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The Composition of the Glycolipids in Dog Intestine*

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ABSTRACT: Whole lipid extracts of dog small intestine were chromatographed on silicic acid, yielding all the glycolipids in three major fractions. Five classes of glycolipid were isolated in high yield from the fractions by column chromatography with Florisil, DEAE-cellulose, and silicic acid and by thin-layer chromatography. Cerebrosides, ceramide di-, tri-, and pentahexosides, and gangliosides were isolated in amounts of 0.162, 0.098, 0.041, 0.070, and 0.171 μmoles per g of fresh tissue, respectively, and 0.026 μmole-/g of sulfatide was found by direct analysis. A minor glycolipid fraction, estimated at 0.014 μmole/g, contained longer oligosaccharide chains. This fraction was separated from all other lipids and appeared to be a mixture of ceramide hexahexosides containing glucose, galactose, hexosamine, and fucose. These seven classes of lipids accounted for 82% of the original whole lipid hexose, and the remaining 18% was distributed among unresolved side fractions. All the isolated lipids were glycosides of glucosylceramide with long-chain fatty acids. The intestine glycolipids are distinguished from those of several other tissues by the absence of ceramide tetrahexosides and presence of penta- and hexahexosides.

The resolution of the glycolipids of dog small intestine into three silicic acid fractions was described in a previous publication (Vance *et al.*, 1966). The glycolipid mixture from one of these was isolated from all other lipids and partially resolved into at least four classes of glycolipid. Two of these were characterized as a novel ceramide pentaglycoside and a monosialoganglioside[1] This paper describes the other glycolipids of this fraction together with those of the other two silicic acid fractions. An estimate is included of the distribution of the types of glycolipid in intestine based on these isolations.

Experimental Section

Analytical Methods. Methods for the determination of phosphorus, primary amine, long-chain nitrogen, chromic acid uptake, ester groups, sialic acid, hexosamine, glucose, total hexose, and the distribution of fatty acids were described previously (Vance *et al.*, 1966). The fatty acid methyl esters were further purified from sphingosine contaminant by silicic acid chromatography modified from the method of Hirsch and Ahrens (1958). Hydroxy fatty acids were then determined by acetylation of the free acids and hydroxamate formation using a modification of the method of Gutnikov and Schenk (1962). Fucose was determined on glycolipid fractions low in phospholipid by the method of Dische and Shettles (1948) applied to a water suspension of the lipid. Sulfatides were determined by the method of Kean (1968) and reducing sugar by the method of Somogyi (1945).

Preparation and Composition of the Glycolipids. The extraction, purification, and initial resolution of the lipids by preparative silicic acid chromatography were all carried out as described previously. The fractions containing glycolipid were eluted from the column by mixtures of 10, 17, and 50% methanol in chloroform, respectively, and were designated S-10, S-17, and S-50.

Preparation and Resolution of the S-10 Glycolipids This fraction contains two glycolipid classes, ceramide mono- and dihexosides. These were isolated by two-stage column chromatography using Florisil.

Florisil Chromatography. Florisil (450 g; Floridin Co., 60–100 mesh) were placed in a 41-mm i.d. column and washed with 2 l. of chloroform. Fraction S-10 lipids containing 9.8 matoms of lipid K and 0.61 mmole of lipid galactose were placed on the column in 500 ml of chloroform solution. The column was then eluted with 1750 ml of 5%, 3150 ml of 38%, and finally 2100 ml of 60% methanol in chloroform, respectively. The

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[1] In this paper the following terminology is used: "ganglioside" is a glycolipid which contains sialic acid, "ceramide oligohexoside" is a ceramide oligoglycoside which does not contain sialic acid. The composition of all solvent mixtures is expressed on a volume per volume basis. The author is indebted to Miss Martha D. Fendley for valuable technical assistance.

glycolipids were completely recovered from the column. Ninety per cent were in the fraction eluted with 38% methanol in chloroform together with only 3.7% of original phospholipid.

TABLE I: Composition of Dog Intestine S-10 Glycolipids.[a]

	Ceramide Monohexoside	Ceramide Dihexoside
Mass (μg)	842	1250
Total anthrone hexose (μmoles)	0.97	1.76
Glucose (μmoles)	0.97	0.76
Galactosamine (μmoles)	0.0	0.0
Sulfate ester (μmoles)	0.008	0.091
Total nitrogen (μatoms)	1.11	1.10
Chromic acid uptake, chloroform-soluble fraction after hydrolysis (μatoms of oxygen	117	121
Phosphorus (μatoms)	0.00	0.08
Distribution of non-hydroxylated fatty acids (%)		
16:0	12	17
18:0	5	13
18:2	0	5
20:0	20	18
20:4	0	4
22:0	34	16
24:0	29	27

[a] Values given per micromole of long-chain base.

The second stage was carried out in a 17-mm i.d. column containing 58 g of Florisil washed with 330 ml of chloroform. The 38% methanol fraction containing 0.66 mmole of lipid galactose was placed on the column in 100 ml of 18% methanol in chloroform. The column was eluted successively with 500 ml of 18%, 350 ml of 25%, 500 ml of 32%, and 500 ml of 40% methanol in chloroform, respectively. A total of 17 fractions was collected in this elution sequence which gave complete recovery of galactolipid from the column; 54.9% of the galactose was found in the fractions eluted by 18% methanol in chloroform. The fractions in this group were examined by thin-layer chromatography using silica gel G with the solvent system chloroform–methanol–water (75:25:3). Only ceramide monohexoside (R_F 0.65) was observed in these fractions and they were pooled. The composition of this preparation is given in Table I. All of the 25% methanol eluate and the first fraction of the 32% eluate, which comprised 11.2% of the lipid galactose, were mixtures of mono- and dihexosides. The remaining 32 and 40% eluates contained only dihexoside (R_F 0.47) and these comprised 34.0% of the lipid galactose. The latter 32% eluates were essentially free of phospholipid and were pooled for analysis (Table I).

The sequence of sugars in the dihexoside was determined by hydrolysis in 0.3 N HCl in chloroform–methanol (2:1) (Sweeley and Klionsky, 1963). After refluxing 4.75 μmoles for 2.5 hr, the hydrolysate was concentrated and partitioned between equal volumes of water and chloroform and both of these fractions contained anthrone-reacting hexose. Calculated as galactose, there were 6.27 μmoles in the chloroform phase and 4.00 μmoles in the aqueous phase. Aliquots of each fraction were then hydrolyzed by refluxing for 3.5 hr in 2 N aqueous HCl on the sand bath. Analyses for glucose based on glucose oxidase and galactose based on corrected total reducing sugar showed that the glucose was present almost exclusively in the hydrolysate of the original chloroform phase, which contained all the ceramide.

TABLE II: Composition of Di- and Trihexoside Preparations Isolated from the S-17 and S-50 Lipids.[a]

	Dihexoside II S-17	Trihexoside I S-17	Trihexoside II S-17	Trihexoside A S-17	Trihexoside B S-17	Trihexoside S-50
Mass (μg)	1026	1177	1510	2067	1470	1246
Corrected total hexose (μmoles)	1.81	2.60	3.42	2.78	3.12	2.64
Glucose (μmoles)	0.84	0.87	0.74	0.82	0.88	0.99
Hexosamine (μmoles)	0.00	0.01	0.03	0.01	0.00	0.136
Total nitrogen (microatoms)	1.07	1.20	1.50	1.27	1.08	1.30
Chromic acid uptake (μatoms of oxygen)	135	159	183			155
Chromic acid uptake, chloroform-soluble fraction after hydrolysis (μatoms of oxygen)	111	103	128	110	122	124
Total phosphorus (μatoms)	0.03	0.08	0.10	0.04	0.02	0.02
Sulfate ester (μmoles)	0.013	0.05	0.069			0.005

[a] Values given per micromole of long-chain base.

TABLE III: Composition of the S-50 Fraction D Oligohexosides.[a]

	Fraction D Oligohexosides	Hexahexoside D-1	Hexahexoside D-2
Mass (μg)	1550	1686	2120
Total anthrone hexose as galactose (μmoles)	5.50	6.52	7.08
Glucose (μmoles)		0.78	0.77
Fucose (μmoles)		1.41	1.76
Hexosamine (μmoles)	1.21	1.21	1.32
Corrected total hexose (μmoles)		4.79	4.99
Galactose (by difference, μmoles)		2.61	2.46
Total nitrogen (μatoms)	2.40	2.05	2.57
Chromic acid uptake chloroform-soluble after hydrolysis (μatoms of oxygen)	119	121	153
Total phosphorus (μatoms)	0.015		
Sialic acid (maximum μmoles)	0.23	0.03	0.04

[a] All values given in microatoms or micromoles per micromole of long-chain base.

Essentially all of the sugar in the hydrolysate of the original aqueous phase was galactose. The dihexoside is therefore a galactosylglucosylceramide, possibly a lactosylceramide, although the linkage between the hexoses has not been established.

Preparation and Resolution of the S-17 Glycolipids. Florisil (522 g) was placed in a 41-mm i.d. column and washed with 1.5 l. of chloroform. Fraction S-17 lipid containing 9.00 matoms of lipid phosphorus and 0.72 mmole of lipid galactose was placed on the column in 280 ml of 25% methanol in chloroform. This was eluted successively with 700 ml of 38%, 2000 ml of 50%, 3000 ml of 60%, and 1500 ml of 70% methanol in chloroform, respectively. A total of 24 fractions was collected in this elution sequence which gave complete recovery of glycolipid from the column. The individual fractions were examined by paper chromatography using silica gel loaded paper (Whatman Chromedia SG 81) with the solvent system chloroform–methanol–water (75:25:3) and detected with Rhodamine 6G under ultraviolet light. The 38% methanol eluates and the first 500 ml of the 50% methanol eluates contained negligible amounts of glycolipid and were discarded. The remaining 50% methanol eluates and the first 250 ml of the 60% eluate were homogeneous and identical and the R_F corresponded to ceramide dihexoside. These fractions comprising 41.2% of recovered lipid galactose were pooled and dialyzed against distilled water. The composition of this preparation designated "dihexoside II" is given in Table II. The remaining fractions of the 60 and 70% methanol eluates contained two components with R_F values corresponding to dihexoside and trihexoside, the latter being by far the major component in all the fractions. These fractions, comprising 53.5% of recovered lipid galactose, were pooled and subjected to silicic acid chromatography.

A 17-mm i.d. column was packed with 74 g of silicic acid in a chloroform slurry. The column was washed with 200 ml of chloroform and charged with the pooled lipids containing 0.194 mmole of lipid galactose in 10% methanol in chloroform. The column was then eluted with 1100 ml of 15%, followed by 500 ml of 38% methanol in chloroform. A total of 29 fractions were collected in this elution sequence giving complete recovery of lipid galactose. The first four fractions of the 15% methanol eluate contained insignificant amounts of glycolipid and were discarded. The next six fractions were homogeneous and identical by silica gel paper chromatography (R_F 0.57, purple fluorescence) and were combined. The composition of this pool, comprising 26.6% of lipid galactose and designated trihexoside I, is given in Table II. The next seven fractions up to the peak contained the same component of the earlier fractions and two others with lower R_F (0.45 and 0.37 with yellow and purple fluorescence, respectively). The last 18 fractions contained the yellow component with much smaller amounts of the lower R_F material and were therefore pooled. The composition of this pool, comprising 69.4% of lipid galactose and designated trihexoside II, is given in Table II.

Trihexosides A and B (Table II) were also obtained in sequential elution from a comparable silicic acid chromatogram of the S-17 trihexoside fraction.

Preparation and Resolution of the S-50 Glycolipids. This is the most complex of the silicic acid fractions and its resolution was reported previously (Vance *et al.*, 1966). The glycolipids were first isolated from the other lipids of this fraction by a combination of solvent fractionation and Florisil chromatography. The mixture was then resolved by use of DEAE-cellulose chromatography which separated the gangliosides from the oligohexosides. The latter were separated into three components with silicic acid chromatography. The composition of the gangliosides and one of the oligohexosides, a ceramide pentaglycoside, was reported in detail. The nature of the other two is reported here.

One of these comprises the first major peak material, "fraction B," tubes 44–70 (Vance *et al.*, 1966). These fractions appeared homogeneous and identical by thin-layer chromatography and were pooled for analysis to-

gether with those from other identical preparations. This glycolipid was relatively insoluble in water and was not dialyzed. The lipid containing 0.57 mmole of galactose was taken up in 23 ml of hot methanol, leaving a small amount of colored residue. Upon cooling the methanol solution, a white precipitate formed. The supernatant was decanted after centrifuging, concentrated to 3.5 ml, and cooled. A small amount of precipitate was formed and this was combined with the first precipitate. The supernatant contained nitrogenous impurities and about 10% of the original lipid galactose and was discarded. On thin-layer chromatograms the R_F of this precipitate lipid was identical with that of trihexoside B and trihexoside I and slightly below that of trihexoside II. The composition of the lipid, designated S-50 trihexoside, is given in Table II.

The sequence of sugars was determined by hydrolysis of 8.4 μmoles with the chloroform–methanol–hydrochloric acid reagent described above for the dihexoside; 84% of the recovered glucose and only 18% of the galactose was found in the chloroform phase together with all the ceramide. The remaining sugar was found in the original aqueous phase. A total of 25.8 μmoles of hexose was recovered in both fractions. The data suggest a galactosylgalactosylglucosylceramide structure for the trihexoside.

The third oligohexoside fraction consisted of all glycolipid material obtained from the column after elution of the trihexoside and pentahexoside. The pooled lipids of tubes 111–200 inclusive, "fraction D" (Vance *et al.*, 1966), comprised 31.5% of the hexoses of the entire fraction S-50 oligohexoside mixture. The composition of this dialyzed preparation, designated fraction D oligohexoside, is given in Table III.

DEAE-cellulose Chromatography of the Fraction D Oligohexoside. A 17-mm i.d. column was packed with DEAE-cellulose in the acetate form to a column height of 43 cm. The column was then washed with 200 ml of chloroform and fraction D containing 0.803 mmole of lipid galactose placed on the column in 40 ml of 15% methanol in chloroform. The column was then eluted successively with 110 ml of 15%, 200 ml of 24%, 200 ml of 30%, 200 ml of 36%, 200 ml of 40%, 200 ml of 45%, 150 ml of 50%, 200 ml of 60%, and 200 ml of 75% methanol in chloroform, respectively. A total of 30 fractions were taken from this sequence which gave 91.8% recovery of lipid galactose. Two separate glycolipid peaks were obtained. The first peak, eluting with 24% methanol in chloroform, comprised 18.5% of the original lipid galactose and was identified as ceramide pentaglycoside by thin-layer chromatography and by analysis. The lipids eluting in the larger second peak comprised 64.8% of the original lipid galactose. Thin-layer chromatograms indicated that the fractions were all mixtures. The first fractions in the sequence contained the higher R_F lipids. These fractions were then arbitrarily divided at the peak and pooled into an early eluting group and a late eluting group. The two preparations were then subjected to repeated DEAE-cellulose chromatography. No homogeneous fractions were obtained, as judged by thin-layer chromatography. The second DEAE-cellulose column fractions were therefore recombined for analysis. The early eluting group is designated "hexahexoside D-1" and the late eluting group, "hexahexoside D-2." These fractions represent 29.7 and 35.0%, respectively, of total glycolipid galactose in the original S-50 fraction D. The composition of these two fractions is given in Table III.

Analysis of Minor Fractions by Thin-Layer Chromatography. A minor oligoglycoside fraction (S-50-P3) containing 5.1% of intestine lipid galactose was resolved by preparative thin-layer chromatography. The phospholipids were first removed by two-stage column chromatography with Florisil and the glycolipid product was dialyzed. Lipid containing 57.0 μmoles of galactose was streaked on four silica gel G plates and the chromatograms were developed in the system chloroform–methanol–water (65:35:8). The plates were sprayed with 0.0005% Rhodamine 6G and examined by ultraviolet light. Three brightly fluorescent zones were observed and the lipids were eluted from the pooled silica gel of each zone with methanol–chloroform (4:1). The lipids appeared homogeneous by thin-layer chromatography and corresponded to gangliosides (R_F 0.51), ceramide pentaglycoside (R_F 0.57), and ceramide trihexoside (R_F 0.75), respectively. They were analyzed for total anthrone-reacting hexose, sialic acid, galactosamine, glucose, and chromic acid uptake by the ceramide fraction (chloroform-soluble material after hydrolysis). These analyses confirmed the chromatographic identification and purity of the isolated lipids. Ganglioside, ceramide pentaglycoside, and ceramide trihexoside were recovered in amounts corresponding to 20.1, 25.8, and 37.8%, respectively, of total lipid hexose. These three glycolipid classes therefore accounted for 83.7% of total lipid hexose in this fraction.

Results

Ceramide Monohexosides. The analyses reported in Table I are consistent with a glucose cerebroside structure with a predominance of long-chain fatty acids. About half of the fatty acids are hydroxylated but proper standards were not available for analysis of this fraction. Wet combustion values (chromic acid uptake) of the total ceramide portion are consistent with rather long chains in both groups. The value of 117 μatoms of oxygen per micromole of the ceramide compares with 121 μatoms for tetracosanoylsphingosine and 115 μatoms for docosanoylsphingosine. The cerebroside is free of phospholipid and contains only traces of sulfatide although the latter comprises about 10% of lipid hexose in the parent mono- and dihexoside fraction. The total cerebroside in the original S-10 lipid was calculated from the yield of homogeneous cerebroside in the 18% methanol eluate and that mixed with dihexoside in the subsequent fractions. The latter was estimated on the assumption that the cerebrosides contain only glucose and that the galactose present in hydrolysates of the mixture may be used as a measure of ceramide dihexoside. Galactose was estimated by subtracting glucose from the total anthrone-reacting hexose corrected for the glucose content. The molar color relationship used for this calculation is 1 μmole of galactose = 0.70 μmole of glu-

TABLE IV: Distribution of Glycolipid Classes in Dog Intestine.

	μmoles/g of Dry Lipid-Free Tissue (14.6% of fresh tissue)[b]					Total μmoles/g of Fresh Tissue
	S-10	S-17	S-50	S-80	Total	
Cerebroside	1.11	0.0	0.0		1.11	0.162
Sulfatide	0.18				0.18	0.026
Dihexoside	0.43	0.24	0.0		0.67	0.098
Trihexoside	0.0	0.18	0.10		0.28	0.041
Pentahexoside	0.0	0.0	0.48		0.48	0.070
Hexahexoside	0.0	0.0	0.10		0.09	0.014
Ganglioside[a]	0.0	0.0	1.17		1.17	0.171
% of total intestine "galactose" as unidentified glycolipid	0.4	2.4	9.4	5.7	17.9	

[a] Based on sialic acid content; calculated as monosialoganglioside. [b] Glycolipid content was related to the original tissue weight by the lipid phosphorus content of the original whole lipid extract and the silicic acid subfractions. A determined average of 95.3 μatoms of lipid phosphorus/g of dry lipid-free tissue was used for this purpose.

cose. The parent fraction S-10, containing 9.8 matoms of lipid phosphorus, contained 279 μmoles of cerebroside.

Ceramide Dihexosides. The analyses reported in Table I are all consistent with a galactosylglucosylceramide structure with sulfatide and phospholipid contaminant. The latter contributed to the excess mass of the preparation (theoretical molecular weight for lignocerylsphingosine dihexoside = 972) as did its insolubility, which precluded the use of dialysis for removal of mineral and other impurities. The fatty acids were similar in character to those of the cerebroside. The original S-10 lipids contained 109 μmoles of ceramide dihexoside calculated with the monohexoside above. Dihexoside isolated from the S-17 lipids (Table II) is more pure than that isolated from the S-10. The fatty acids are also similar in character to those of the cerebrosides and are about 70% hydroxylated. Ceramide dihexoside (124 μmoles) was isolated from the original S-17 lipids containing 9.00 matoms of lipid phosphorus.

Ceramide Trihexosides. The excessive mass values (molecular weight of lignocerylsphingosine trihexoside = 1134) for preparations IIA,B (Table II) are due largely to inorganic impurities. None of these isolated lipids were dialyzed because of their low water solubility. The phosphorus contaminant must be largely inorganic in all preparations except II since chromic acid uptake by the ceramide portions are in the expected range. Values for corrected total hexose and for total chromic acid oxidation are also high for preparation II, and it has a slightly higher R_F in the thin-layer chromatograms than do other trihexosides. The S-10 and S-17 lipids are devoid of sialic acid and galactosamine although small amounts of galactosamine were present in the S-50 trihexoside. Trihexoside (95.8 μmoles) was isolated from the S-17 lipids containing 9.00 matoms of lipid phosphorus. Other S-17 fractions containing 15.3% of the lipid galactose were not included in the dihexoside and trihexoside recovery because of phospholipid contaminant. Trihexoside (26.6 μmoles) was isolated from the oligohexoside fractions (S-50-P3, -P4, and -P5) obtained from whole intestine lipids containing 25.8 matoms of lipid phosphorus.

Ceramide Hexahexosides Preparations D-1 and D-2 (Table III) contain an average of 6.00 and 6.31 μmoles of hexoses and amino sugars per μmole of ceramide, respectively. They are mixtures of higher ceramide oligohexosides but are essentially free of phospholipid, ganglioside, and ceramide pentahexoside. Assuming that all fraction D hexose other than the 18.5% isolated as ceramide pentahexoside belongs to this group, there were present 25.6 μmoles of hexahexoside from original intestine lipids containing 25.8 matoms of lipid phosphorus.

The distribution of the seven classes of intestine glycolipid is summarized in Table IV. Amounts listed for cerebroside, dihexoside, trihexoside, and pentahexoside were largely obtained from isolated lipid and do not include unresolved side fractions. The value for ganglioside was based on the sialic acid content of fraction S-50, calculated as monosialoganglioside. The sulfatide value was obtained from direct analysis of the ceramide mono- and dihexoside fraction (S-10) after Florisil treatment and is probably minimum. However, other fractions prepared from S-10 and S-17 where low enough in phospholipid to permit analysis for sulfatides contained negligible amounts of this lipid. The estimation of hexahexosides was described above.

These results are based on the fractionation of large pools of tissue taken from many dogs and are subject to minor errors arising from variation in content of those glycolipids present in more than one silicic acid fraction. The content of all the various types of glycolipid is 3.98 μmoles/g of dry lipid-free tissue and this accounts for 82.1% of the total lipid hexose in the original silicic acid subfractions. Assuming a similar distribution in

the remaining 17.9% of unidentified lipid hexose, there would be 4.85 μmoles of glycolipid/g of dry lipid-free tissue and 5.09 μmoles of glycolipid/100 μatoms of phospholipid phosphorus.

Discussion

The isolation and identification of ceramide mono-, di-, and trihexosides in dog small intestine is consistent with the general distribution of these substances in animal tissues (Carter *et al.*, 1965). It is of interest that the ceramide tetrahexoside, "aminoglycolipid" or "globoside," which has been found in a number of human tissues is absent from dog intestine and replaced by the larger pentahexoside and hexahexoside. However, the two latter lipids may be more widely distributed than is apparent at present since their water solubility and chromatographic properties make it probable that they have been discarded in some fractionation procedures. The pentahexoside, for example, is a major constituent of both dog small intestine mucosa and smooth muscle and of dog stomach mucosa (J. M. McKibbin, 1967, unpublished data). A different ceramide pentahexoside containing fucose has recently been isolated from hog intestine by Suzuki *et al.* (1967).

The pattern of distribution of the other glycolipids in dog small intestine is distinct from that of the few tissues that have been analyzed. Molar ratios of either dihexoside or trihexoside to cerebroside are 2–12-fold higher in human kidney (Makita, 1964; Martensson, 1963), spleen (Svennerholm and Svennerholm, 1963), and erythrocytes (Vance and Sweeley, 1967) than in dog small intestine. The molar ratio of dihexoside to cerebroside in human liver (Svennerholm and Svennerholm, 1963) is also much higher than in dog small intestine although the ratios of the latter are very similar to those of human blood serum (Vance and Sweeley, 1967). Perhaps the more significant differences in glycolipid pattern will be seen in the distribution of the glycolipids with longer oligosaccharide chains. The larger molecules offer greater chemical diversity with their greater variety of sugars, long-chain bases, and linkages. Variation in tissue oligoglycosides is becoming apparent in the oligohexosides as reported above, and is well established in the gangliosides, which show striking differences in distribution and types in nervous tissue from a number of animal species (Svennerholm, 1965). Although data are limited it appears that the glycolipids show more organ and species specificity than do the other classes of lipids. This fact coupled with the haptenic activity of several glycolipids suggests that they could play some part in cellular recognition.

The intestine monohexosides appear to be exclusively glucocerebrosides and resemble those of human blood serum, red blood cells, spleen, liver, and the accumulating cerebrosides of Gaucher's disease in this respect, whereas the cerebrosides of human kidney, brain, and spinal cord are predominantly galactocerebrosides (Carter *et al.*, 1965). It is of interest that rat intestine, alone among a number of rat tissues, contains a cerebroside glycosidase which is active for both gluco- and galactocerebrosides (Brady *et al.*, 1965).

The intestine ceramide di- and trihexosides are monoglucosyl lipids and in this respect resemble those isolated from tissues of other species outside the central nervous system (Carter *et al.*, 1965). The structure of the normal ceramide trihexoside of human kidney is *O*-D-galactopyranosyl-(1→4)-*O*-D-galactopyranosyl-(1→4)-*O*-D-glucopyranosyl-(1→1)-ceramide (Makita and Yamakawa, 1964) and is identical with the accumulating ceramide trihexoside of Fabry's disease (Sweeley and Klionsky, 1966). An enzyme catalyzing hydrolysis of the terminal galactosyl linkage of this lipid has been obtained in purified form from rat intestine (Brady *et al.*, 1967a). It is present in normal human small intestine but virtually absent from that of patients with Fabry's disease (Brady *et al.*, 1967b).

The hexahexoside is unique among oligohexosides in the average length of the oligosaccharide chain and in its high fucose content. The fraction contains a glycolipid which has two or more fucose residues, suggesting the possibility of branching in the oligohexoside chain. A ceramide pentahexoside containing one fucose residue was isolated from human adenocarcinoma by Hakomori and Jeanloz (1965).

The glycolipids found in small intestine are a composite of those contributed by the lipoproteins of the mucosa and smooth muscle. It was convenient to use whole intestine extracts for isolation and identification of the glycolipids. Work on the identification and determination of these lipids in the muscle and mucosa and in their cellular subfractions is in progress.

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Phosphorylation of 2-D-Deoxyglucose and Associated Inorganic Phosphate Uptake in Ascites Tumor Cells*

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ABSTRACT: The kinetics of 2-D-deoxyglucose and inorganic orthophosphate consumption, deoxyglucose 6-phosphate accumulation, adenine mononucleotide transformation, and respiration by Ehrlich ascites carcinoma cells incubated in 2.7–5.4 mM phosphate at 23° have been measured over a 3-min period after addition of 2-D-deoxyglucose. The estimate of P:O ratios based on deoxyglucose phosphorylation varies with time, being near 3 in the first 30 sec and declining thereafter. Consistent discrepancies between deoxyglucose phosphorylation and inorganic phosphate disappearance suggests either an unidentified reservoir of "high-energy phosphate" available for adenosine triphosphate regeneration or a release of inorganic phosphate from endogenous phosphate esters during the initial periods of rapid sugar phosphorylation. The latter interpretation is favored as being consistent with Lynen's phosphate cycle concept and other known features of ascites tumor cell metabolism. In these terms, the initial P:O ratio calculated from d(deoxyglucose 6-phosphate)/d*t*, after correction for phosphate derived from the declining adenosine triphosphate level, approaches 3 because the phosphorylation of deoxyglucose almost completely interrupts the preceding phosphorylation of endogenous substrates. As the phosphorylation of endogenous substrates increases again to compete effectively with deoxyglucose phosphorylation, the apparent P:O ratio declines. The very low P:O ratios calculated from inorganic phosphate and oxygen uptake over a 15-min interval are shown to be invalid because of the sharp decline in the rate of deoxyglucose phosphorylation after 3 min.

Recently, Morton and Lardy (1967a–c) described a technique for estimating intracellular oxidative phosphorylation by means of 2-deoxyglucose phosphorylation. The 2-deoxyglucose acted as a trap for the terminal phosphate of ATP and the deoxyglucose 6-phosphate thus formed neither inhibited hexokinase nor underwent further metabolism. A measurement of the inorganic orthophosphate taken up and the oxygen consumed could then be used to estimate the P:O ratio. The technique proved successful with bovine spermatozoa (1967a) and the P:O ratio approached the theoretical value of 3.0 in epididymal spermatozoa which had been treated to render the cell membrane permeable to ATP and protein (1967b). Application of the technique to ascites tumor cells yielded uniformly low P:O ratios (1.0 or less) which could be increased somewhat by addition of fluoride to the incubation medium (1967a). These last results raise some questions about the assumption that the intracellular P:O ratio is near 3 in ascites cells, which assumption has been used in calculation of theoretical rates of ATP synthesis in Ehrlich ascites cells (Coe, 1966a,b; Lee and Coe, 1967). A series of experiments was therefore undertaken to determine whether the low ratios estimated by the method of Morton and Lardy are valid for the ascites tumor cell system.

Experimental Procedures

Tumor Preparation. A hyperdiploid strain of Ehrlich ascites carcinoma cells was grown for 7–11 rays in Swiss white or strong A mice (*cf.* Table I) and was prepared and incubated in a tricine–phosphate buffer as described previously (Ibsen *et al.*, 1958, 1960).

Buffer Composition. Phosphate–Locke solution was modified by replacing the 54 mM phosphate buffer with 50 mM tricine (trishydroxymethylmethylglycine; General Biochemicals, Inc., Chagrin Falls, Ohio) and either 2.7 or 5.4 mM orthophosphate. Sodium chloride concentration was adjusted to make the solution isotonic, and final pH was adjusted to 7.4. The final concentrations of constituents were: tricine, 50 mM; sodium phos-

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