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Biochemical Characteristics, Metabolism, and Antitumor Activity of Several Acetylated Hexosamines

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We have synthesized several potential inhibitors and/or modifiers of the carbohydrate portion of plasma membrane glycoconjugates. These include fluorinated and actylated analogs of D-glucosamine, D-galactosamine, and D-mannosamine. These compounds have been tested to determine their effects on both [^{14}C]glucosamine and [^{3}H]leucine incorporation into glycoconjugate and on cell growth and viability using P-288 murine lymphoma cells maintained in tissue culture. The most cytotoxic agent tested was 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranose or simply β-pentaacetylglucosamine which prevented cell growth at $10^{-4}-10^{-3}$ M. β-Pentaacetylglucosamine cytotoxicity was correlated with its high lipid solubility, having an octanol/water partition coefficient of 0.424 as compared with 0.278 for the α-anomer and 0.017 for N-acetylglucosamine. In vitro metabolism studies with [^{14}C]- and/or [^{3}H]-labeled pentaacetylglucosamine have indicated intracellular de-O-acetylation leading to the biosynthesis of UDP-N-acetylglucosamine, followed by the incorporation of this sugar into cellular glycoprotein. Concomitant with the formation of increased amounts of this nucleotide sugar, intracellular UTP and CTP pools fell to one third normal within 3 h after the administration of 1 mM pentaacetylglucosamine. At present it is unclear whether the cytotoxicity of β-pentaacetylglucosamine or other similar agents is due to alterations in nucleotide and nucleotide-sugar pools causing a decrease in energy charge and polynucleotide biosynthesis or is due to a direct effect on membrane glycoconjugate biosynthesis.

Key words: glucosamine, glycoproteins, chemotherapy, nucleotide sugars, ribonucleotide pools, lymphoma

A large body of evidence exists describing differences in cellular plasma membrane composition that develop following oncogenic transformation by viruses or carcinogens (1, 2). One of the major constituents of the membrane that is altered after transformation is the membrane glycoconjugate (3, 4). These membrane glycoconjugates have been implicated in the control of cell division, intercellular associations, and metastasis (5–9). These implications are based on the observations that changes in the biochemical and organizational structures of membrane-bound carbohydrates or related membrane-bound glycosyltransferases (10) occur during cell aggregation, mitotic division, cell surface capping, malignant transformation, and in response to stimuli such as contact inhibition of cell movement. Therefore it may be therapeutically advantageous to alter or inhibit the biosynthesis

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of these tumor cell surface constituents. This might result in 1) tumor cell death caused by the inhibition of the biosynthesis of vital membrane components, 2) a change in tumor cell surface architecture leading to a reversion in cellular behavior to a more normal state, or 3) an increase in tumor cell immunogenicity due to the incorporation of sugar analogs into cell glycoconjugates.

Initial studies aimed at accomplishing these goals have utilized high concentrations of naturally occurring membrane sugars in hopes of creating a feedback inhibition on the biosynthesis of membrane glycoconjugates (11). The daily in vivo administration of large amounts of D-glucosamine to mice bearing experimental tumors resulted in an inhibition of tumor growth (12) while a continuous intravenous infusion of L-fucose resulted in a decrease in mammary tumor size in rats (13). The tumor toxicity of glucosamine has been attributed to a build-up of high concentrations of UDP-N-acetylhexosamines with a concomitant drop in intracellular UTP and ATP pool sizes (14). The effects of fucose on tumor growth have been attributed to an increase in tumor macromolecular-bound fucose. This increase is thought to effect a change in cell to cell communication resulting in a type of contact inhibition of tumor cell growth (15).

In addition to these studies using naturally occurring membrane sugars as antitumor agents, several reports have appeared describing the antitumor activity of various fluoro sugar analogs. 6-Deoxy-6-fluoro-D-glucose and 2-deoxy-2-fluoro-D-glucose affected leukemic cell growth in vitro but had little effect on tumor cell growth in vivo. The cytotoxic effects of these compounds may have been due to their inhibition of hexokinase since both were found to inhibit yeast hexokinase (16). Another fluoro sugar analog, 2-deoxy-2-(2-fluoroacetamido)-2-D-glucopyranose, has been shown to affect glycoprotein biosynthesis (17, 18) and increase the median life span of mice inoculated with L1210 leukemia by 25% (19). The only other sugar analog reported to have some antitumor activity was 2-deoxy-D-glucose (DOG), a glucose, mannose or, glucosamine analog.

DOG has been shown to lower intracellular adenine nucleotide pools (20) and to inhibit glycosylation in viruses (21) and mammalian cells (22). It has also been shown to be cytotoxic to tumor cells at low concentrations (23) and to alter cell surface architecture as detected by differences in plant-lectin induced agglutination (24). Along with these findings there have been reports that DOG is metabolized to a nucleotide-sugar and incorporated into membrane glycoconjugate (25).

We have extended these studies by synthesizing several fluorinated or acetylated sugar analogs such as 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranose or simply pentaacetylglucosamine (PAG), and assessing their antitumor activity, metabolism, and effects on ribonucleoside phosphate pools and on macromolecular biosynthesis in P288 murine lymphoma cells, in vitro.

METHODS

Syntheses of the Hexosamine Analogs

2-Trifluoroacetamido-2-deoxy-D-glucopyranose (F_3-GlcNac) was prepared according to Wolfrom and Conigliaro (26).

2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranose (β-PAG) was prepared by the method of Horton (27).

2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-galactopyranose (PAGAL) was prepared by the method of Stacey (28).

2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-mannopyranose (PAMAN) was prepared by the method of Levene (29).

Radioactive Syntheses

[U-^{14}C]-2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-glucopyranose [^{14}C-uniformly labeled on the sugar carbons]. A solution of D-[U-^{14}C]glucosamine hydrochloride, (0.049 mg, specific activity 232 mCi/mmole) in ethanol-water, 0.5 ml, (supplied by New England Nuclear Corporation, Boston, Massachusetts) was transferred to a round bottom flask and evaported to dryness on a rotary evaporator and then dried in vacuo over phosphorus pentoxide. The residue was dissolved in dry pyridine (0.7 ml) and mixed with anhydrous sodium acetate (2 mg) to convert the hydrochloride to the free base. After 15 min acetic anhydride (0.2 ml) was added, mixed thoroughly, and left overnight. Ice (0.5 g) was added and after 10 min the solution was evaporated to dryness in vacuo until free from pyridine. The residue was extracted several times with chloroform solution and evaporated to dryness. The product was further purified by thin layer chromatography over silica gel (HF-254, E. Merck, Darmstadt, Germany) by developing the plate with methanol:ethyl acetate (1:9). The product (R_f 0.65) was extracted from the scrapings of the silica gel zone between R_f of 0.5 and 0.8 with methanol:chloroform (1:9). After removal of the solvent the residue was taken up in chloroform, filtered and evaporated to dryness to afford pure [U-^{14}C]-2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-D-glucopyranose. Finally it was dissolved in ethanol (95%, 0.5 ml) as a stock solution.

In order to determine optimal synthetic conditions and the anomeric nature of the product, the synthesis was carried out with cold D-glucosamine hydrochloride and its NMR spectrum determined. The NMR spectrum indicated the presence of only the α-anomer in the reaction mixture as determined by the position and coupling constants of the anomeric proton (27). Further confirmation was obtained by the crystallization of the oil and comparison of its mp with an authentic sample of "α-PAG."

2-Acetamido[N-acetyl-1-^{14}C]-2-deoxy-1,3,4,6-tetra-O-acetyl-[O-acetyl-^{3}H]-α-D-glucopyranose. A solution of D-[1-^{14}C]-N-acetylglucosamine (0.049 mg, specific activity 31.6 mCi/mmole) in ethanol-water, 0.5 ml (supplied by New England Nuclear Corporation), was evaporated to dryness in a round bottom flask on a rotary evaporator and then dried in vacuo over phosphorus pentoxide. The residue was dissolved in dry pyridine (0.5 ml) and [^{3}H] acetic anhydride (2.56 mg, specific activity 400 mCi/mmole, as an 80% benzene solution supplied by New England Nuclear Corporation) and cold acetic anhydride (0.1 mg) was added. The thoroughly mixed solution was left at room temperature overnight. Ice (0.5 g) was added and after 15 min the solution was evaporated to dryness in vacuo. A trap of solid KOH was inserted between the flask and the pump. The residue, freed from acetic acid and pyridine, was extracted several times with chloroform. The chloroform solution was evaporated to dryness and purified by thin layer chromatography as described in the previous experiment. A stock solution was made by dissolving the compound in ethanol (95%, 0.5 ml). The ratio of ^{3}H:^{14}C was found to be 3:1.

Octanol/Water Partition Coefficients

One hundred milligrams of the compound were added to octanol (20 ml) and water (20 ml). The mixture was shaken in a separatory funnel and allowed to stand for 48 h at

21°C to reach equilibrium. The resulting water solution (19.0 ml) was evaporated to dryness and the solid dried over P_2O_5 to a constant weight. The concentration of the solute in octanol was determined by the difference and the ratio of octanol to water concentrations was calculated.

Cell Cultures

Murine P288 lymphoma cells were maintained as an ascites tumor in DBA/2J female mice. Periodically cells were aseptically removed from mice, washed twice in RPMI 1640 medium, and cultured in RPMI 1640 containing 10% fetal calf serum. These cultures were grown in a 90% air/10% CO_2 incubator. These cells maintained their viability in culture and in log phase had a generation time of approximately 13 h. Cells were maintained in culture for periods of 1–3 months whereupon new in vitro cultures were initiated from the in vivo line. In this manner some constant selective genetic pressure was maintained on the cell line.

Generally, cell cultures were initiated at concentrations of $5 \times 10^4 - 1 \times 10^5$ cells/ml. These cultures grew logarithmically to about 2×10^6 cells/ml without the addition of fresh medium. Routinely these cell cultures were fed fresh medium containing 100 μg/ml neomycin every 2–3 days. Mycoplasma contamination was monitored by using the fluorescent dye, 4′,6-diamidino-2-phenylindole, (DAPI), provided by Professor Otto Dann of the Institut für Angewandte Chemie der Friedrich-Alexander-Universität, Schuhstrasse 19, 852 Erlangen, West Germany, as an indicator of plasma membrane associated nucleic acids found in mycoplasma (30). No indication of mycoplasma contamination was evident. Mycoplasma contaminated TA3 mammary cells were used as a positive control. They were provided by Drs. J Laskin and M. T. Hakala.

Biological Testing Systems

A. Cell growth and viability. For routine drug testing P288 murine leukemic cells were suspended at approximately 10^5 cells/ml in fresh RPMI 1640 minus glucose containing 10% fetal calf serum. One milliliter aliquots were then transferred to disposable polyethylene test tubes and placed in the CO_2 incubator. One hour later sugar analogs were added to a final concentration of 1 mM (or as otherwise stated). Cell growth and viability were monitored at later times by use of a Coulter cell counter and Trypan blue dye exclusion, respectively.

B. Macromolecular biosynthesis. [^{14}C] Glucosamine, 2 μM (1.1×10^6 DPM, Amersham Corporation, Arlington Heights, Illinois, and [^{3}H] leucine, 0.37 mM, (2.6×10^6 DPM, New England Nuclear Corporation) were added to cell cultures to assess the effects of sugar analogs on glycoprotein and protein biosynthesis. Two, five, or twenty-four hours later incubations were terminated by the addition of 2 ml of 10% trichloroacetic acid (TCA). The acid insoluble radioactivity was washed twice with 10% TCA and the resulting pellet was dissolved in NaOH and its radioactivity quantitated by scintillation counting methods (31).

Metabolism of 2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-glucopyranose (PAG)

The metabolism of PAG in P288 cells was studied utilizing freshly prepared [U-^{14}C] PAG prepared by acetylation of D-[U-^{14}C] glucosamine hydrochloride (232 mCi/mmole, NEN) or by use of penta [^{3}H] acetyl [^{14}C] glucosamine, prepared by acetylation of D-[1-^{14}C]-N-acetylglucosamine (31.6 mCi/mmole, NEN) with [^{3}H] acetic anhydride (400 mCi/mmole, NEN) as previously described.

The incorporation of these radiolabeled PAG analogs into glycoconjugate was monitored as previously described. Chromatographic analysis of intracellular soluble radioactivity was performed on both chloroform and ethanol extracts of cells incubated in the presence of both these radiolabeled analogs. S + S orange ribbon paper was spotted with aliquots of these extracts and developed in pyridine:HAc:EtAc:H_2O (5:1:5:3). Radioactivity was detected by scintillation counting methods (31).

Ribonucleotide Pool Size Analysis

Ribonucleotide pool size analysis was performed on P288 cells treated with 1 mM glucosamine, α-PAG, β-PAG, N-acetylglucosamine, 2-trifluoroacetamido-2-deoxy-D-glucopyranose or 2-deoxyglucose for various periods of time. Approximately $5 \times 10^6 - 10^7$ treated cells were washed twice with 2 ml of RPMI 1640 and extracted with 6% perchloric acid (100 μl/10^7 cells) and centrifuged at 800 × g for 2 min. The supernatant (acid soluble fraction) was neutralized with 2 N KOH to a pH of 7.0. Formed precipitate was removed by centrifugation and an aliquot of the resulting supernatant was analyzed for its ribonucleotide content using a Dupont 830 high pressure liquid chromatographic system equipped with a 254 and 280 nm detector. Ten microliters of the supernatant was absorbed onto an ABX column (1 m × 2 mm, U shaped) and eluted at room temperature using a phosphate buffer gradient (0.0025 M, pH 3.0 to 0.5 M) at a flow rate of about 1 ml/min (Y. Rustum, unpublished results). The retention time of each cellular peak was compared to the retention times of a mixture of authentic ribonucleotide and nucleotide-sugar standards. Each peak was identified and automatically integrated on a Spectra Physics Model 23, 000-010 Autolab Minigrator. The sum total of all peak areas was also recorded.

RESULTS

Effects of Glucosamine Analogs on Tumor Growth and Synthesis

P288 cells incorporated 4–5 times more [^{14}C]glucosamine in medium depleted of glucose (Table I). Incorporation increased more than fivefold from 864 to 4,815 dpm in a 5-h incubation and more than fourfold in a 24-h incubation from 4,815 to 21,030 dpm. The viability, growth, and incorporation of leucine by cells maintained in RPMI 1640 minus glucose plus 10% fetal calf serum remained at approximately the same level as cells grown in RPMI 1640 containing 2 mg/ml glucose plus 10% fetal calf serum. Cells maintained in the complete absence of glucose (none provided by serum) stopped dividing within 24 h and their viability dropped to 79%. Therefore, all further testing was performed in RPMI 1640 minus glucose plus 10% fetal calf serum.

The effects of the addition of various hexosamine analogs (1 mM) on cell growth, viability, and the incorporation of glucosamine and leucine was investigated (Table II). It was found that most of the compounds were relatively noncytotoxic with the exception of β-PAG (III) which reduced cell number to one half and cellular viability to 83% within 24 h. Most of the other compounds eventually reduced cell number within 48 h as compared with the control (Fig. 1) with the exception of N-acetylglucosamine (II) which had no effect on cell growth or viability (not shown). All the pentaacetylhexosamines [α-and β-pentaacetylglucosamine (III and IV), pentaacetylgalactosamine (V) and pentaacetylmannosamine (VI)] and 2-trifluoro-N-acetylglucosamine (VII) specifically depressed

TABLE I. P288 Cell Growth and Incorporation of Glucosamine and Leucine in Glucose Free Medium

Medium[a]	Incubation time (h)	Cell number (cells/ml × 10^{-5})	Incorporation[c] (dpm)	
			[^{14}C]glucosamine	[^{14}C]leucine
RPMI 1640 + 10% FCS	5	1.18	864	5,313
RPMI 1640 + 10% FCS	24	2.87	4,815	37,582
RPMI 1640 + 10% HI dialyzed FCS	24	2.57	5,242	38,742
RPMI 1640 minus glucose + 10% FCS	5	1.13	4,573	4,914
RPMI 1640 minus glucose + 10% FCS	24	2.25	21,030	31,475
RPMI 1640 minus glucose + 10% HI dialyzed FCS	24	2.04	28,420	32,472

[a]Approximately 1 × 10^5 P288 cells were suspended in 1 ml of RPMI 1640 medium containing 10% fetal calf serum (FCS), or RPMI 1640 medium free of glucose plus 10% FCS or 10% heat-inactivated (HI, 56°C, 1 h), dialyzed (glucose free) FCS at time zero. Five or twenty-four hours later the incubation was terminated and the cell number determined with a Coulter counter.

[b]Cells were clumped when dialyzed FCS was used.

[c][U-^{14}C]glucosamine, 2 μM (1.1 × 10^6 dpm) or [1-^{14}C]leucine, 0.37 mM (1.1 × 10^6 dpm), was added to separate cultures at time zero. Incubations were terminated by the addition of 10% TCA. Acid insoluble radioactivity was quantitated.

All data are the average of 3–4 experiments performed with duplicate 1-ml cultures.

[^{14}C]glucosamine incorporation into glycoprotein while, with the exception of β-PAG, all had very little effect within 5 h on [^{3}H]leucine incorporation into protein. Depression of [^{14}C]glucosamine incorporation was as follows: β-PAG > α-PAG > F_3-GlcNAc > α-PAGAL > β-PAMAN ≫ GlcNAc and 2-deoxyglucose which had little effect on [^{14}C]-glucosamine incorporation.

Any of the sugar analogs manifesting effects in these test systems at 1 mM were then tested at lower concentration of 10^{-4} and 10^{-5} M. In all cases no effects were noted at 10^{-5} M after 24 h and slight effects were noted at 10^{-4} M. As an example, the effects of β-PAG at 10^{-3}, 10^{-4}, and 10^{-5} M on cell growth and macromolecular synthesis are shown in Table III. The effect of β-PAG on cell growth was not influenced by the concentration of glucose in the medium while its effect on glucosamine incorporation may have been amplified in low glucose containing medium. Little effect on cell growth was noted at 10^{-4} M after 24 h but became more apparent at 48 h.

Increasing incubation times to 48 h in the presence of 1 mM glucosamine or α- or β-PAG resulted in both a further decrease in cell growth and viability for cells treated with either α- or β-PAG but also with D-glucosamine (Fig. 1). At 24 h, cell viabilities for the control were 98%, glucosamine 98%, α-PAG 98%, and β-PAG 83%; at 48 h viabilities were control 95%, glucosamine 67%, α-PAG 92%, and β-PAG 19%. Forty-eight-hour exposures to 1 mM 2-deoxy-D-glucose also resulted in a decrease in cellular growth and viability intermediate between the effects of glucosamine and α-PAG (Fig. 1).

TABLE II. Effects of Various Sugar Analogs on P288 Mouse Lymphoma Cells

Compound[a]		Incorporation (% control)[b]				Growth[c] (% control)
		[^{14}C]glucosamine		[^{3}H]leucine		
		2 h	5 h	2 h	5 h	24 h
I.	2-Amino-2-deoxy-D-glucopyranose (Glc NH_2)	–	–	97	95	99
II.	2-Acetamido-2-deoxy-D-glucopyranose (GlcNAc)	95	97	93	97	103
III.	2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D-glucopyranose (β-PAG)	33	10	77	62	53
IV.	2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-glucopyranose (α-PAG)	30	23	90	91	88
V.	2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-galactopyranose (α-PAGAL)	64	59	103	98	85
VI.	2-Acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-β-D mannopyranose (β-PAMAN)	76	77	96	91	83
VII.	2-Trifluoroacetamido-2-deoxy-D-glucopyranose (F_3-GlcNAc)	–	31	97	88	87
VIII.	2-Deoxy-D-glucopyranose (DOG)	90	98	89	96	92

[a]Approximately 1 × 10^5 P288 cells were suspended in 1 ml of glucose free RPMI 1640 medium containing 10% fetal calf serum and the indicated compound to a final concentration of 1 mM.
[b][^{14}C]glucosamine, 2 μM (1.1 × 10^6 dpm) and [^{3}H]leucine, 0.37 mM (2.6 × 10^6dpm) were added at time zero. At the indicated times cells were extracted twice with 2 ml of 10% trichloroacetic acid and the acid insoluble radioactivity was quantitated and expressed as a percent of control incorporation.
[c]Growth was monitored by counting aliquots of cells with a Coulter counter.

Partition Coefficients

The octanol/water partition coefficient for β-PAG was found to be higher [0.424] than that of α-PAG [0.278] and much higher than that of GlcNAc [0.017].

Metabolism of α-Pentaacetylglucosamine (PAG)

The incorporation and metabolism of [U-^{14}C]-2-acetamido-2-deoxy-1,3,4,6-tetra-O-acetyl-α-D-glucopyranose, specific activity 232 mCi/mmole, and 2-acetamido[N-acetyl-1-^{14}C]-2-deoxy-1,3,4,6-tetra-O-acetyl [O-acetyl-^{3}H]-α-D-glucopyranose, specific activity ^{14}C (31.6 mCi/mmole) and ^{3}H (95 mCi/mmole), was studied with P288 lymphoma cells.

Cells incubated with trace amounts of [^{14}C]PAG, 0.1 μCi/ml, for various time periods were analyzed for incorporation of the label into putative cellular glycoprotein (Fig. 2). Incorporation into lipid-free macromolecular material was nearly linear for 20 h. Examination of the soluble pool of radioactivity following a 48 h exposure to [^{14}C]PAG indicated that most of the ethanol soluble label cochromatographed with authentic UDP-N-acetylglucosamine (UDP-GlcNAc), Fig. 3, while the cellular chloroform extract contained authentic [^{14}C]PAG. Chromatography of the extracellular tissue culture medium revealed only authentic [^{14}C]PAG.

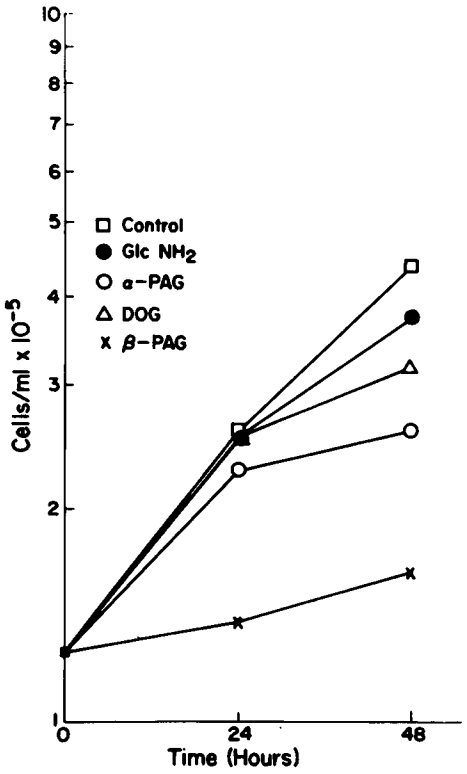

Fig. 1. The effects of various sugar analogs on P288 lymphoma cell growth. Glucosamine ($GlcNH_2$) and the other sugar analogs α- or β-pentaacetylglucosamine (PAG) and 2-deoxyglucose (DOG) were added at a final concentration of 1 mM to duplicate 1 ml P288 leukemic cell cultures. Cell growth and viability was monitored at 24 and 48 h as indicated in the text.

Similar experiments were performed with the double labeled [^{14}C,^{3}H]PAG. Cellular medium contained 2 μCi ^{3}H and 0.65 μCi ^{14}C per ml. Incorporation into acid insoluble material and chloroform or ethanol soluble pools were examined. A very rapid incorporation or exchange of some ^{3}H-labeled material into the acid insoluble pool occurred within the first few minutes of drug exposure. Overlooking this the ratio of incorporated ^{14}C:^{3}H increased with time (2–24 h) suggesting intracellular de-O-acetylation occurred before its incorporation into cellular glycoconjugate. This was later confirmed by chromatograms of soluble ethanol extracts (Fig. 4A). Only ^{14}C was detected in areas corresponding to UDP-GlcNAc while ^{14}C and ^{3}H were detectable in areas corresponding to authentic PAG. ^{14}C and ^{3}H were detectable in chloroform extracts (Fig. 4B) in areas corresponding to authentic [^{14}C,^{3}H]PAG. The ratio of ^{14}C:^{3}H in the partially de-O-acetylated PAG or front region of the chromatogram of the cellular chloroform extract was diminished as compared with the authentic PAG. This suggests some rapid exchange of ^{3}H in the cell.

Ribonucleotide Pool Size Analysis

Ribonucleotide pool size analysis was performed in P288 murine leukemic cells treated with 1 mM glucosamine ($GlcNH_2$), α- or β-pentaacetylglucosamine (α- or β-PAG), N-acetylglucosamine (GlcNAc), 2-trifluoro-N-acetylglucosamine (F_3-GlcNAc) and 2-deoxyglucose (DOG) for periods of 1, 3, 5, and 24 h.

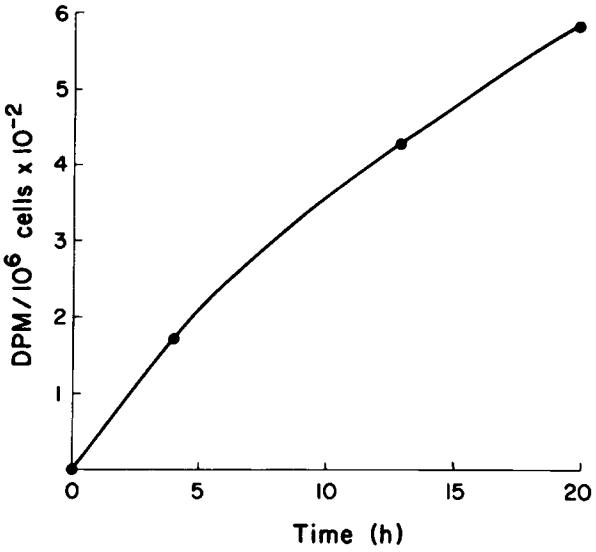

Fig. 2. Incorporation of [^{14}C]PAG in P288 murine lymphoma cells. Cells were seeded at 1×10^5/ml in RPMI 1640 medium plus 10% fetal calf serum containing 0.1 μCi [^{14}C]PAG/ml (specific activity, 232 Ci/mole). After 4, 12, and 20 h 25-ml cell aliquots were removed. The cell suspensions were centrifuged and extracted for 20 min with 2 ml of 1% phosphotungstic acid dissolved in 0.5 N HCl at 4°C. The mixtures were centrifuged and the resulting pellets washed with 2 ml of 5% TCA, centrifuged, and extracted with 2 ml of chloroform:methanol:ether (2:1:1). The resultant lipid free insoluble material was dissolved in 1.0 N NaOH and the radioactivity in this material was determined by scintillation counting methods.

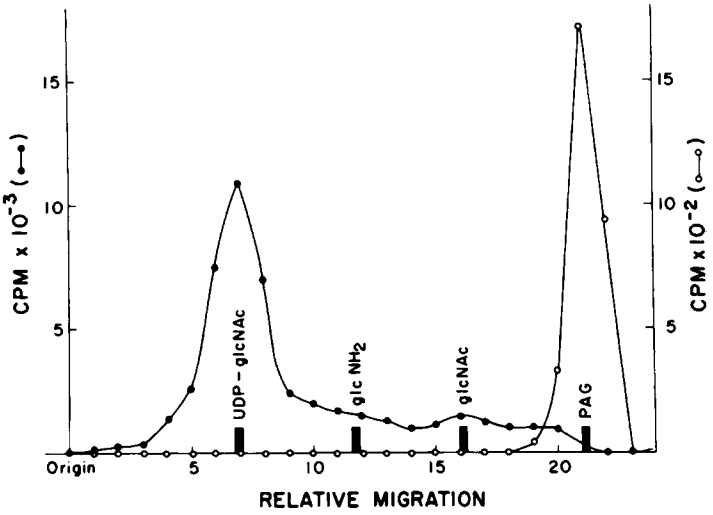

Fig. 3. Chromatography of P288 cell extracts. P288 murine lymphoma cells were incubated in the presence of [^{14}C]PAG (0.1 μCi/ml) for 48 h. Cells were harvested, washed twice with PBS, and extracted with 1 ml chloroform, centrifuged, and the pellet reextracted with 1 ml of 70% ethanol. Aliquots of these extracts were analyzed with descending paper chromatography using pyridine:HAc: EtAc:H_2O (5:1:5:3). Radioactivity was detected by scintillation counting methods. (•—•)ethanol extract; (○—○) chloroform extract.

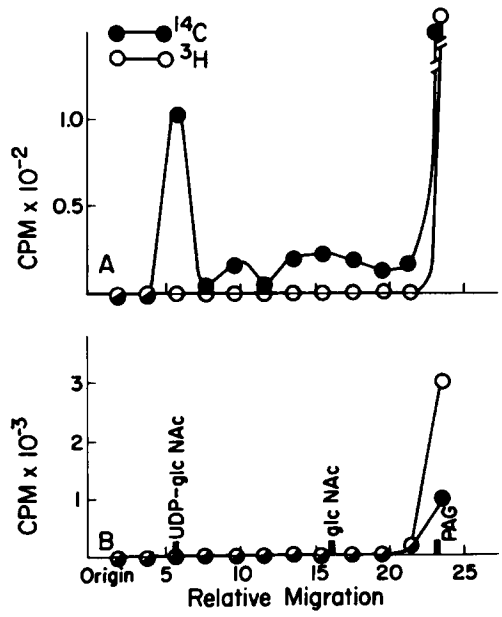

Fig. 4. Chromatography of P288 cell extracts. P288 murine lymphoma cells (10^5/ml) were incubated in the presence of [^{14}C,^{3}H]PAG, (^{14}C, 0.65 μCi/ml; ^{3}H, 2 μCi/ml) for 6 h. The ^{14}C is located in the N-acetyl group while the ^{3}H is located in the O-acetyl groups. Cells were harvested, washed twice with PBS, and extracted with 1 ml of chloroform, centrifuged, and the organic layer removed. The cellular pellet was then extracted with 1 ml 70% ethanol/H_2O. Chromatography of these extracts were performed as described in Fig. 3. A: Chromatogram of ethanol extract, (•—•) ^{14}C; (○—○) ^{3}H. B: Chloroform extract, (•—•) ^{14}C; (○—○) ^{3}H.

Treatment of P288 leukemic cells with 1 mM glucosamine for 3 h resulted in a large increase in the nucleotide-sugar pools (Fig. 5B and Table IV). The twin peaks probably consist mainly of UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine and some UDP-glucose. After 3 h this pool was increased fourfold over control and after 24 h an eightfold increase was evident. Other early changes in pool sizes were a large decrease in the UTP pool (to 36% control) and a concomitant large decrease in the CTP pool size (to 23% control). Smaller decreases in the purine triphosphate pools were seen (GTP, 52% and ATP, 57%) but these decreases were more variable and were not discernable within 1 h of incubation. Both β-PAG (Fig. 5C) and α-PAG (Fig. 5D) treatment resulted in increases in the nucleotide-sugar pools. After 3 h the nucleotide-sugar pool in the cells treated with β-PAG was 167% of control while α-PAG treated cells had 202% of control nucleotide sugars (Table IV). Decreases in the UTP and CTP pools to one third of the control were evident in cells treated with β-PAG while little change in these pools was evident at 3 h in cells treat with α-PAG. However specific decreases in UTP and CTP pools were evident at 5 h and very prominent within 24 h in cells treated with α-PAG.

N-Acetylglucosamine (GlcNAc) treatment of P288 leukemic cells resulted in little change in pool sizes while F_3-N-acetylglucosamine (F_3-GlcNAc) administration resulted in more than doubling of the intracellular UDP-sugar content (Fig. 6D and Table IV). The only apparent pool size affected by a 3-h exposure to 1 mM GlcNAc was an increase in the UDP-ADP pool size (Fig. 6B and Table IV) to twice control. Again this change was variable

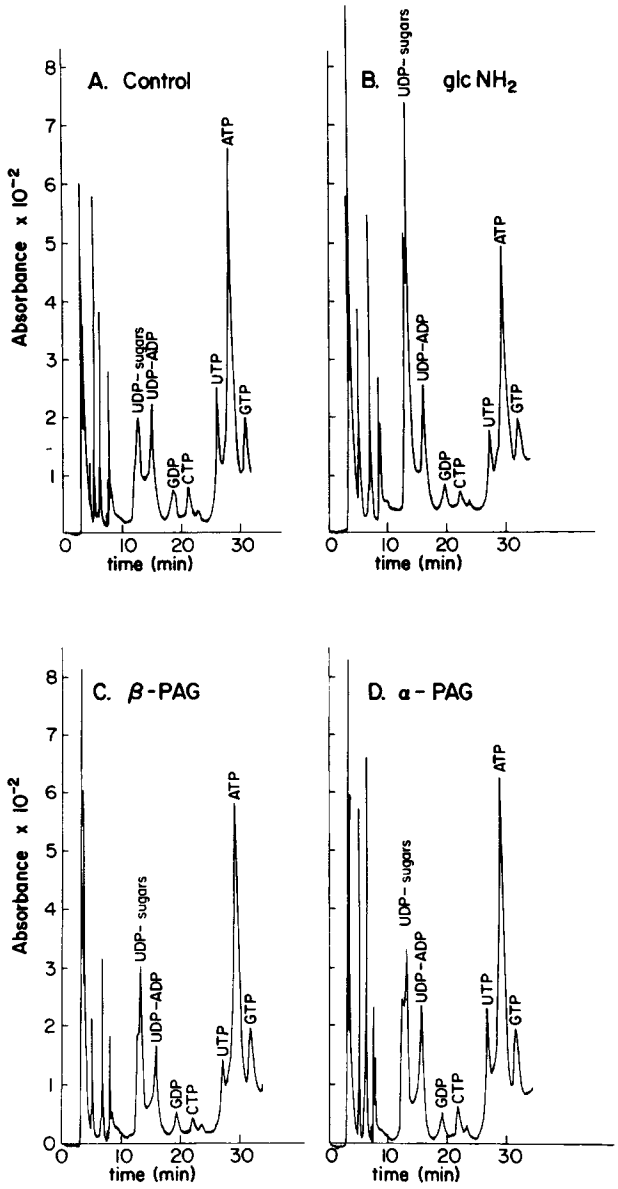

Fig. 5. High pressure liquid chromatography separation of intracellular nucleotide pools. Acid soluble extracts were prepared from 5 X 10^6 P288 leukemic cells. A) Control; B) treated with 1 mM glucosamine ($GlcNH_2$); C) β pentaacetylglucosamine (β-PAG); D) α-PAG for 3 h. Absorbance at 254 nm (0.0–0.08 units full scale) was monitored versus column retention time (min). Each peak was automatically integrated, normalized, and listed as the % control nucleotide pool (Table IV).

and was not evident in the other times studies. 2-Deoxy-D-glucose (Fig. 6C) treatment did affect pool sizes dramatically. Generally the ribonucleoside triphosphates were reduced (ATP and CTP to half of control) and the ribonucleoside diphosphates increased (GDP to more than 11 times control and UDP-ADP to more than eightfold higher). No increase in

TABLE III. Effects of β-PAG on P288 Lymphoma Cell Growth and Glucosamine Incorporation

	Incorporation of glucosamine (% control)[a]						Growth, % cell no.[c] β-PAG / control	
Time	2 h		5 h		24 h		24 h	
Glucose	+	−	+	−	+	−	+	−
β-PAG concentration (M)								
0	100[b]	100	100	100	100	100	100	100
10^{-3}	63	33	30	10	27	7	61	69
10^{-4}	82	99	74	73	85	50	95	86
10^{-5}	100	100	100	100	94	100	98	98

[a]Approximately 1×10^5 cells were suspended in 1 ml RPMI 1640 medium containing 11.1 mM glucose (+) or glucose free (−) plus 10% FCS. One hour later PAG was added to the desired final concentration and an additional hour later [^{14}C]glucosamine, 2 μM, (1.1×10^6 dpm), was added. Incubations were terminated with 2 ml 10% TCA and acid insoluble radioactivity was determined. Data are expressed as % control incorporation.

[b]Incorporation was calculated for each culture tube in duplicate and expressed as % dpm (drug treated/control). About a five-fold increase in glucosamine incorporation was apparent when glucose-free medium was employed. Growth was slightly lower in glucose-free medium as compared with control.

[c]Cells were seeded at 1×10^5 cells/ml at time zero. Twenty-four hours later cell number was determined with a Coulter counter. Data are expressed as % cell number (drug treated/control). Control cultures increased about 2.5 times in 24 h. All data are the average of 2–3 experiments.

TABLE IV. Effect of Various Sugars or Sugar Analogs on Nucleotide Pool Sizes

	Nucleotide pool size (% control)[a]					
	Sugar or sugar analog					
Nucleotide	$GlcNH_2$	GlcNAc	β-PAG	α-PAG	DOG	F_3-GlcNAc
GTP	52	73	80	88	83	88
ATP	57	103	91	86	53	98
UTP	36	117	33	82	80	103
CTP	23	110	32	86	47	110
GDP	84	122	73	95	1,122	83
UDP and ADP	100	200	100	165	845	118
UDP-sugars	434	108	167	202	83	252

[a]The % control nucleotide pool sizes were calculated from the integrated peak areas obtained from the high pressure liquid chromatograms scans, Fig. 5 and 6. To normalize the data each integrated peak was divided by the total integrated area and this value set to 100% for control cultures.

nucleotide-sugar pools was noted but this may be due to an inherent instability of UDP-2-DOG in acid extracts. Breakdown of large amounts of UDP-2-DOG may account for the high level of UDP and ADP found (Fig. 6C and Table IV). The high amount of GDP (11 times above normal) may also be accounted for by the instability of GDP-2-deoxyglucose in perchloric acid (21). At 0°C all the GDP-2-deoxyglucose is broken down to free 2-deoxyglucose within 5 min. Schmidt et al. (21) also concluded that after the administration of 2-deoxyglucose to virus-infected mammalian cells it is the GDP-2-deoxyglucose that is the agent responsible for the inhibition of glycosylation of viral glycoproteins.

DISCUSSION

It is evident that glucosamine rapidly enters tumor cells and is metabolized to UDP-N-acetylglucosamine and UDP-N-acetylgalactosamine resulting in a drop in both UTP and CTP pools (Fig. 5). The dramatic decrease in the CTP pool is not surprising since UTP

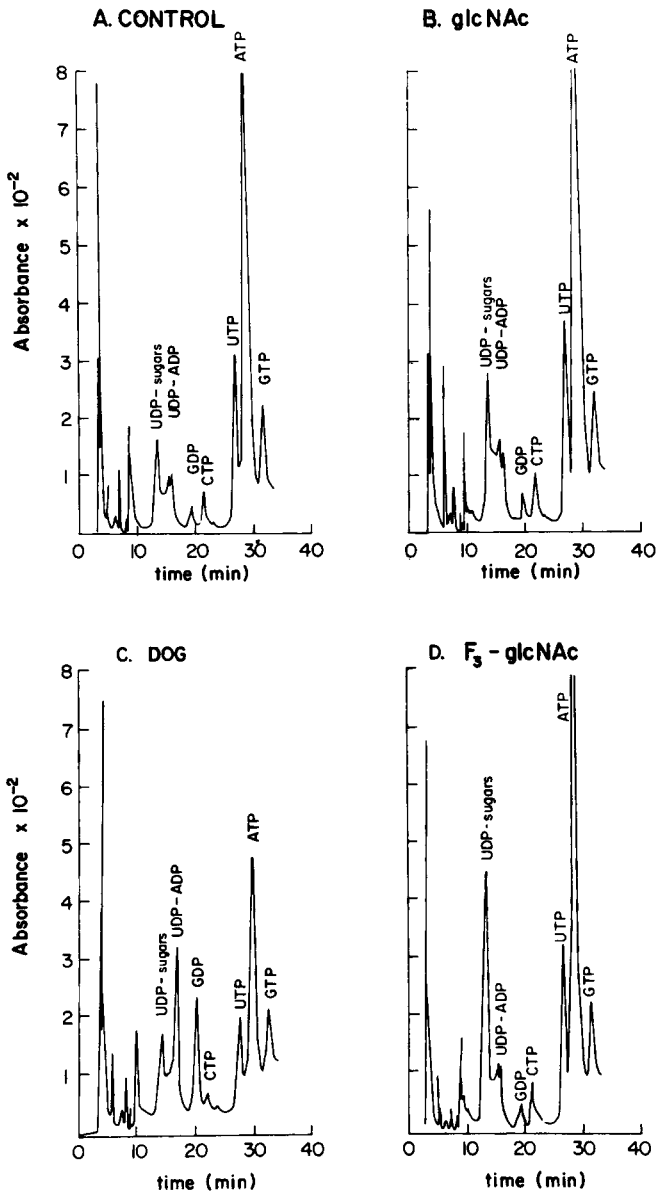

Fig. 6. High pressure liquid chromatography separation of intracellular nucleotide pools. Acid soluble extracts were prepared from 5 X 10^6 P288 leukemic cells, A) Control; B) treated with 1 mM N-acetylglucosamine (GlcNAc); C) 2-deoxy-D-glucose (DOG); D) 2-trifluoro-N-acetylglucosamine (F_3-GlcNAc). Absorbance at 254 nm was monitored versus column retention time (min). Each peak was automatically integrated, normalized, and listed as % control nucleotide pool (Table IV).

provides the de novo source of cytidine nucleotides through the CTP synthetase reaction (UTP + ATP + NH_3 = ADP + orthophosphate + CTP, E.C. 6.3.4.2). It is surprising that P288 murine leukemic cells are still able to grow at near normal levels (Fig. 1) for up to 48 h after the addition of 1 mM glucosamine even though the UTP and CTP pool sizes are diminished to one third normal within 3 h (Table IV). Recently this type of hexosamine induced nucleotide pool size reduction has been exploited in the chemotherapy of hepatic tumor cells in vitro (32). The addition of galactosamine and 3-deazauridine resulted in pharmacological synergism. This was a result of the enhancement of the toxicity of 3-deazauridine caused by the depletion of the normal intracellular UTP and CTP pools. These reductions were caused by the formation of large amounts of UDP-sugars (33). Therapeutic effects such as these may be further enhanced by agents such as β-PAG, a fully acetylated amino sugar analog which is more lipid soluble and has a greater inherent toxicity as compared to glucosamine or galactosamine.

It was found that β-PAG is de-O-acetylated and further metabolized to UDP-N-acetylglucosamine (Fig. 7) resulting in a concomitant lowering of the UTP and CTP pools. Its enhanced toxicity as compared to glucosamine or N-acetylglucosamine may be due to greater cellular uptake via passive diffusion since this fully acetylated sugar is more lipid soluble. The octanol/water partition coefficient for β-PAG was higher than α-PAG and much higher than that of GlcNAc. It is also possible that the enhanced toxicity of β-PAG may be due to its action as a potent inhibitor of hexokinase (34) although the addition of high concentrations of glucose to the cell medium did not reverse its toxicity (Table III). Whatever the case may be there is a difference in tumor toxicity between α- and β-anomers of PAG with the β form being much more toxic than the α form (Fig. 1). This may be due to the higher octanol/water partition coefficient for β-PAG compared to α-PAG resulting in the higher uptake of the former due to passive diffusion.

A comparison of the biological effects of F_3-GlcNAc (VII, Table II) and GlcNAc (II, Table II) is noteworthy. The addition of fluorine to the N-acetyl group permitted the compound to interfere with [^{14}C] glucosamine incorporation, decreasing it to 31% of control within 5 h (Table II). GlcNAc at a similar concentration of 1 mM did not interfere with [^{14}C] glucosamine incorporation and both compounds did not inhibit [^{3}H]-leucine incorporation. Ribonucleotide pool size analysis of P288 cells treated with 1 mM GlcNAc (Fig. 6B) or 1 mM F_3-GlcNAc demonstrated that F_3-GlcNAc was converted to nucleotide sugar whereas GlcNAc conversion was not detectable (Table IV), although it was probably occurring at a low rate. In 1 mM concentration GlcNAc was not

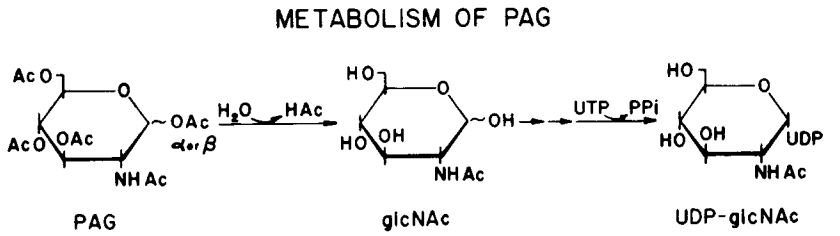

Fig. 7. Metabolism of PAG. The results of our studies have indicated that PAG (pentaacetylglucosamine) rapidly enters cells, and is de-O-acetylated to form naturally occurring N-acetylglucosamine (GlcNAc). The GlcNAc is then phosphorylated and later conjugated with UTP to form large amounts of UDP-GlcNAc.

cytoxic to P288 cells while F_3-GlcNAc (1 mM) did decrease cell number to 87% of control within 24 h and to 78% by 48 h. The differences in biological effects and metabolism of these compounds may be due to differences in cellular permeability or uptake as indicated by Bekesi et al. (35) or in rate of enzymatic phosphorylation reactions necessary in the formation of nucleotide sugar. Cellular uptake of glucosamine is carrier mediated as indicated by decreased macromolecular incorporation in the presence of glucose (Table I) while PAG and GlcNAc seem to rely on passive diffusion for entry into the cells. F_3-GlcNAc may be able to take advantage of the carrier mediated transport since it did compete with and was able to lower cellular glucosamine incorporation (Table II).

2-Deoxy-D-glucose, at 1 mM concentrations, was not a specific inhibitor of glycoprotein biosynthesis in P288 murine leukemic cells. In fact up to 24 h it had very little effect on any parameter studied except intracellular ribonucleotide pools which showed a dramatic decrease in ribonucleoside triphosphates along with a sharp increase in ribonucleoside diphosphates (Fig. 6, Table IV). There was no indication of further metabolism of 2-deoxy-D-glucose phosphate to UDP-2-deoxy-D-glucose as compared with the increases observed in UDP-sugars after glucosamine or α- or β-PAG administration (Figs. 5, 6). Since UDP-2-deoxy sugars and GDP-2-deoxyglucose are not stable in acid extracts, any nucleotide sugar formed might have broken down by the conditions used for the quantitation of intracellular ribonucleotide pools (21, 33).

Chemotherapy based on the inhibition or modification of plasma membrane glycoconjugate may indeed be feasible. Alterations in membrane glycoconjugate may stimulate immune responsiveness or inhibition of glycoconjugate biosynthesis may alter cellular behavior. Our first approach to this goal has been the synthesis of several simple acetylhexosamines. We were initially very excited when we observed that PAG caused a specific inhibition of macromolecular glucosamine incorporation with little or no inhibition of leucine. Further studies with acetylated sugars such as PAG indicated that once these moieties enter cells they are de-O-acetylated and further metabolized to nucleotide sugars (Fig. 7). Once deacetylated they can easily compete with and essentially dilute the radiolabeled GlcNAc pool synthesized from [^{14}C]glucosamine explaining the specific inhibition of [^{14}C]glucosamine incorporation. Once O-deacetylated, the GlcNAc can be converted to UDP-GlcNAc resulting in a reduction of the UTP and CTP pools. This specific ribonucleotide pool size reduction caused by PAG may be further exploited in combination chemotherapy with pyrimidine antagonists.

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REFERENCES

1. Nicolson GL: Biochim Biophys Acta 458:1, 1976.
2. Nicolson GL, Poste G: New Engl J Med 295:253, 1976.
3. Gahmberg CG, Hakomori S: Proc Natl Acad Sci USA 70:3329, 1973.
4. Warren L, Fuhrer JP, Buck CA: Proc Natl Acad Sci USA 69:1838, 1972.

5. Kraemer PM: In Manson LA (ed): "Biomembranes." New York: Plenum Press, 1964, vol 1, p 67.
6. Roseman S: In Weissman G, Claiborne R: "Cell Membranes, Biochemistry, Cell Biology and Pathology." New York: Hospital Practice, 1975, p 55.
7. Curtis ASG: In Butler JAV, Noble D (eds): "Progress in Biophysics and Molecular Biology." Oxford: Pergamon Press, 1973, vol 27, p 317.
8. Bosmann HB, Bieber GF, Brown AE, Case KR, Gersten DM, Kimmerer TW, Lione A: Nature (London) 246:487, 1973.
9. Bernacki RJ, Kim U: Science 195:577, 1977.
10. Porter CW, Bernacki RJ: Nature (London) 256:648, 1975.
11. Kornfeld S, Kornfeld R, Neufeld EF, O'Brien PJ: Proc Natl Acad Sci USA 52:371, 1964.
12. Quastel JH, Cantero A: Nature (London) 171:252, 1953.
13. Seltzer MH, Roseman JM, Wolfe DE, Tsou KC, Miller EE, Rosato FE: Growth 33:353, 1969.
14. Bekesi JG, Winzler RJ: J Biol Chem 244:5663, 1969.
15. Cox RP, Gessner BM: Cancer Res 28:1162, 1968.
16. Bessel EM, Courtenay VC, Foster AB, Jones M, Westwood JH: Eur J Cancer 9:463, 1973.
17. Schultz AM, Mora PT: Carbohydr Res 40:119, 1975.
18. Kent PW, Mora PT: In Kent PW (ed): "Membrane Mediated Information." New York: American Elsevier Publishing Company, 1973, vol 1, p 129.
19. Pero RW: Babiarz P, Rittman L, Simon P, Fondy TP: 170th National Meeting, American Chemical Society, Chicago, Illinois, August, 1975.
20. McComb RB, Yushok WD: Cancer Res 24:193, 1964.
21. Schmidt MFG, Schwartz RT, Scholtissek C: Eur J Biochem 70:55, 1976.
22. Havell EA, Vilcek J, Falcoff E, Berman B: Virology 63:475, 1975.
23. Myers MW, Sartorelli AC: Biochem Biophys Res Commun 63:164, 1975.
24. Hwang KM, Sartorelli AC: Biochem Pharmacol 24:1149, 1975.
25. Steiner M, Somers K Steiner S: Biochem Biophys Res Commun 61:795, 1974.
26. Wolfrom ML, Conigliaro PJ: Carbohydr Res 11:63, 1969.
27. Horton DJ: J Org Chem 29:1776, 1964.
28. Stacey M: J Chem Soc 272, 1944.
29. Levene PA: J Biol Chem 57:323, 1923.
30. Russell WC, Newman C, Williamson DH: Nature (London) 253:461 (1975).
31. Bernacki RJ: Eur J Biochem 58:477, 1975.
32. Jackson RC, Williams JC, Weber G: Cancer Chemother Rep 60:835, 1976.
33. Keppler DO, Rudigier JFM, Bischoff E, Decker KFA: Eur J Biochem 17:246, 1970.
34. Korytnyk W, Bernacki RJ, Danhauser L, Hanchak M, Paul B, Rustum Y, Sharma M, Sufrin J: Fed Proc Fed Am Soc Exp Biol 35:1639, 1976.
35. Bekesi JG, Molnar Z, Winzler RJ: Cancer Res 29:353, 1969.