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Glycolipids as Indicators of Tumorigenesis

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Hyperplastic liver nodules and hepatocellular carcinomas were induced in rats by oral administration of the carcinogen N-2-fluorenylacetamide. Neoplastic tissue was compared with control, fetal, neonatal, and precancerous liver tissues. The development of the tumors was slow, such that temporal changes in the biochemical and morphologic development of carcinogenesis could be identified. Ganglioside sialic acid levels were elevated in all but the most poorly differentiated tumors. Experiments to monitor individual enzymes suggested that the alterations in glycolipid composition were a direct effect of alterations in biosynthetic activities. The pattern during tumorigenesis was the inverse of that during normal development. Also, ganglioside patterns showed a progressive simplification from hyperplastic nodules to well-differentiated hepatomas and through two grades of poorly differentiated hepatomas. An increase in the activity of the branchpoint enzyme of ganglioside biosynthesis preceded both a decrease in the branchpoint enzyme of the disialoganglioside pathway and a marked increase in the galactosyltranferase of G_{M1} formation. The results indicate that ganglioside deletions are the end result of a *cascade* of events in the tumorigenic transformation. The onset of ganglioside deletions but not of the cascade per se may correlate with the onset of malignancy.

Glycolipid levels are elevated early in certain surrounding tissues especially in the blood. In rats bearing transplantable hepatomas, serum levels of lipid-bound sialic acid were elevated 2.5-fold. Similar results were obtained with sera of mice bearing transplantable mammary carcinomas and of cancer patients. These findings provide new emphasis for gangliosides in both cancer detection and as regulatory signals for growth and multiplication of cells.

Key words: hyperplastic liver nodules, hepatoma, N-2-fluorenylacetamide, ganglioside, sialic acid, carcinogenesis, cancer detection

Abbreviations: UDP-Gal) uridine diphosphate galactose, GalNAc) N-acetylgalactosamine; CMP) cytosine monophosphate; NAN) N-acetylneuraminic acid; LacCer) lactosylceramide.

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Glycolipids, and especially gangliosides (sialoglycosphingolipids), emerge as useful models for the study of cell surface heteroglycans. They are enriched at the surface of the cell [1–7] and function as specific membrane receptors [8] where their biological role is beginning to be identified [9]. Yet there has appeared no simple correlation between ganglioside composition and the oncogenic transformation.

As part of an investigation into the early biochemical events of tumorigenesis, we have studied ganglioside composition and biosynthesis during normal liver development and in experimental liver tumors induced by the carcinogen N-2-fluorenylacetamide in the rat.

Ganglioside is a generic term for a family of glycosphingolipids which contain sialic acid in addition to hexoses and, in most, N-acetylated hexosamines (Fig. 1). The hydrophobic portion of the ceramide moiety of the ganglioside is anchored in the membrane and consists of a long-chain fatty acid linked through an amide bond to sphingosine. The carbohydrate residues are then linked via the C-1 of sphingosine.

Ganglioside terminology is sometimes confusing even to the specialist. We use the terminology of Svennerholm [10], in which monosialogangliosides are designated as G_M, disialogangliosides as G_D, and trisialogangliosides as G_T. The simplest of the major monosialogangliosides, designated G_{M3}, is formed by adding sialic acid to the oligosaccharide terminus of lactosylceramide (galactose-glucose-ceramide). Further addition of N-acetylgalactosamine results in G_{M2}; the addition of a terminal galactose yields G_{M1} (Fig. 1). A second sialic acid added to the terminal galactose results in a disialoganglioside designated G_{D1a}. If, instead of N-acetylgalactosamine, a second sialic acid is added directly to the first sialic acid of G_{M3}, the disialoganglioside G_{D3} results. Sequential additions of N-acetylgalactosamine, galactose, and sialic acid to G_{D3} result in formation of G_{D2}, GD_{1b}, and G_{T1b}, respectively. These various relationships of the component gangliosides of the mono- and disialoganglioside pathways are illustrated in Figure 2. Although first identified in brain, where they are most concentrated, gangliosides are ubiquitous constituents of surface membranes of most, if not all, extraneural tissues.

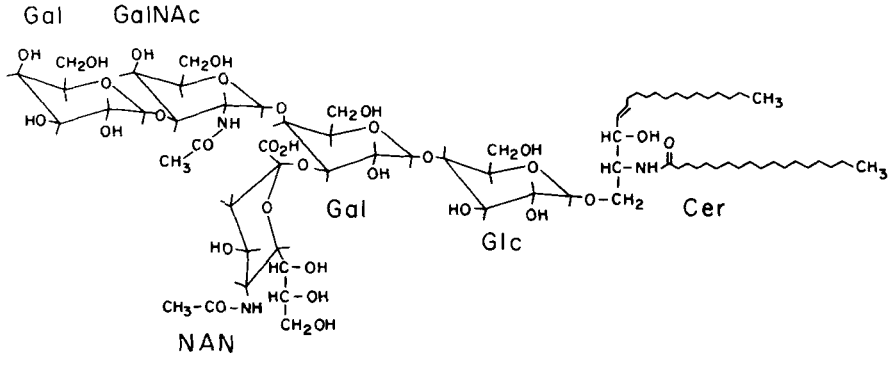


Fig. 1. Structure of the monosialoganglioside G_{M1}. The most complex of the monosialogangliosides, G_{M1}, consists of a ceramide moiety (Cer) linked through a glycosidic bond to an oligosaccharide. The oligosaccharide consists of glucose (Glc), galactose (Gal), N-acetylgalactosamine (GalNAc) and sialic acid (NAN = N- acetylneuraminic acid).

Ganglioside biosynthesis is catalyzed by glycosyltransferases [11–13], associated in liver with the Golgi apparatus [14]. Monosaccharide units are transferred from their nucleotide derivatives to appropriate glycolipid acceptors, and each of these transfers is catalyzed by a different glycosyltransferase reaction (Fig. 3). The product of one reaction in the sequence is the substrate for the next, so that if any step in the sequence is blocked all higher homologs are deleted.

This report is a summation of a continuing research effort to elucidate the nature of the ganglioside changes which accompany experimental liver tumorigenesis. While a causal relationship between ganglioside changes and altered social behavior of tumor cells has not been established, glycolipid, and especially ganglioside, changes may provide useful indicators of the tumorigenic transformation with possibilities for application to early diagnosis. New work is focusing on the regulatory events associated with modulations in glycosyltransferase activities and the cascade of biochemical events which may precede the cell surface changes.

MATERIALS AND METHODS

Hyperplastic liver nodules and hepatocellular carcinomas were induced in livers of Wistar rats by feeding a low-protein diet containing 0.05% of the carcinogen N-2-fluorenylacetamide (2-FAA) according to a modification [15] of the Farber schedule [16]. Rats

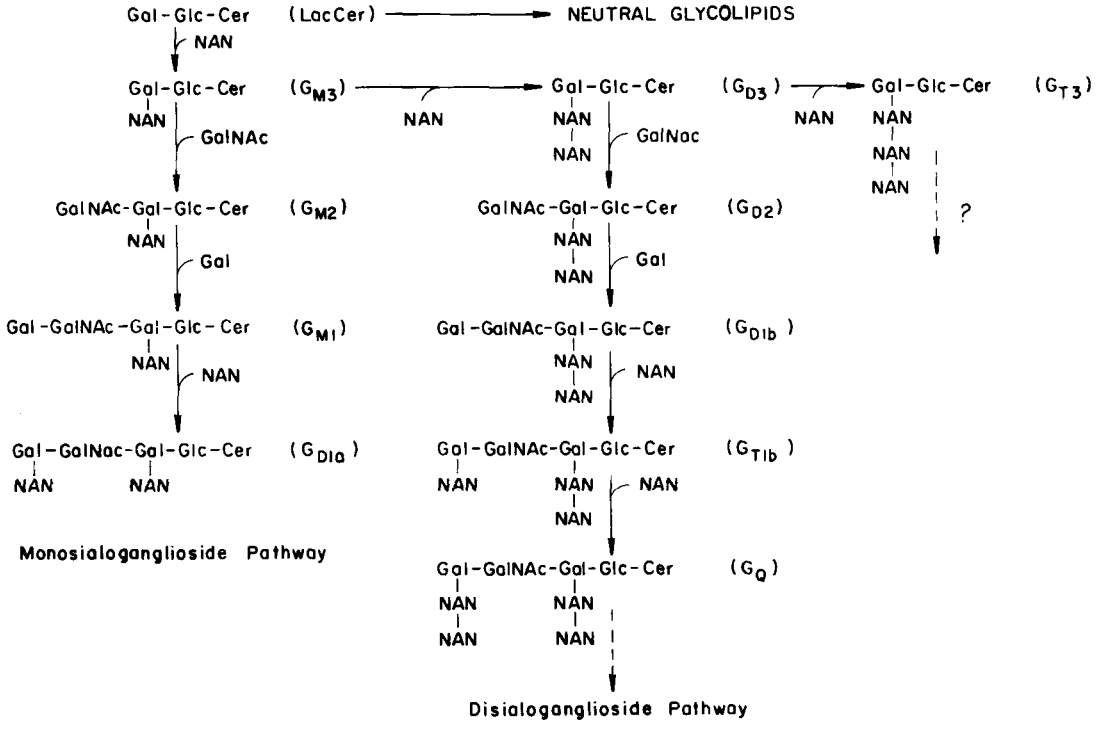


Fig. 2. The ganglioside biosynthetic pathways of rat liver. Cer = ceramide (N-acylsphingosine): Gal = galactose; GalNAc = N-acetylgalactosamine. NAN = N-acetylneuraminic acid (sialic acid). Based on studies of Keenan et al [14] and Merritt et al [15, 25].

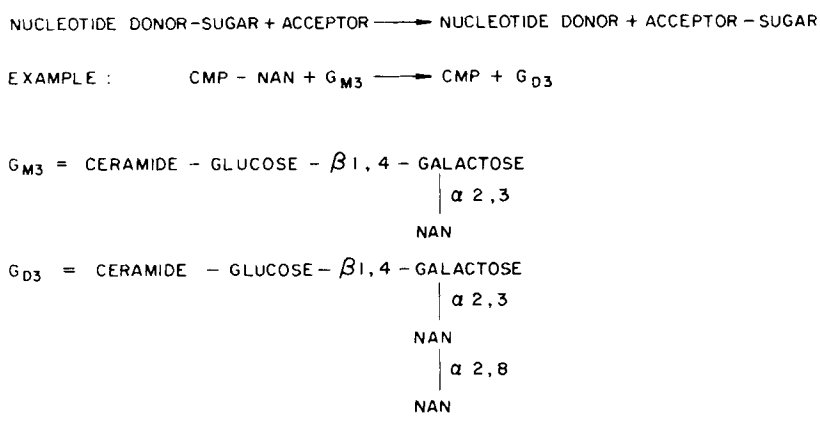


Fig. 3. The glycosyltransferase reaction. Sugars are added sequentially from appropriate sugar nucleotide donors to individual acceptor molecules in reactions catalyzed by specific glycosyl transferases. The example shown is the reaction catalyzed by CMP-NAN: G_{M3} sialyltransferase. The sugar nucleotide donor is CMP-NAN. The acceptor is the monosialoganglioside G_{M3}. The products of the reaction are CMP and a disialoganglioside G_{D3}.

were sacrificed at 4- to 8-week intervals over 200 days to obtain both hyperplastic nodules and hepatocellular carcinomas in varying stages of development. The bases for histopathologic classification of hepatocellular carcinomas [15] and nodules [17] have been summarized. Transplantable hepatocellular carcinomas were initiated in an inbred strain (Wistar CDF) of rats by feeding 2-FAA at a level of 0.025% for 10–12 months. Tumors were aseptically removed and were subsequently maintained in syngeneic recipients via subcutaneous implantation.

Ganglioside amounts and composition were determined for histologically different hepatocellular carcinomas and compared with those for control livers, hyperplastic nodules, and liver and tissue surrounding hepatomas and nodules as well as livers of fetal, newborn, week-old, weanling, and adult rats as described [15]. Regenerating liver was obtained following partial hepatectomy. Gangliosides were extracted by the Ledeen procedure [18], ganglioside mixtures were resolved by multiple developments using thin-layer chromatography, and individual gangliosides were visualized and quantitated by reaction with resorcinol and densitometry. Both total and ganglioside sialic acid were estimated by the Warren procedure [19].

Assays of ganglioside glycosyltransferases were modified from conditions for UDP-Gal: G_{M2} galactosyltransferase [20], UDP-GalNAc: G_{M3} N-acetylgalactosaminyltransferase [21], and CMP-NAN: G_{M1} [14] and CMP-NAN: LacCer [22] sialyltransferases as characterized from rat liver. CMP-NAN was prepared by the method of Kean [23]. Proteins were determined by the procedure of Lowry et al [24]. Total particulate fractions were utilized as the enzyme source with homogenization in 0.32 M sucrose containing 14 mM 2-mercaptoethanol [25].

For determination of lipid-soluble sialic acid of serum samples, the simplified procedure described by Kloppel et al [26] was utilized. Approximately 1 ml of serum or plasma was extracted with chloroform-methanol (2:1). Sialic acid-containing lipo- and glycoproteins were precipitated by addition of 0.1% solution of tripotassium citate to the con-

centrated chloroform-methanol extracts. With this method, recovery of [^{3}H]G_{M1} added to serum samples averaged 92% [26]. Sialic acid contents of the citrate supernatants were analyzed by the Warren method [19] using the Warren formula [19] to correct for variations due to unspecific absorbance.

RESULTS

As outlined in the introduction, the general significance of sialic acid alterations during tumorigenesis has been questioned because, while elevations have been recorded for solid tumors, a frequent change in transformed cells in culture is a lowering of sialic acid content [27, 28]. Our findings, taken together with previous work from our laboratory and work of others, is summarized in Figure 4. Sialic acid levels are elevated in fetal liver and at birth, drop sharply in the week after birth, and remain more or less constant in the adult. Following administration of carcinogen, sialic acid values increase with nearly a twofold elevation in hyperplastic liver nodules. Maximum values are attained in poorly differentiated, well-circumscribed hepatomas with subsequent decline in invasive, poorly differentiated and poorly circumscribed hepatomas.

On a protein basis, levels of total sialic acid were elevated to 1.3–3.6 times normal levels for liver in both autocthonously growing and transplantable hepatomas (Table I).

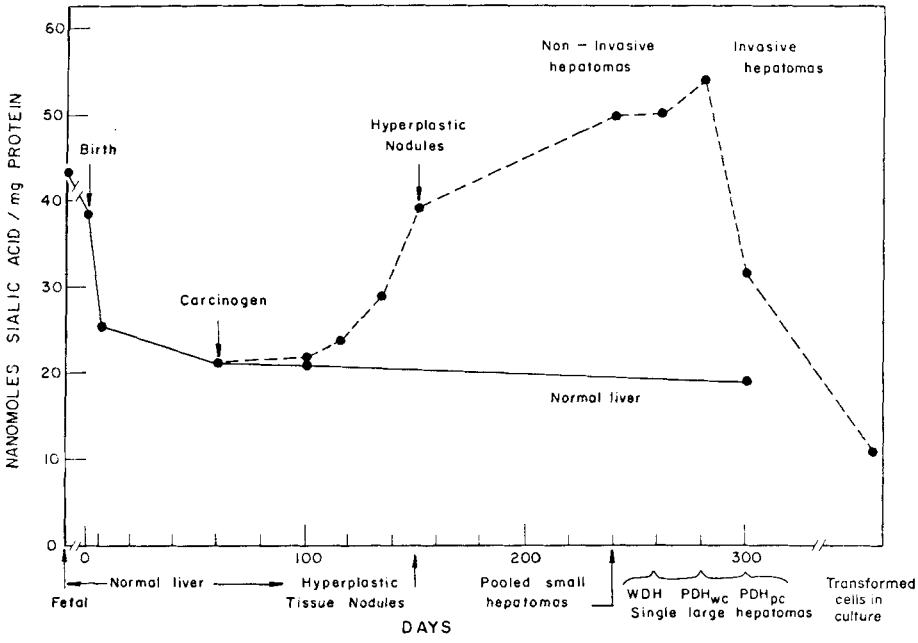


Fig. 4. Summary of changes in total specific sialic acid content during liver development and N-2-fluorenylacetamide-induced tumorigenesis in rat liver and for cells in culture. WDH) well-differentiated hepatocellular carcinoma (hepatoma); PDH$_{pc}$) poorly circumscribed, poorly differentiated hepatocellular carcinoma (hepatoma); PDH$_{wc}$) well-circumscribed, poorly differentiated hepatocellular carcinoma (hepatoma). Indications for cells in culture are based on information from the literature (see Discussion). Other values are from the studies of Merritt et al [15].

TABLE I. Sialic Acid Levels in Experimental Liver Tumors and Control Tissues

Tissue source	Sialic acid (nmoles/mg protein)	
	Total	Ganglioside
Normal liver	20	0.44
Hyperplastic nodules	40	1.40
Tissue surrounding nodules	23	1.00
Small hepatomas	50	1.28
Tissue surrounding hepatomas	24	0.72
Large hepatomas: Noninvasive	50	2.20
Invasive	30	0.90
Transplanted hepatomas: T-1	72	0.86
T-2	65	2.08
T-3[a]	25	0.56
Liver from animals bearing transplanted hepatomas	26	–
Regenerating liver	16	–

[a]Transplanted hepatoma T-3 was metastatic to the lung. Data from Merritt et al [15]; Kloppel TM, Sarles D, Jacobsen LB, Morré DJ (Manuscript submitted).

Additionally, sialic acid levels were significantly elevated in livers from carcinogen-treated animals prior to the appearance of either hepatomas or hyperplastic nodules and even in apparently normal livers of animals with transplanted hepatomas growing subcutaneously (Table I). In contrast, regenerating liver showed no elevation in total sialic acid (Table I).

The levels of ganglioside sialic acid reflected those of total sialic acid (Table I). Livers of fetal and newborn rats contained nearly twice the amount of ganglioside sialic acid on a protein or DNA basis, as did livers of adult rats. Subsequently ganglioside sialic acid levels were found to be elevated above those of normal adult liver in all liver tissues following the carcinogen-treatment regimen. Analyses of individual nodules and hepatomas revealed two populations of tumors in which the levels of ganglioside sialic acid were 2.3 and 3.8 times normal [15]. Ganglioside sialic acid content was at hepatoma levels even in very small nodules [15].

Individual gangliosides were evenly distributed between products of the mono- and disialoganglioside pathways (Fig. 2) in normal liver. The monosialogangliosides predominated in liver tissues following administration of the carcinogen (Fig. 5). Increased levels of specific monosialogangliosides were present in nodules, in liver of carcinogen-treated animals prior to the appearance of tumors, and in the liver tissues surrounding nodules and hepatomas. Quantitative increases in monosialogangliosides were accompanied by a gradual decline in products of the disialoganglioside pathway (Fig. 6) that was suggestive of the deficiency in the enzyme that converts hematoside (G_{M3}) to disialohematoside (G_{D3}) subsequently observed (see also Merritt et al [25]). The ratio ($G_{M1} + G_{D1a}$)/($G_{D1b} + G_T$), used as an index of the relative predominance of the mono- or disialoganglioside pathways, fell from 2.7 for fetal liver to 0.4 for adult liver (Fig. 6). When hyperplastic nodules and hepatocellular carcinomas were compared, a reverse pattern was observed. The ratio ($G_{M1} + G_{D1a}$)/($G_{D1b} + G_T$) rose steadily to values of 2.7 and 11.8 in well-circumscribed and poorly circumscribed, poorly differentiated hepatomas, respectively. These results suggest that the monosialoganglioside pathway is repressed during adult development. During the tumorigenic transformation, this pathway appears to be restored and the disialoganglioside pathway repressed.

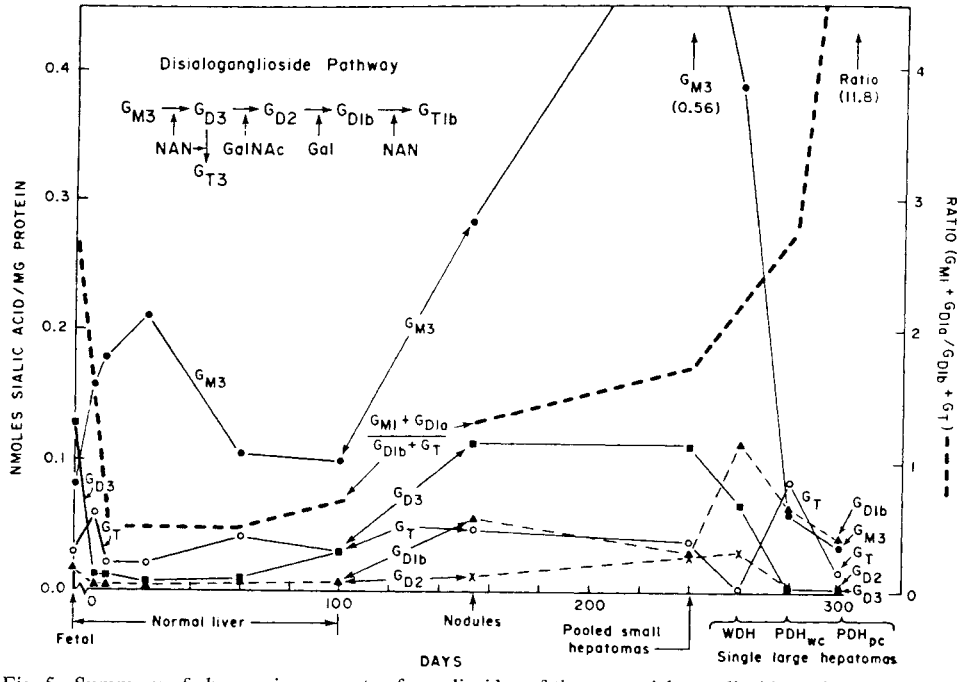


Fig. 5. Summary of changes in amounts of gangliosides of the monosialoganglioside pathway of rat liver during normal development and tumorigenesis induced by feeding the carcinogen N-2-fluorenylacetamide. Ganglioside nomenclature is that summarized in Figure 2; hepatoma designations as in Figure 4. Figures 5–7 from Merritt et al [15].

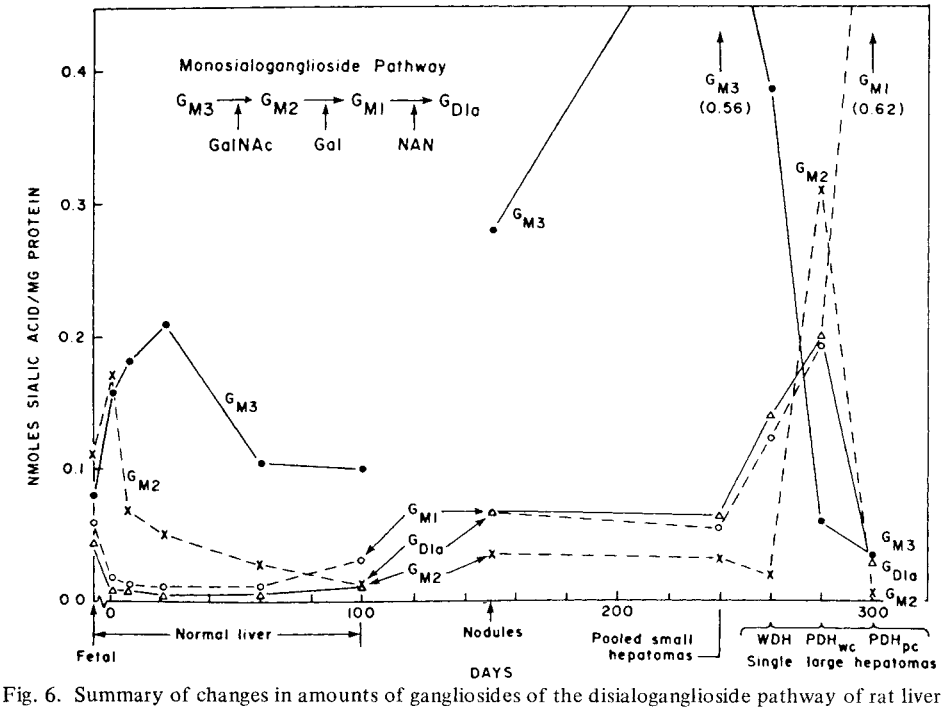


Fig. 6. Summary of changes in amounts of gangliosides of the disialoganglioside pathway of rat liver during normal development and tumorigenesis induced by feeding the carcinogen N-2-fluorenylacetamide. Ganglioside nomenclature is that summarized in Figure 2; hepatoma designations as in Figure 4.

The pattern of ganglioside alteration during experimental liver tumorigenesis is summarized in Figure 7. The complex ganglioside pattern of normal liver is retained except in the most poorly differentiated tumors. The initial change, observed in carcinogen-treated liver, is an elevation in the precursor gangliosides hematoside (G_{M3}) and disialohematoside (G_{D3}). These two gangliosides increase in parallel in hyperplastic nodules and small hepatomas to a 5+-fold elevation over control levels. At the same time, all other gangliosides except G_{M2} and GT also increase (Fig. 7). In large hepatomas the elevated levels of G_{M1} and G_{D1a}, end products of the monosialoganglioside pathway (Fig. 2), predominate and trisialogangliosides are deficient (Table II). With progressive tumorigenesis (decreasing degree of differentiation), monosialogangliosides tend to dominate. In poorly differentiated hepatomas, this pattern is extended further in that disialohematoside is much reduced. Finally, in the poorly circumscribed, poorly differentiated hepatomas, 80% of the total ganglioside is G_{M1} (Table II).

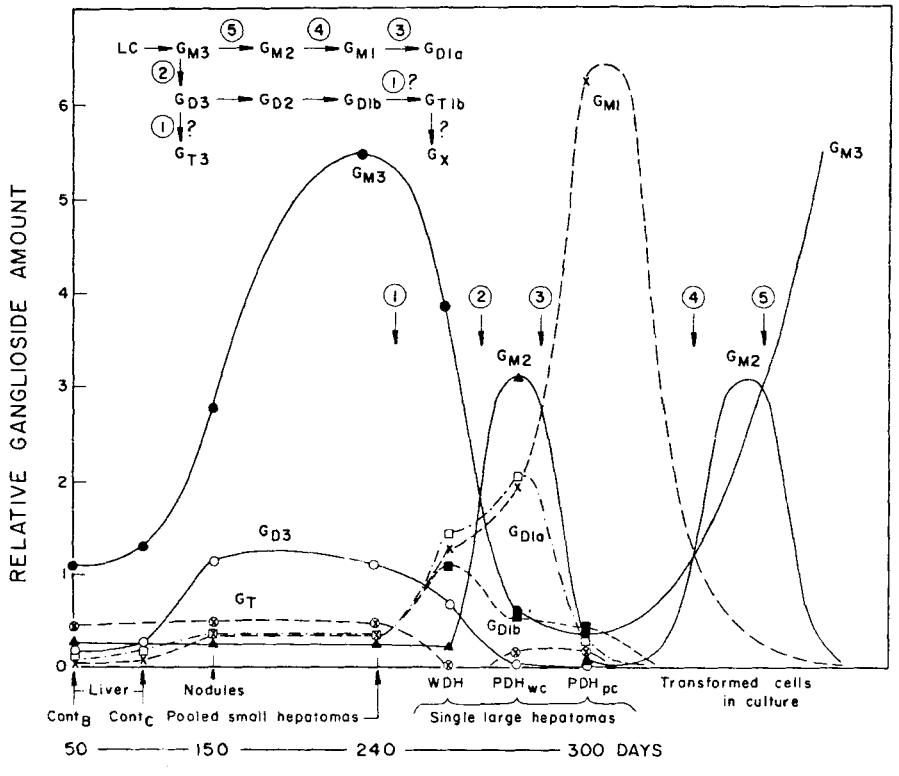


Fig. 7. Summary of glycolipid changes during N-2-fluorenylacetamide-induced tumorigenesis in rat liver and cultured cells. Indications for cells in culture are based on information in the literature (see Discussion). Other values were determined experimentally (Figs. 5 and 6). Numbers at crossover points refer to potential enzyme deficiencies or enzyme blocks in the ganglioside biosynthetic sequence for rat liver (Fig. 2); hepatoma designations as in Figure 4.

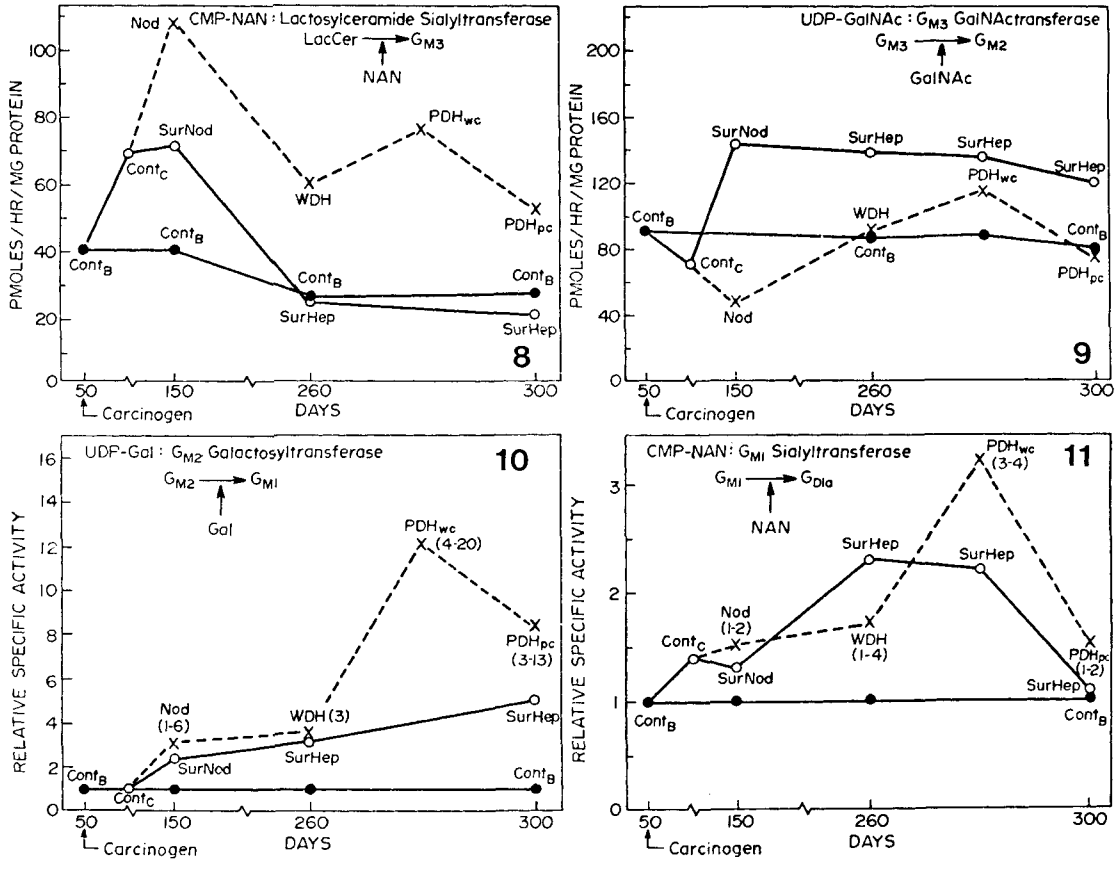
TABLE II. Summary of Gangliosides and Biosynthetic Enzymes of Large Hepatomas [15, 25]

Ganglioside		Well-differentiated		Poorly differentiated			
				Well-circumscribed		Poorly circumscribed	
Monosialoganglioside pathway							
LC							
↓	←NAN		(2.0)[b]		(2.7)		(1.7)
G_{M3}		390[a]		58		35	
↓	←GalNAc		(1.0)		(1.3)		(0.9)
G_{M2}		19		311		6	
↓	←Gal		(3.8)		(12.0)		(8.8)
G_{M1}		126		196		620	
↓ ↓	←NAN		(1.6)		(3.3)		(1.5)
G_{D1a}		143		202		30	
Disialoganglioside pathway							
G_{M3}							
↓	←NAN		(0.5)		(0.4)		(0.2)
G_{D3}		69		ND		ND	
↓	←GalNAc				(0.5)		
G_{D2}		32		26		28	
↓	←Gal				(0.6)		
G_{D1b}		114		65		41	
↓	←NAN				(0.6)		
G_T		20		82		14	
↓	←NAN				?		
G_X		53		20		ND	
Ratio: Monosialogangli-osides/disialogangliosides		2		3		12	

[a] pmoles ganglioside per milligram protein.
[b] Numbers in parentheses are specific activity relative to liver.

Further investigations revealed that glycolipid alterations during tumorigenesis were a direct reflection of alterations in the activities of their respective biosynthetic enzymes ([25]; Table II). Some of these results will be summarized to illustrate major points.

Activities of the enzymes of the monosialoganglioside pathway (lactosylceramide → G_{D1a}) were elevated progressively above those of control liver during 2-FAA-induced tumorigenesis in both precancerous tissues and well-differentiated hepatomas (Figs. 8–11). Activities of the first enzyme in the biosynthetic sequence CMP-NAN: lactosylceramide sialyltransferase were elevated 2.5-fold in precancerous liver tissues and hyperplastic nodules and remained near that level in well-differentiated and poorly differentiated hepatomas (Fig. 8). The UDP-N-acetylgalactosamine: G_{M3} N-acetylgalactosaminyltransferase was elevated in the tissues surrounding nodules and hepatomas as well as in well-circumscribed, poorly differentiated hepatomas, but was depressed in nodules (Fig. 9). Activities of UDP-galactose: G_{M2} galactosyltransferase (Fig. 10) and to a lesser extent the CMP-NAN: G_{M1} sialyltransferase (Fig. 11) were correlated directly with early tumor growth [17]. CMP-NAN was not incorporated with G_{D1a} as substrate in any of the tissues investigated, suggesting that G_{D1a} is an end product of the monosialoganglioside pathway. The relative levels or activities of biosynthetic enzymes of the monosialoganglioside pathway correlated with glycolipid levels in this branch of the pathway in all tumor types as summarized in Table II and Figure 13.



Figs. 8–11. Specific activities of glycosyl transferases of ganglioside biosynthesis of the monosialoganglioside pathway comparing liver (Cont), nodules (Nod), hepatomas, and tissues surrounding nodules (SurNod) and hepatomas (SurHep). $Cont_B$) control liver from rats fed basal diet without carcinogen; $Cont_C$) control liver from rats fed basal diet with added carcinogen – liver tawny with prominent sinusoids: PDH_{pc}) poorly circumscribed, poorly differentiated hepatocellular carcinoma (hepatoma); PDH_{wc}) well-circumscribed, poorly differentiated hepatocellular carcinomas (hepatoma); WDH) well-differentiated hepatocellular carcinoma (hepatoma). Fig 8. CMP-NAN: Lactosylceramide sialytransferase. Fig. 9. UDP-GalNac: G_{M3} N-acetylgalactosaminyltransferase. Fig 10. UDP-Gal: G_{M2} galactosyltransferase. Fig. 11. CMP-NAN: G_{M1} sialyltransferase. Numbers in parenthesis give the range of values. Figures 8–13 from Merritt et al [25].

Specific activities of the enzyme at the branchpoint between the mono- and di-sialoganglioside pathways (Fig. 2) were generally lower in hepatomas than in normal liver (Fig. 12); a loss of CMP-NAN: G_{D1b} sialyltransferase was observed in two of three well-circumscribed, poorly differentiated hepatomas [25]. Specific activities of other biosynthetic enzymes of the disialoganglioside pathway, UDP-N-acetylgalactosamine: G_{D3} N-acetylgalactosaminyltransferase and UDP-galactose: G_{D2} galactosyltransferase, were either near or somewhat above control levels in these same tumors [25]. Deficiency of the branchpoint enzyme may be a potentially important contributor to the progressive rise in the $(G_{M1} + G_{D1a}) / (G_{D1b} + G_T)$ ratio that is characteristic of hepatomas (Table III).

In all of these experiments, glycolipid changes in the early stages of tumorigenesis were found not only in tumor tissues themselves but in the liver tissue surrounding the tumors (Table I). Even the livers of animals bearing subcutaneously transplantable liver tumors showed increased levels of sialic acid (Table I). We have recently determined that levels of lipid-bound sialic acid in blood, also a tissue which surrounds the tumors, are increased.

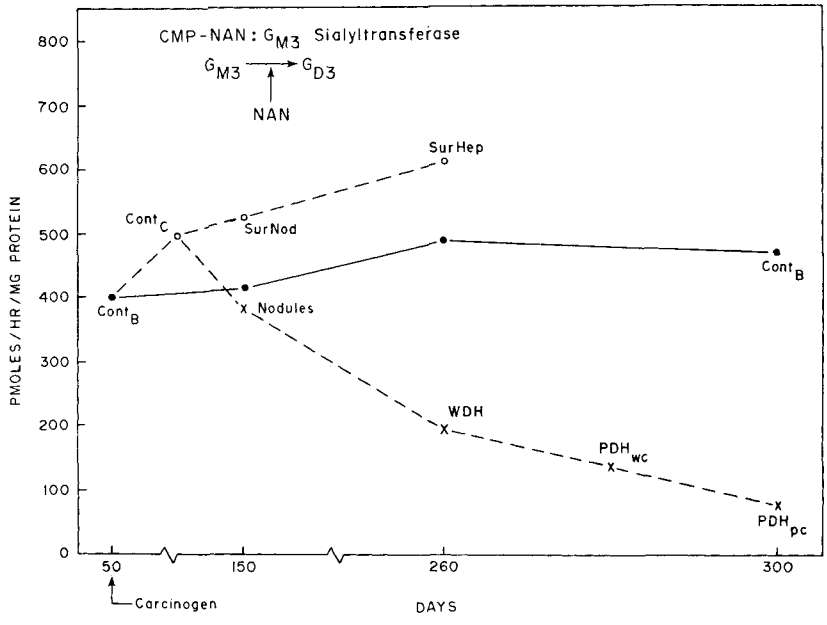


Fig. 12. CMP-NAN: G_{M3} sialyltransferase activity in liver, nodules, hepatocellular carcinomas, and tissues surrounding nodules and hepatomas. This is the branchpoint enzyme leading into the disialoganglioside pathway illustrated in Figure 3 which seems to be progressively reduced or blocked during liver tumorigenesis. Abbreviations are the same as for Figures 8–11.

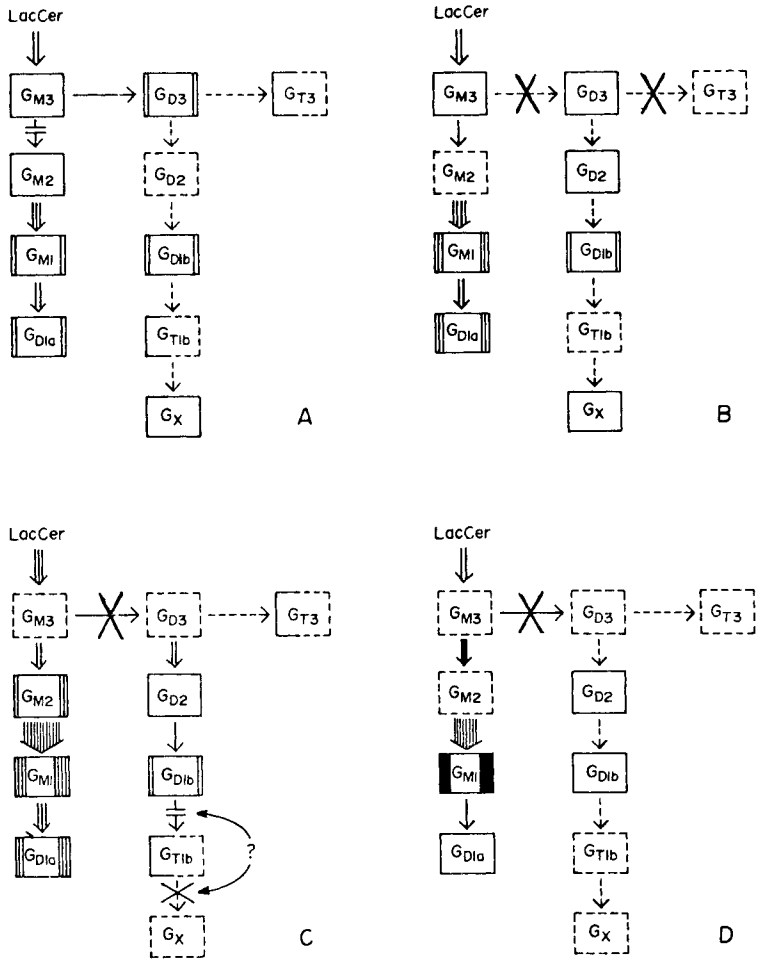


Fig 13. Summary of alterations in ganglioside biosynthesis in nodules and hepatomas. Boxes around ganglioside symbols indicate the relative levels of gangliosides [15]. A dash-line box indicates a ganglioside that is proportionately reduced in amount or absent. The number of bars within a box indicates the relative increase of ganglioside level over that of control liver. A single box with solid lines represents no change from control. A box with two extra bars represents twice control. A box with three extra bars represents three-times control, etc. Arrows between boxes indicate relative specific activities of the corresponding biosynthetic enzymes. The number of solid arrows indicates the relative increase in specific activities of the corresponding biosynthetic enzymes. A single solid arrow is control level, etc. Hatches through arrows indicate a potential site of a missing or depressed glycosyl transferase activity. A) Hyperplastic nodules; B) well-differentiated hepatoma; C) well-circumscribed, poorly differentiated hepatoma; D) poorly circumscribed, poorly differentiated hepatoma.

In studies of Kloppel et al [26], the level of total gangliosides, determined on the basis of sialic acid, was elevated 2.5 times above control values in sera of mice (Table IV) or rats (not shown) carrying a transplantable tumor or either drug-induced and hepatic or spontaneous and mammary (Table II) origins. Elevations were observed for the transplanted mammary carcinomas at 11, 21, and 35 days after transplantation; at 11 days palpable tumor masses could not be detected. These increases were not shown by whole-serum sialic acid or by sialic acid content of precipitated lipoproteins and glycoproteins, which account for more than 90% of the sialic acid of the serum.

Later studies employed a simplified extraction and purification procedure (citrate procedure) that permitted analyses on 0.5–1 ml of serum. Elevated serum levels of sialic acid were shown by all tumor-bearing mice (Table V) as well as rats (Table VI) when compared with age- and litter-matched controls. The degree of elevation varied among individuals from 40 to 230% but each was significantly different from controls at the 99% confidence limit.

Data with human subjects are still limited [26]. As with the animal model, serum sialic acid of the glycolipid fraction was elevated nearly two-fold in carcinoma patients and appeared to decline after surgery. Within the normal population, the variation was ± 20% (Table VII).

TABLE III. Ratio of Mono- to Disialogangliosides Comparing Fetal and Normal Adult Liver, Hyperplastic Nodules, and Hepatomas

Tissue	Ratio
Fetal liver	2.7
Adult liver	0.6
Liver tumors:	
Hyperplastic nodules	1.2
Small hepatomas	1.6
Single large hepatomas	
Well-differentiated	1.8
Poorly differentiated, well-circumscribed	2.5
Poorly differentiated, poorly circumscribed	11.8

TABLE IV. Levels of Ganglioside-Bound Sialic Acid in Pooled Samples of Sera From Tumor-Bearing Mice 60 Days After Transplantation

Group	Sex	Sialic acid, (nmoles/ml serum)	Experimental/control
Control	Male	14.7	
	Female	16.0	
Tumor-bearing	Male	32.6	2.21
	Female	42.4	2.65

Gangliosides were extracted and purified by column chromatography from pooled sera of 25 mice prior to determination of ganglioside sialic acid. From Kloppel et al [26].

TABLE V. Lipid-Soluble Sialic Acid in Sera From Individual Male Mice Bearing Transplantable Mammary Carcinomas

Degree of vascularization	Tumor mass (g)	Sialic acid (nmoles/ml serum)	Experimental/ control
Low	0.51	29	1.5
	0.98	44	2.2
	2.00	27	1.3
	3.20	34	1.7
	3.25	50	2.5
	5.20	61	3.0
High	5.10	35	1.7
	5.45	64	3.2
	6.00	31	1.5
	8.00	39	1.9
	8.80	43	2.1
	9.40	53	2.7

Gangliosides were determined on lipid extracts following precipitation of lipo- and glycoproteins by the citrate procedure for sera eight weeks after transplantation. Experimental values are averages from duplicate assays on 0.5 ml of serum. Average control value (± SD) for four mice was 20 ± 4 nmoles sialic acid per milliliter of serum. All individual experimental values were significantly different from controls at a 99% confidence interval. Degree of vascularization was judged subjectively from number and size of prominent blood vessels associated with the tumor mass. From Kloppel et al [26].

TABLE VI. Levels of Ganglioside-Bound Sialic Acid and Lipid Phosphorus in Pooled Samples of Sera From Control Rats and Rats Bearing Transplantable Hepatomas

Group	Sialic acid (nmoles/ml serum)		Lipid phosphorus (nmoles/ml serum)
	Citrate procedure	Column procedure	
Control	34	3.7	30
Transplantable hepatomas			
T_1	99	–	–
T_2	62	5.3	80

TABLE VII. Lipid-Soluble Sialic Acid in Sera of Human Carcinoma Patients

Sex	Tumor status	Individuals	Sialic acid, (nmoles/ml serum ± SD)
Female	Normal	10	16 ± 3
	Mammary carcinoma	7	49 ± 20
	after surgery	6	31 ± 8
	Colonic carcinoma	5	49 ± 8
	after surgery	3	39 ± 1
Male	Normal	8	24 ± 4
	Colonic carcinoma	5	47 ± 9
	after surgery	3	24 ± 9

Control value for pooled transfusion blood (four different lots) was 20 ± 2 nmoles sialic acid per milliliter of serum. From Kloppel et al [26].

DISCUSSION

Constituents of cell surfaces of mammalian cells which may be important to cancer-related properties are glycoproteins and glycolipids [9, 11, 31, 34]. Sialic acid is a common terminal saccharide on many of these glycoproteins and glycolipids.

Several tumors have been reported to have elevated sialic acid content. These include human tumors of the colon, stomach, breast, and other tissues [35, 36] and experimental liver tumors of the rat [15, 27, 29]. Yet, in cultured transformed cells a frequent change is a lowering of the membrane sialic acid content [37–39]. In light of this paradox and other considerations, some authors have concluded that specific cell surface sialic acid changes are not a general property of neoplastic cells [27, 28].

Our results indicate changes in sialic acid of opposite sign during a tumorigenic continuum. Sialic acid levels are elevated in fetal liver and at birth, drop sharply in the week after birth, and remain more or less constant in the adult. Following administration of carcinogen, sialic acid values once again begin to increase, and maximum values are attained in poorly differentiated, well-circumscribed hepatomas. The lowered sialic acid level in the poorly circumscribed hepatoma relative to the well-circumscribed hepatoma may be related to the rapid rate of growth of the cells of the poorly differentiated hepatomas. This pattern of change is shown by cell lines of fibroblast origin which already exhibit an extremely rapid rate of growth. Transformation yields an even more rapid rate of growth and a decrease in sialic acid. This same pattern is observed with transplantable hepatomas (Table I). Also, in hepatoma cells grown in culture, both G_{M1} and G_{M3} are increased over levels in hepatocytes but the ratio of G_{M3} over G_{M1} is higher in hepatoma cells than in hepatocytes [30]. This accumulation of a lower monosialoganglioside is similar to transformation-related events in cells transformed with DNA viruses, in which gangliosides more complex than G_{M3} are lost, and in cells transformed by chemical carcinogens, in which gangliosides more complex than G_{M2} are lost [9, 31–33].

The alterations in sialic acid are largely due to "bound" sialic acids, ie, those serving as prosthetic groups of heteroglycan (glycoproteins or glycolipids). Less than 10% of the total sialic acid of liver or of liver tumors occurs in forms not accounted for by heteroglycans [15]. Of the bound sialic acid, the bulk is in the form of glycoprotein; ganglioside sialic acid represents only about 2–5% of the total bound sialic acid [15]. Yet, gangliosides and other glycolipids offer an important experimental advantage over glycoproteins in that they can be isolated readily and characterized chemically while showing approximately the same degree of carbohydrate complexity [11, 40] and degree of alteration in tumor cells [9] as their glycoprotein counterparts. Thus, glycolipids, and especially gangliosides, emerge as useful models for the study of cell surface heteroglycans. They are enriched at the surface of the cell [1–7] and function as specific membrane receptors [8]. The structure, biosynthesis, and function of gangliosides and alterations in ganglioside biosynthesis during development and in transformed cells have been reviewed [9].

Some functions of glycolipids are just beginning to be identified. They were originally speculated to be important in membrane structure, and several human blood group antigens, such as the antigens, A, B, H, Le^a, Le^b, P, and p^k, have been characterized as glycosphingolipids [41]. More recently, the specific oligosaccharide sequences of glycolipids have become important candidates as receptors for toxins, drugs, hormones, and transmitter substances [8, 9]. Van Heyningen and co-workers [42] first showed that brain gangliosides bound cholera toxin and blocked its physiologic effect. There is evidence that

receptors for serotonin [9, 43, 44] are glycolipids. Glycolipids from brain bind viruses [45]. A variety of results summarized by Fishman and Brady [46] on the interactions of thyroid-stimulating hormone (TSH) with gangliosides implicate a role for the gangliosides in the biological effects of glycoprotein hormones of the TSH group. Many of these diverse biological effects are believed to be mediated through cyclic AMP after activation of adenylate cyclase bound to the plasma membrane [9, 47] via a ganglioside-mediated interaction.

In most but not all transformed cells and tissues, the patterns of glycolipid composition are altered. The alterations are usually in the direction of a simplified pattern of glycolipid distribution (Fig. 13; [48–58]), where the higher homologs of the gangliosides are decreased or absent and the lower homologs (precursor gangliosides) are frequently increased in amount [9, 15, 33, 59]. Various hamster, chick embryo, mouse, and human fibroblast cell lines all exhibit ganglioside simplification when transformed by viruses [19, 31–33, 59]. Yet it remains unclear whether there is a correlation between the observed chemical changes and the altered growth properties of the transformed cells [9]. Siddiqui et al [60] and others [61] demonstrated that the simplification of gangliosides did not result from rapid growth of the tumor. By comparing both slowly and rapidly growing cell lines, they found the same reduction in the higher glycolipid homologs and increases in the simpler precursors. Additionally, several Morris minimal deviation hepatomas have been examined both in vitro [62] and in vivo [60, 63, 64]. The normal controls consistently contained a higher concentration of complex gangliosides. The same trend in alteration of ganglioside patterns has been found for solid tumors induced by chemical carcinogens [15, 29, 58]. However, as exemplified by our studies with rat hepatomas, these simplifications in glycolipid pattern emerge as late events in tumorigenesis.

The simplification of the pattern of gangliosides in neoplasia, ie, depletion of complex gangliosides and an accumulation of simpler precursor gangliosides, has been related to one or more enzymatic deficiencies rather than to an increase in degradative enzymes [51, 52, 56, 65, 66] (Fig. 14). For example, a reduction in higher ganglioside homologs and in the activity of UDP-N-acetylgalactosamine:hematoside N-acetylgalactosaminyltransferase after transformation with DNA viruses, either SV40 or polyoma, has been shown by Brady et al [65]. Similarly, accumulation of the ganglioside G_{M1} in chemically induced mammary carcinomas of the rat was due to depression of the sialyl transferase which converts G_{M1}, a monosialoganglioside, to G_{D1a}, a disialoganglioside [58]. Fishman et al [57] related reductions in G_{M1} and G_{D1a} and an increase in G_{M2}, the immediate precursor of G_{M1}, to an absence of a galactosyltransferase in a mouse cell line transformed by murine sarcoma virus, an RNA virus. In the chemically induced rat hepatoma studied here, we find an overall depression in synthesis of higher homologs of the disialoganglioside pathways ($G_{D3} \rightarrow G_{T1b}$) [25]. This appears to be due to a deficiency in the branchpoint enzyme, the sialyltransferase of G_{D3} formation (Fig. 11). Thus, in all examples studied, the simplification in ganglioside pattern with malignant transformation is due to a depression or absence of a transferase which interrupts the ganglioside biosynthetic pathway. The chain of events leading to this depression or absence of one or more glycosyltransferases is only now beginning to be understood.

An important factor which contributes to increases in lower ganglioside homologs are increases in biosynthetic enzymes giving rise to precursor gangliosides [24, 53, 58]. This is especially evident in our studies with rat hepatomas. The in vitro enzyme data correlate well with ganglioside patterns to account for more than a 10-fold elevation of certain monosialogangliosides (Figs. 4–6). As with the enzyme deficiencies, the mechanisms

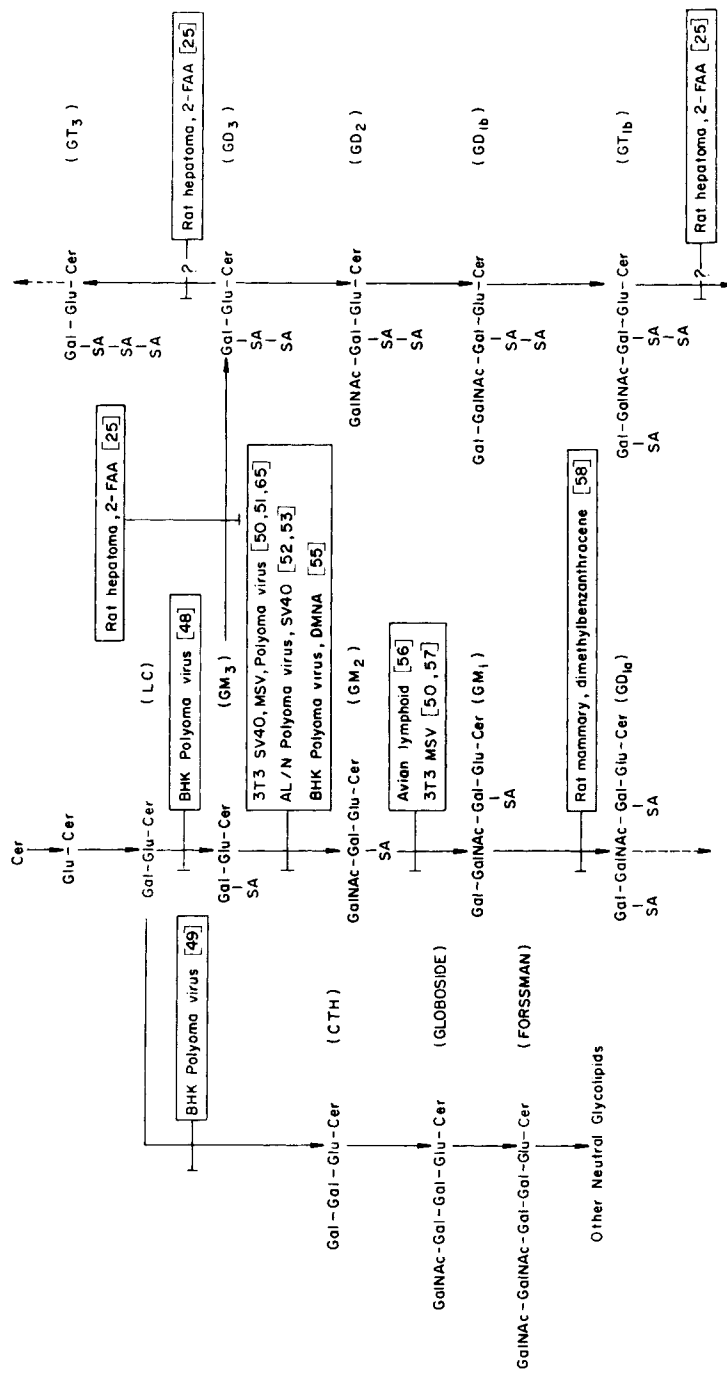


Fig. 14. Summary diagram showing specific glycosyl transferases within the glycolipid biosynthetic pathways which may be blocked as a result of transformation by different oncogenic agents. SA = sialic acid (N-acetylneuraminic acid = NAN). Other abbreviations are as in Figure 2. Modified from Richardson et al [33].

whereby activities of ganglioside biosynthetic activities are increased twofold to 20-fold in tumors have been little studied.

Exceptions to altered ganglioside synthesis have been encountered in spontaneously transformed cells in culture [52, 65, 67, 68]. Additionally, Fishman et al [69] report a normal ganglioside composition and normal glycosyltransferase activities in BALB/3T3 cells transformed by a monkey sarcoma virus. Thus, there appears to be no simple correlation between ganglioside composition and the oncogenic transformation and little doubt that the ganglioside changes are secondary to the primary transforming event.

With liver tumors, one has the experimental advantage that the development is slow, so that temporal changes leading to altered ganglioside compositions can be identified. As outlined in Results, our findings point to a temporal sequence of alterations beginning with hyperplastic nodules and extending to poorly differentiated hepatocellular carcinomas. Gross simplifications in ganglioside patterns and depletion of glycosyltransferases are preceded by marked elevations in ganglioside biosynthetic activities. These findings indicate that ganglioside deletions, although late events in the tumorigenic transformation, are related to some earlier events, perhaps a minimum deviation event. The lactosylceramide sialyltransferase of hematoside production, the enzyme at the branchpoint between the neutral glycolipid and ganglioside biosynthetic pathways, is a potentially important regulatory enzyme and its early and sustained elevation in liver tumorigenesis may be a significant minimum deviation event. On the other hand, the elevation of the lactosylceramide sialyltransferase may simply be part of a *cascade* of biochemical events stemming from initial transformation. This cascade appears to set in motion a series of changes which result first in an elevation of gangliosides and then in their depletion. This would explain why some minimum-deviation hepatomas have a ganglioside composition more nearly normal than hyperplastic nodules and may explain some of the aforementioned "exceptions" to altered ganglioside biosynthetic patterns. Extreme simplifications of ganglioside pattern apparently are not expressed until a high degree of malignancy is attained.

Of particular interest are the events in the cascade which preceed the elevation in the branchpoint enzyme at the beginning of the ganglioside biosynthetic pathway. To continue our investigation of the ganglioside biosynthetic cascade, enzymes of CMP-NAN production and of uridine triphosphate (UTP) formation have been monitored (Table VIII). Preliminary studies indicate that they, too, become elevated but the precise time course of this elevation has not been determined.

Despite encouraging progress to indicate that a glycolipid cascade is an important contributor to the events of tumorigenesis, there is little or no evidence to relate loss of gangliosides to malignancy or loss of normal growth restraints. Some evidence has come from studies of spontaneous revertants in cell culture. Additionally, attempts to "add back" missing glycolipids and restore normality have been only partially successful. Den et al [55] report that both SV40- and dimethylnitrosamine-transformed hamster cells and their nonmalignant revertants have simplified ganglioside patterns and markedly reduced levels of UDP-N-acetylgalactosamine: GM_3 N-acetylgalactosaminyltransferase activity. The revertant properties of these cells include both a return to saturation density-dependent growth and a lack of ability to form tumors in animals. On this basis, Den et al [55] suggested that altered ganglioside synthesis is not associated with growth regulation. In contrast, Cumar et al [52] found normal ganglioside patterns and synthetic ability in flat revertants of SV40-transformed 3T3 cells. Laine and Hakomori [70] showed a reduction in saturation density and growth rate of transformed hamster cells cultured in the presence of globoside; additionally the globoside-treated cells exhibited a greater adhesion for each

TABLE VIII. Specific Activities of CMP-Sialic Acid Synthetase and UDP Kinase of a Transplantable Hepatoma

Enzyme	Specific activity		Ratio:
	Control liver	RLT-2 hepatoma	Tumor/control
CMP-sialic acid synthetase (nmoles/h/mg protein)	80 ± 8	114 ± 10	1.43
UDP kinase (μmoles/h/mg protein)	50 ± 12	218 ± 43	3.7

Unpublished results of K. Creek and W. Elliott, Purdue University

other than for the substrate. With both SV40-transformed and untransformed 3T3 cells, Keenan et al [71, 72] found that gangliosides added to culture media reduced both the saturation density and growth rate; especially effective were monosialogangliosides and the tetrahexosylceramide formed by removal of sialic acid from G_{M1}. The significance of these findings is still uncertain, since it is doubtful that the gangliosides added back interact with the surface membranes in the same way as endogenously synthesized gangliosides [73, 74]. A high degree of nonspecificity is indicated from the findings of Brailovsky et al [75, 76], who found that addition of glycolipids derived from Salmonella minnesota mutants to culture media inhibited growth of transformed rat embryo fibroblasts.

The major significance of ganglioside alterations at the moment, however, may reside in their value as diagnostic aids and as indicators of the tumorigenic transformation. The early elevations during tumorigenesis are of interest. Our results show that elevations in UDP-galactose: G_{M2} galactosyltransferase specific activity, in particular, correlates with increasing tumorigenicity. Whereas relative specific activities in hyperplastic nodules averaged approximately three times those of controls, poorly differentiated hepatomas had relative specific activities 20 times those of controls. Furthermore, in studies in which relative galactosyltransferase specific activities in individual nodules were related to tumor wet weight [17], activity increased with increasing nodule size in two populations. One population contained nodules in which relative specific activities were at the lower range found in poorly differentiated hepatomas; the other population contained nodules in which relative specific activities were at the lower range found in well-differentiated hepatomas. The results are believed to be the first to relate specific populations of nodules to specific grades of hepatocellular carcinomas, and therefore suggest the specificity of this enzyme as an indicator of early tumorigenesis.

From a diagnostic viewpoint, alterations in blood serum lipid-bound sialic acid are particularly significant in view of the ease of monitoring such changes in the clinic. Serum levels of lipid-bound sialic acid are elevated 2.5-fold, based on pooled serum samples purified by column chromatography and by a simplified extraction procedure in mice bearing transplantable mammary carcinomas [26], in rats bearing transplanted hepatomas (Table V), with sera of carcinoma patients [26], and with client-owned dogs received by the Purdue University Veterinary Clinic bearing a variety of autochthonously growing carcinomas and sarcomas [77]. In the last-named study, statistically significant elevations of serum sialic acid were observed in 22 of 24 tumor-bearing dogs. The potential of these observations as the basis of a biochemical method for early detection of cancer is under investigation.

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REFERENCES

1. Klenk HD, Choppin PW: Proc Natl Acad Sci USA 66:57, 1970.
2. Weinstein DB, Marsh JB, Glick MC, Warren L: J Biol Chem 245:3828, 1970.
3. Dod BJ, Gray GM: Biochim Biophys Acta 150:397, 1968.
4. Renkonen O, Gahmberg CG, Simons K, Kaariainen L: Acta Chem Scand 24:733, 1970.
5. Gahmberg CG: Biochim Biophys Acta 249:81, 1971.
6. Yogeeswaran F, Sheinin R, Wherrett JR, Murray RK: J Biol Chem 247:5146, 1972.
7. Keenan TW, Huang CM, Morré DJ: Biochem Biophys Res Commun 47:1277, 1972.
8. Cuatrecasas P: Ann Rev Biochem 43:169, 1974.
9. Fishman PH, Brady RO: Science 194:906, 1976.
10. Svennerholm L: In Florkin M, Stotz EH (eds): "Comprehensive Biochemistry." Amsterdam: Elsevier, vol 18, p 201, 1970.
11. Roseman S: Chem Phys Lipids 5:270, 1970.
12. Basu S, Kaufman B, Roseman S: J Biol Chem 248:1388, 1973.
13. Stiegerwald JC, Basu S, Kaufman B, Roseman S: J Biol Chem 250:6727, 1975.
14. Keenan TW, Morré DJ, Basu S: J Biol Chem 249:310, 1974.
15. Merritt WD, Richardson CL, Keenan TW, Morré DJ: J Natl Cancer Inst 60:1313, 1978.
16. Merkow LP, Epstein SM, Farber E, Pardo M, Bartus B: J Natl Cancer Inst 43:33, 1969.
17. Merritt WD, Morré DJ, Doak RL, Keenan TW: Cancer Res (Manuscript submitted).
18. Ledeen RW, Yu RK, Eng LF: J Neurochem 21:829, 1973.
19. Warren L: J Biol Chem 234:1971, 1959.
20. Wilkinson FE, Morré DJ, Keenan TW: J Lipid Res 17:146, 1976.
21. Yeung KK, Moskal JR, Chien JL, Gardner DA, Basu S: Biochem Biophys Res Commun 59:252, 1974.
22. Richardson CL, Keenan TW, Morré DJ: Biochim Biophys Acta 488:88, 1977.
23. Kean EL: J Biol Chem 245:2301, 1970.
24. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ: Biol Chem 193:265, 1951.
25. Merritt WD, Morré DJ, Keenan TW: J Natl Cancer Inst 60:1329, 1978.
26. Kloppel TM, Keenan TW, Freeman MJ, Morré DJ: Proc Natl Acad Sci USA 74:3011, 1977.
27. Nicolson GL: Biochim Biophys Acta 458:98, 1976.
28. Weiss L: J Natl Cancer Inst 50:3, 1973.
29. Merritt WD, Keenan TW, Morré DJ: Cancer Biochem Biophys 1:179, 1976.
30. Brady RO, Borek C, Bradley RM: J Biol Chem 244:6552, 1970.
31. Hakomori S: Colloq Gesell Biol Chem 22:65, 1971.
32. Hakomori S: Biochim Biophys Acta 417:55, 1975.
33. Richardson CL, Baker SR, Morré DJ, Keenan TW: Biochim Biophys Acta 417:175, 1975.
34. Wallach DFH: "Membrane Molecular Biology of Neoplastic Cells." Amsterdam–Oxford–New York: Elsèvier Scientific, 1975.
35. Barker SA, Stacey M, Tipper DJ, Kirkham JH: Nature 184:BA68, 1959.
36. Mabry EW, Carubelli R: Experentia 28:182, 1972.
37. Bryant ML, Stoner GD, Metzger RP: Biochim Biophys Acta 343:226, 1974.
38. Ohta N, Pardee AB, Mcauslan R, Burger MM: Biochim Biophys Acta 158:98, 1968.
39. Perdue JF, Kletzien R, Wray VL: Biochim Biophys Acta 266:505, 1972.
40. Weigandt H: Hoppe Seyler's Z Physiol Chem 354:1049, 1973.
41. Rapport MM, Graf L: Prog Allergy 13:273, 1969.
42. van Heyningen WE, Carpenter CCJ, Pierce NF, Greenough WG: J Infect Dis 124:415, 1973.
43. Wolley DW, Gommi BW: Proc Natl Acad Sci USA 53:959, 1965.
44. van Heyningen WE: Nature 249:415, 1974.
45. Loh HH, Cho TM, Wu YC, Way EL: Life Sci 14:2231, 1974.
46. Mullin BR, Fishman PH, Lee G, Aloj SM, Ledley FD, Winand RL, Kohn LD, Brady RO: Proc Natl Acad Sci USA 73:842, 1976.
47. Finklestein RA: Crit Rev Microbiol 2:533, 1973.
48. Den H, Schultz A, Basu M, Roseman S: J Biol Chem 246:2721, 1971.
49. Kijimoto S, Hakomori S: Biochem Biophys Res Commun 44:557, 1973.
50. Brady RO, Fishman PH: Biochim Biophys Acta 355:121, 1974.

51. Mora PT, Fishman PH, Bassin RH, Brady RO, McFarland VW: Nature (New Biol) 245:226, 1973.
52. Cumar FA, Brady RO, Kolodny EH, McFarland VW, Mora PT: Proc Natl Acad Sci USA 67:757, 1970.
53. Fishman PH, McFarland VW, Mora PT, Brady RO: Biochem Biophys Res Commun 48:48, 1972.
54. Mora PT, Cumar FA, Brady RO: Virology 46:60, 1971.
55. Den H, Sela B, Roseman S, Sachs L: J Biol Chem 249:659, 1974.
56. Keenan TW, Doak RL: FEBS Lett 37:124, 1973.
57. Fishman PH, Brady RO, Bradley RM, Aaroson SA, Todaro GJ: Proc Natl Acad Sci USA 71:298, 1974.
58. Keenan TW, Morré DJ: Science 183:935, 1973.
59. Hakomori S: Adv Cancer Res 18:265, 1973.
60. Siddiqui B, Hakomori S, Vogt PK, Saito T: Fed Proc 29:928, 1970.
61. Kijimoto S, Hakomori S: FEBS Lett 25:38, 1972.
62. Brady RO, Borek C, Bradley RM: J Biol Chem 244:6552, 1969.
63. Siddiqui B, Hakomori S: Cancer Res 30:2930, 1970.
64. Cheema P, Yogeeswaran G, Morris HP, Murray RK: FEBS Lett 11:181, 1970.
65. Brady RO, Fishman PH, Mora PT: Fed Proc 32:102, 1973.
66. Dnistrian AM, Skipski VP, Barclay M, Essner ES, Stock CC: Biochem Biophys Res Commun 64:367, 1975.
67. Mora PT, Brady RO, Bradley RM, McFarland VW: Proc Natl Acad Sci USA 63:1290, 1969.
68. Mora PT, Cumar FA, Brady RO: Virology 46:60, 1971.
69. Fishman PH, Moss J, Vaughan V: J Biol Chem 251:4490, 1976.
70. Laine RA, Hakomori S: Biochem Biophys Res Commun 54:1039, 1973.
71. Keenan TW, Franke WW, Weigandt H: Hoppe Seyler's Z Physiol Chem 355:1543, 1974.
72. Keenan TW, Schmid E, Franke WW, Weigandt H: Exp Cell Res 92:259, 1975.
73. Schmid E, Keenan TW, Franke WW, Wiegandt H: J Cell Biol 70:389a, 1976.
74. Tomich MM, Mather IH, Keenan TW: Biochim Biophys Acta 433:357, 1976.
75. Brailovsky C, Trudel M, Lallier R, Nigam VN: J Cell Biol 57:124, 1973.
76. Brailovsky C, Lallier R, Nigam VN: J Cell Biol 63:34a, 1974.
77. Kloppel TM, Franz CL, Morré DJ, Richardson RC: Am J Vet Res (In press).