INDUCTION OF TYROSINE AMINOTRANSFERASE BY D-GLUCOSAMINE IN RAT HEPATOMA CELL CULTURE

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Summary. Tyrosine aminotransferase (TA) was induced 5-8 fold by D-glucosamine (0.025M) in rat hepatoma cells grown in culture. This effect was observed only in a D-glucose deficient incubation medium. Under these conditions D-glucosamine cannot replace D-glucose as a carbon source as evidenced by the marked reduction in protein and DNA in these cultures over a 72 hour period. Although total protein per culture decreased, there was a net increase in TA which was sensitive to actinomycin-D and cycloheximide treatment. N-acetylglucosamine was without effect under these conditions.

Several studies have indicated that tyrosine aminotransferase (TA) in rat hepatoma cells grown in culture responds to glucocorticoid hormones in a manner similar to TA in rat liver (1,2). Other characteristics shared by the intact liver and these hepatoma cells with respect to TA activity include the following: (a) elevated enzyme activity is maintained as long as hormone is present (3,4); (b) rapid loss of TA activity occurs after hormone washout (3,4); and (c) inhibitors of protein synthesis such as actinomycin-D and puromycin prevent TA induction (3,5). Based upon such observations, it has been assumed that the regulatory mechanisms operative for TA in rat liver also exert significant control of this enzyme under tissue culture conditions. The hepatoma cells offer a distinct advantage for studying regulation of

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this enzyme in that carbon sources (sugars) and nitrogen sources (amino acids) required for maintenance and growth of these cells can easily be adjusted. In one phase of our work, in which we were studying the relationship between carbohydrate metabolism and TA activity, it was observed that D-glucosamine could increase the activity of this enzyme about 5-8 fold. The present report concerns itself with this observation and the cytotoxic effects of glucose starvation and D-glucosamine on Reuber H-35 rat hepatoma cells.

Materials and Methods. Approximately 4×10^6 rat hepatoma cells (Reuber H-35) were inoculated into glass culture bottles containing 6 ml of enriched Dulbecco's Modified Eagle Medium* (Grand Island Biological Co., Grand Island, New York) and incubated at 37°C in an atmosphere of 10% CO_2 and 90% balanced air. After 2 days the medium was decanted and replaced with fresh medium. Incubation was continued as before for 2 additional days. At the end of this period the regular medium in each culture bottle was replaced with an experimental medium.

Addition of either D-glucosamine or N-acetylglucosamine to glucose-free Dulbecco medium (the glucose-free medium was also obtained from Grand Island Biological Co.) was accomplished by first passing a concentrated solution of these substances through a Millipore filter to sterilize them. A sufficient quantity of each was added to the various media to make a final concentration of 0.025 M. When D-glucosamine and D-glucose were used in com-

*The medium was supplemented so that to 85 ml of medium was added; 15 ml fetal calf serum, 10,000 units penicillin, and 10 mg streptomycin. When glucose deficient medium was used dialyzed fetal calf serum replaced regular fetal calf serum.

bination the concentration of each was 0.0125 M. The D-glucosamine containing medium was neutralized by adding sterile $NaHCO_3$ dropwise (per 6 ml of medium, approximately 0.1 - 0.2 ml of a solution containing 75 mg/ml was used) until the pH was adjusted to about 7.4 using phenol red as an indicator.

After incubating the cells in the desired medium for various periods of time they were harvested by first decanting the medium and then rinsing the cultures with 6 ml of warm (37°C) buffer containing 8 g NaCl, 0.4 g KCl, and 0.4 g $NaHCO_3$/liter. The cells were readily detached from the culture bottles by treating them for 5 min with a warm (37°C) solution of the above buffer containing 0.033% trypsin and 0.02% EDTA. After decanting the cell suspensions they were centrifuged for 3 min at 1000 rpm. The packed cells were suspended in 1.5 ml of ice cold sodium phosphate buffer (0.1M, pH 7.5)and centrifuged for 1.5 min at 1000 rpm. This wash procedure was repeated three times. After the last centrifugation the supernatant was discarded and the packed cells stored at -15°C.

For analysis, the cells were suspended in 2.0 ml of the above phosphate buffer and sonicated with two 15 sec bursts (65 watts) with a Bronson Sonifier. The disrupted cell suspension was centrifuged at 20,000 x g for 20 min. The supernatant was analyzed for TA activity by the spectrophotometric method of Lin et al. (6) and for protein by the Lowry procedure (7). The residue was analyzed for DNA content by the Burton procedure (8) as outlined by Grossman and Mavrides (4).

Results and Discussion. The data in figure 1 indicate that D-glucosamine causes approximately an 8-fold increase in TA activity when hepatoma cells are incubated for 72 hours in a D-glucose deficient medium. In the presence of D-glucose the

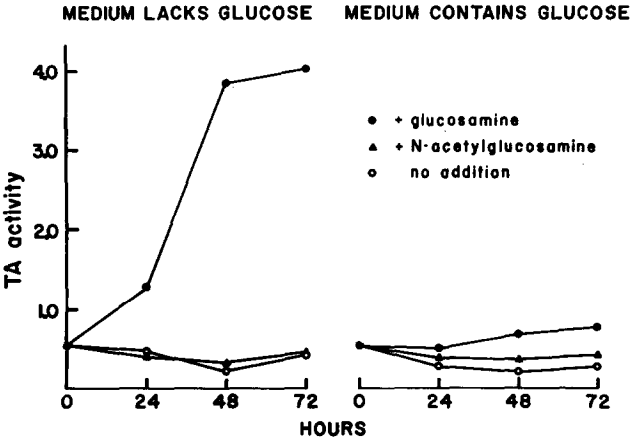

Figure 1. Equal numbers of cells (approx. 4×10^6) were inoculated into a series of culture bottles and grown in enriched Dulbecco medium for 4 days at 37°C. The medium was changed at the end of the second day of this initial incubation period. Following this 4 day period two cultures were collected (t_0) and analyzed as described in the Methods Section. The remaining cultures were incubated for an additional 24, 48 and 72 hours in media which contained: (a) regular medium, D-glucose content 0.025M; (b) medium with 0.0125M D-glucose plus 0.0125M D-glucosamine; (c) medium without D-glucose; and (d) medium without D-glucose plus 0.025M D-glucosamine. The data reported are averages of duplicate cultures. Tyrosine aminotransferase (TA) activity is expressed as μmoles of p-hydroxyphenylpyruvate formed per hour per 100 μg DNA.

effect of D-glucosamine is considerably diminished. N-acetylglucosamine showed no significant effect on TA activity in regular or D-glucose deficient media. Bekesi et al. (9) reported that N-acetylglucosamine did not alter viability or transplantability of Sarcoma 180 ascites tumor cells, while D-glucosamine markedly inhibited both parameters. Their tentative conclusion was that neoplastic cells are relatively impermeable to N-acetylglucosamine. The present results are consistent with this interpretation.

The data in Table 1 indicate that rat hepatoma cells are sensitive to D-glucose deficiency as evidenced by the significant reduction in protein and DNA per culture. To a great extent this

represents death of cells rather than a reduction of these substances within the entire cell population since microscopic examination of these cultures indicated a diminished cell density. The absence of D-glucose, however, did not induce TA activity (Table 1). To the contrary, TA activity diminished more or less proportionately to the decline in total protein. Only after substitution of D-glucose with D-glucosamine was there a substantial increase in TA activity which occurred despite a considerable decrease in cell population. It is particularly interesting to note that D-glucosamine caused a disproportionate decline in DNA content as compared to protein content of these cultures. As shown in Table 1 the ratio of these substances (mg protein per 100 μg DNA)

Table 1. The effects of glucosamine on tyrosine aminotransferase activity, protein, and DNA content of rat hepatoma cells incubated in the presence and absence of glucose.

Medium	Time (hr)	TA activity (μmoles/hr)	Protein (mg)	DNA (ug)	mg protein per 1000 ug DNA
	t_0	0.98	2.67	180	1.49
	t_{24}	0.73	3.21	236	1.36
+glucose	t_{48}	0.55	3.18	254	1.26
(regular)	t_{72}	0.84	3.60	283	1.27
	t_{24}	0.89	3.00	185	1.70
+glucose	t_{48}	1.18	2.94	169	1.79
+D-glucosamine	t_{72}	1.53	3.03	195	1.56
	t_{24}	0.79	2.23	179	1.25
-glucose	t_{48}	0.38	1.81	113	1.60
	t_{72}	0.46	1.69	100	1.70
	t_{24}	1.90	2.36	150	1.61
-glucose	t_{48}	2.64	1.86	70	2.70
+D-glucosamine	t_{72}	1.34	0.88	33	2.67

The details of the experimental conditions are the same as those indicated in the legend of Figure 1. Tyrosine aminotransferase (TA) activity, protein, and DNA values are expressed as averages of duplicate cultures. The means of the duplicate cultures did not vary on the average by more than 6% for TA activity, 3% for protein and 8% for DNA.

increased, suggesting reduced DNA synthesis and/or enhanced DNA lability which would cause loss of DNA during incubation, cell collection and subsequent sonication. The former possibility seems more likely since Bekesi et al. (9) refer to investigations in press which demonstrate inhibition of DNA synthesis by D-glucosamine in neoplastic tissue.

The inhibition of DNA synthesis (or enhanced degradation) by D-glucosamine was also observed in the presence of D-glucose (Table 1). In these experiments there was relatively little change in total protein content while DNA decreased about 20-30%. Under these conditions, however, there was only a modest increase in TA activity. The extent to which synthesis of TA is stimulated by D-glucosamine may be related to DNA turnover within the cells. As shown in Table 11, both actinomycin-D and cycloheximide inhibited D-glucosamine induction of TA, and also prevented extensive reduction of DNA content within these cells.

Table 11. Inhibition of glucosamine induction of tyrosine aminotransferase by actinomycin-D and cycloheximide.

Medium	TA activity μmoles/hr	Protein mg	DNA μg	Activity per 100 μg DNA
-D-glu	1.04	3.12	164	0.64
-D-glu + D-glu NH_2	2.11	2.81	112	1.99
-D-glu + D-glu NH_2 + Act D[1]	0.96	2.65	134	0.66
-D-glu + D-glu NH_2 + Cyclo[2]	0.86	3.10	142	0.60

The details of this experiment are similar to those indicated in the legend of Figure 1. The average values for duplicate cultures were obtained 24 hours after changing the medium from regular Dulbecco Modified Eagle Medium to a similar medium containing or lacking the indicated constituents. -D-glu indicates a D-glucose deficient medium. +D-glu NH_2 indicates the addition of D-glucosamine. The concentrations of actinomycin-D[1] and cycloheximide[2] were both 0.2 μg/ml medium.

Although the mechanism of D-glucosamine induction of TA is unclear, it is apparent that this enzyme is not only preserved, but also stimulated, during periods of extensive cell catabolism. In this respect D-glucosamine induction is similar to glucocorticoid induction of TA. It is also possible that TA is a glycoprotein and the presence of D-glucosamine allows for more rapid completion of enzyme molecules. The diminished effectiveness of D-glucosamine in the presence of D-glucose may merely represent competition for transport into the cell. Investigations in these areas are being continued.

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