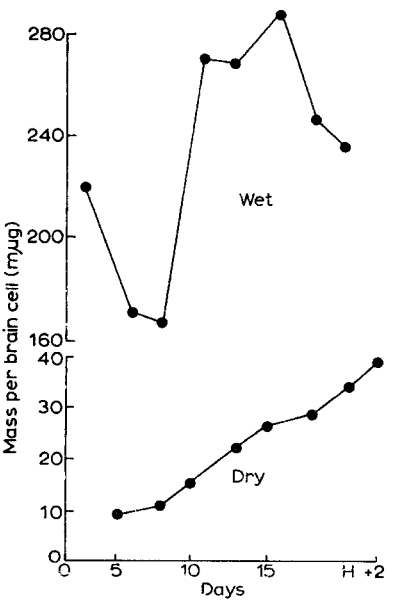

Fig. 2. Changes in the average mass per cell in chick-embryo brain during development. The figures refer to mμg per brain cell. Pooled fresh tissue is the basis for the wet weight, dry weight being the sum of the solid residues of the extraction and partition procedure. The number of cells was calculated from a determination of the DNA phosphorus in samples of the tissue residue after extraction of the lipids. The abscissa is explained in Fig. 1.

of adult-chicken brain. The sphingosine content of adult-chicken brain is 21.4 pg per cell in cerebrosides and 1.0 pg per cell in mucolipids. It must be admitted that data recorded as average amounts per cell are based on an abstraction, namely, the average cell. There is, of course, no justification for the assumption that all cells do contain the component in question, nor that those that do and do not multiply at the same rate. The expedient has, however, the advantage of rendering the findings independent of the fluctuations of water and other cell constituents.

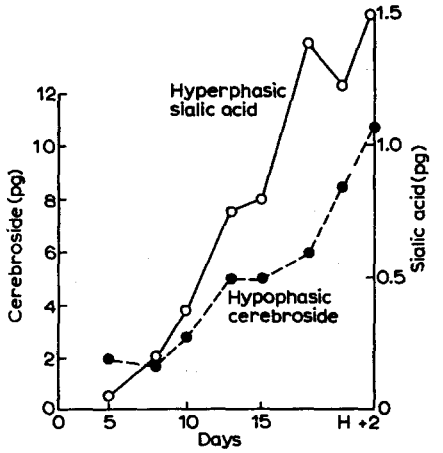

Fig. 3. Changes in the concentration of hyperphasic sialic acid (right ordinate) and hypophasic cerebroside (left ordinate) in chick-embryo brain during development. The figures refer to $\mu\mu$g of compound per cell. The abscissa is explained in Fig. 1.

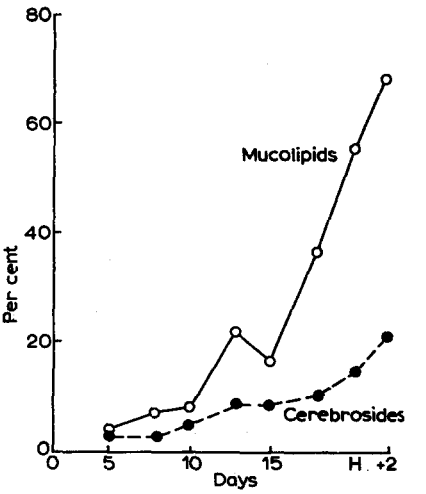

Fig. 4. Changes in the concentration of hyperphasic and hypophasic sphingosine in chick-embryo brain during development. Hyperphasic sphingosine is from the crude mucolipid fraction, hypophasic sphingosine from isolated cerebrosides. The figures are expressed as percent of the corresponding levels in adult brain. For the abscissa, compare Fig. 1.

DISCUSSION

General remarks

The attempt to establish a chemical inventory of the cell in molecular terms meets with several difficulties; difficulties that are compounded when the cell is not regarded as a static entity, as probably no cell is, but as undergoing innumerable continuous changes in the quality and quantity of its components: changes that are presumably orderly, but cannot be synchronous with regard to different molecular species. An even higher level of uncertainty is reached when an entire organ is to be considered, especially, one in which the processes of differentiation are superimposed on those of growth. In trying to reflect on the biochemistry of embryonic development, we may come to the conclusion that even our power of formulating the problems is still in an early embryonic stage. It must be admitted that in many studies on the microbial cell some of these difficulties appear to be removed by being disregarded.

As concerns a developing organ, such as the embryonic brain of the chicken under investigation here, it would be perfectly feasible, though probably not particularly relevant, to answer the question of the rate of appearance of a simple monomer in the differentiating and growing tissue. For instance, the total amount and the rate of accumulation of stearic acid at different stages of development could easily be

ascertained, within the limits of the analytical methods available. Less easy, and already open to much controversial interpretation, would be the attempt to follow stearic acid through its various modes of attachment: glyceride, phosphatidic acid, phosphatide, and more complex forms of lipid or protein derivatives. If such data could be established for every known monomeric component of the tissue and the information fed into a suitably programmed computer, grids of possible combinations or conjugations would emerge that would probably be not far from the truth, though it could be argued that the application of a currently fashionable principle, namely, that what is possible is probable, will not contribute much to a real understanding of the mechanisms of differentiation.

Even less susceptible of an entirely satisfactory solution is the problem that we have set ourselves here, namely, the comparison of two complex lipid derivatives, the cerebrosides and the mucolipids, as they appear at different embryonic stages. The interest is obvious: the lipids are among the foremost plastic constituents of the nerve cell and are presumably intimately connected with its specific functions. The formulation of the concept of a molecule becomes, however, unexpectedly difficult in developmental biochemistry. A cerebroside is, by definition, composed of a fatty acid, a long-chain amino alcohol of the type of sphingosine, and a hexose, usually galactose. To these elements of a cerebroside are added, in a mucolipid, varying proportions of another hexose, an amino sugar, sialic acid, and perhaps other minor constituents. Applying the molecular concept of organic chemistry, we shall rightly conclude that we are dealing with a different molecule if, in a cerebroside, one fatty acid is replaced by another or if the sugar is glucose instead of galactose. But this kind of information is, perhaps, not useful to the biologist, since other properties, —physical, coordinative, etc.—often may carry a greater functional weight. It is, indeed, the parameter which we choose that preordains the answer which we may expect. An indeterminacy besets investigations of this type: we can determine the quantity of a class or the quality of a molecule, but only rarely both in the same observational framework.

The determination of the quantity of a class is usually based on the estimation of a characteristic functional group or of an outstanding constituent, and we have made use, in the present study, of the estimation of both hydrophobic and hydrophilic—or, betters, hypophasic and hyperphasic—sphingosine and of hyperphasic sialic acid. We prefer these non-committal terms, since it is quite clear that the material extracted with chloroform–methanol from the early stages of brain development and then transferred to the aqueous phase is very far from the mucolipids encountered at the late embryonic stages and in the adult organ. To this reservation another one must be added, relating to the comparison between hypo- and hyperphasic sphingosine. As has been shown recently[31], the long-chain aminodiol fraction of the mucolipids of ox brain consists of roughly equal parts of two compounds, sphingosine and its C_{20} homologue, cosisphingosine. Whether this is also true of fowl brain, is not yet known.

Lipids in developing brain; pre-myelin cerebrosides

Early studies on the developing rat brain[32] distinguished three periods: (a) pre-myelin, in the first 10 days after birth, with a high level of water, a low level of lipids; (b) active myelination, from 10 to 40 days, with a gradual loss of water and the deposition of large amounts of lipids; (c) transition to the adult organ, with a steadily diminishing rate of change. Similar observations were later made in mice[33].

In the chick, the corresponding changes must in part take place during the development of the embryo, though the pre-myelin period is generally considered to extend through the major part of the embryonic life. Demonstrable myelin is first encountered not before the 12th or 14th day in spinal cord, ganglia or nerves[34-36]. In the present studies, the rapid loss of tissue water between the 5th and 10th day can be seen in Table II and Fig. 2. A period of active cerebroside formation, and presumably of myelination, which is accompanied by desiccation, begins at least around the 18th day and continues through hatching (Fig. 1, Table II). It is only after the last stage studied here (two days after hatching) that the transition period initiating a lower rate of synthesis may be assumed to begin.

It has, in general, been assumed that cerebrosides do not occur in foetal brain. They have not been found in the brain of newborn mice[33,37] and are thought to be absent before the appearance of stainable myelin[38]. It is, however, difficult to decide this question through rather indirect methods often based on unspecific reducing values. The present study, in which the cerebroside fraction was isolated chromatographically, has extended to less mature organs than had been examined previously. Though the highest rate of cerebroside accumulation does coincide with the time of most rapid myelin formation (18 days through hatching), cerebrosides can, nevertheless, be demonstrated in chick embryo brain as early as 4.5 days after the egg is laid. If the amount per cell is taken into account (Fig. 3), it will be seen that the cerebroside level at this early stage has already reached one-fifth of the level recorded at the time of hatching. If the quantity of cerebroside is expressed as milligrams cerebroside per mg DNA phosphorus, the values are: 8.7 at 5 days, 34.4 at 20 days, 51.5 at 22.5 days. When regarded in this manner, the quantity of cerebrosides in the early embryonic brain is not negligible.

It is, in fact, not unlikely that, before a morphological structure such as myelin can be recognized, all its components must accumulate, and become organized, in a state which is no longer a solution and not yet a solid. In the absence of an understanding of the biochemical foundations of biological shapes, the nature of a protomyelin cannot be discussed profitably. But it may be of interest to remember that, as one of us pointed out a long time ago[39], there exists an area in which the biological necessity of such oscillations between solute and solid is brought into the open, namely, the coagulation of blood.

Abnormal composition of the hyperphasic fraction of the early embryonic brain

As has been mentioned before, the earliest stage of the chick-embryo brain at which the extract was studied in detail was the 4.5-day organ. The hyperphasic fraction collected at this stage deviates considerably from what would be expected of a mucolipid, as it consists of roughly 1/5 carbohydrate, 1/8 lipid, 1/2 amino acid, together with unidentified substances containing nitrogen, phosphorus and sulfur (Table III). It is difficult to see how material of this composition could have been soluble in the extracting solvent which consisted of chloroform–methanol–water (20:10:3). Another surprising feature of this fraction is the change in its solubility after dehydration; whereas prolonged contact with chloroform did not render it insoluble in water, lyophilization did. In this respect, the fraction bears some resemblance to the proteolipid found at the interface between chloroform and water in the partition extraction method[40].

Mucolipids and cerebrosides

A noteworthy feature of the present study is the observation that, in general, the rate of formation of mucolipids outranks that of cerebrosides. In view of the recent finding[31] that, in ox brain, the mucolipids are distinct from the cerebrosides in the type of aliphatic base and fatty acid that they contain, thus indicating a certain degree of anabolic autonomy, the metabolic hierarchy as regards these two classes of lipids cannot yet be established.

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