The inhibitory effect of D-glucosamine on thymidine kinase in chick embryo retinas and HeLa cells

G. Tesoriere, L. Tesoriere, R. Vento, M. Giuliano and G. Cantoro

Istituto di Chimica Biologica, Università di Palermo, Policlinico, I-90127 Palermo (Italy), 2 August 1983

Summary. D-Glucosamine markedly inhibits thymidine incorporation into the TCA-insoluble fraction and thymidine kinase activity in HeLa cells. Both the inhibitory effects are also observed in isolated retinas of chick embryos. In this case the inhibitory effects are age-dependent and the magnitude of the responses decreases with embryonic development. In addition the time of exposure to D-glucosamine which is necessary to reveal the inhibitory effect on thymidine kinase increases with the age of the embryos.

Many authors[1-7] have observed that D-glucosamine has a selective toxic effect on neoplastic tissues. In mouse leukemic cells[4] and in sarcoma 180 ascites tumor cells[3,5], the amino sugar inhibits protein and nucleic acid biosynthesis and reduces the levels of uridine and adenine nucleotide pools. It has been suggested that the inhibitory effect of D-glucosamine might be a consequence of 2 distinct phenomena: a) a marked utilization of ATP for amino sugar phosphorylation, greater than the capacity of cells to regenerate ATP via glycolysis[6,7]; b) a marked fall in UTP level caused by intracellular accumulation of UDP-N-acetyl-glucosamine[4,8]. Friedman et al. observed that in glial tumor cells the amino sugar caused a decreased rate of utilization of exogeneous thymidine for cellular DNA synthesis[9] and inhibits thymidine kinase, the initial enzyme in the thymidine salvage pathway[10]. In chick embryo retina, also, the amino sugar inhibits the protein and nucleic acid biosynthesis[11,12] and these effects seem to be a consequence of a marked decrease in the uridine nucleotide pool.

In the work described in this paper we intended to ascertain whether in embryonal retina, as in tumor cells, D-glucosamine reduces the utilization of exogeneous thymidine and the level of thymidine kinase activity.

Materials and methods. Biochemical compounds were supplied by Sigma Chemical Co., Missouri, USA. Radioactive thymidine was obtained from Sorin, Saluggia, Italy. Anion exchange resin AG 1-X8, 200–400 mesh, formate form, was obtained from BioRad Lab., California, USA.

HeLa TK cells were grown in Eagle's minimum essential medium, containing 10% calf serum, in a 37 °C incubator equilibrated with a 5% CO_2-95% humidified air atmosphere. Cells were harvested for the experiments by replacing the growth medium with a solution of Versene for 15 min at 37 °C and collecting the cells by centrifugation at 500 × g for 5 min. Aliquots of 5×10^5 cells were employed for each incubation sample.

Neural retinas were dissected from chick embryos incubated for 9, 10, 12, 14, 16 and 18 days. HeLa cells or individual retinas were placed in 3 ml of Krebs bicarbonate Ringer pH 7.6 supplemented with 5 mM glucose. Samples were incubated in the presence or absence of neutralized D-glucosamine (20 mM) for various intervals of time at 37 °C.

To evaluate the incorporation of thymidine into the TCA-insoluble fraction, at the end of the incubation time 2 μCi of [Me-^{3}H] thymidine were added and the incubation was continued for 15 min at 37 °C. At the end 2 identical samples were pooled and centrifuged at 10,000 rpm for 5 min at 4 °C. TCA

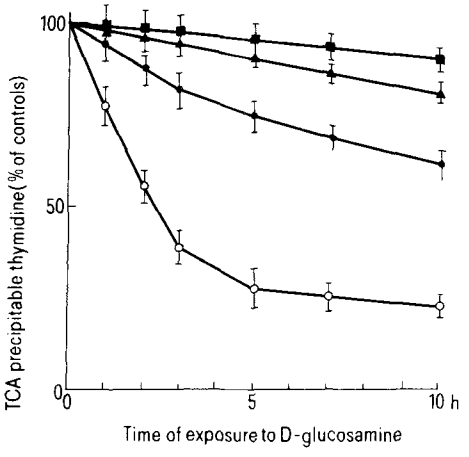

Figure 1. Influence of D-glucosamine on thymidine incorporation into TCA-insoluble fraction, in chick embryo retinas and HeLa cells. Retinas from chick embryos on the 9th (●—●), 14th (▲—▲) and 18th (■—■) day or HeLa cells (○—○) were incubated at 37 °C for various times in the presence of 20 mM D-glucosamine under the conditions reported in the text. Incorporation expressed as cpm/mg of protein is referred as percentage of the non-glucosamine-treated controls. Each point represents the mean ± SE of 4 separate experiments.

Effect of D-glucosamine and its metabolites on thymidine kinase activity in cell-free extracts of retinas from 9- or 14-day chick embryos and HeLa cells. Values are the mean ± SE of 5 separate experiments

Addition	mM	nmoles of d-TMP formed/mg of protein					
		9-day chick embryo	% inhibition	14-day chick embryo	% inhibition	HeLa cells	% inhibition
None		2.80 ± 0.15		1.10 ± 0.05		104 ± 5.2	
D-Glucosamine	5	2.70 ± 0.14	3.5	1.10 ± 0.05	0.0	100 ± 5.2	3.8
	10	2.60 ± 0.14	7.1	1.05 ± 0.05	4.5	85 ± 4.2	18.2
	20	2.40 ± 0.12	14.3	1.05 ± 0.05	4.5	74 ± 3.7	28.8
D-Glucosamine-6-phosphate	5	2.75 ± 0.14	1.7	1.10 ± 0.05	0.0	90 ± 4.5	13.4
	10	2.70 ± 0.14	3.5	1.05 ± 0.05	4.5	75 ± 3.7	27.8
	20	2.25 ± 0.10	20.0	0.90 ± 0.04	18.1	65 ± 3.2	37.5
N-Acetylglucosamine	20	2.70 ± 0.14	3.5	1.10 ± 0.05	0.0	100 ± 5.0	3.8
UDP-N-Acetylglucosamine	20	2.85 ± 0.15	0.0	1.10 ± 0.05	0.0	105 ± 5.2	0.0

precipitable thymidine, measured as described previously[12], was expressed as cpm/mg of protein.

To evaluate thymidine kinase activity, after the incubation, 6 retinas for each determination were homogenized at 1000 rev/min (20 strokes) in 1 ml of 10 mM Tris HCl buffer pH 8.0. HeLa cells resuspended in the same buffer were sonicated in a MSE Mullard Sonifier at 4 °C. 100 µl of homogenate or 50 µl of sonicate were incubated for 60 min at 37 °C in a final volume of 250 µl with 20 mM ATP, 40 mM Tris HCl buffer pH 8.0, 5 mM $MgCl_2$, 40 µM [Me-^{3}H] thymidine (0.5 µCi). The reaction was stopped by means of 100 µl of 10% TCA and the d-TMP formed was measured as previously reported[13]. Activity was expressed as nmoles of d-TMP/mg of protein. Protein was estimated by the method of Lowry et al.[14] using serum albumin as standard.

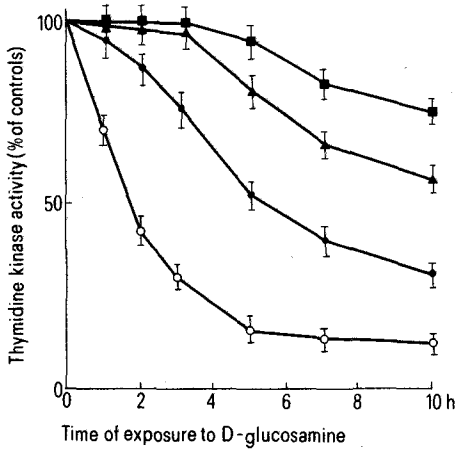

Figure 2. Influence of D-glucosamine on thymidine kinase from chick embryo retinas and HeLa cells. Retinas from chick embryos on the 9th (●—●), 14th (▲—▲) and 18th (■—■) day or HeLa cells (○—○) were incubated at 37 °C for various times in the presence of 20 mM D-glucosamine under the conditions reported in the text. Values, expressed as nmoles of d-TMP formed/mg of protein, are referred to as a percentage of the non-glucosamine-treated controls. Each point represents the mean ± SE of 4 separate experiments.

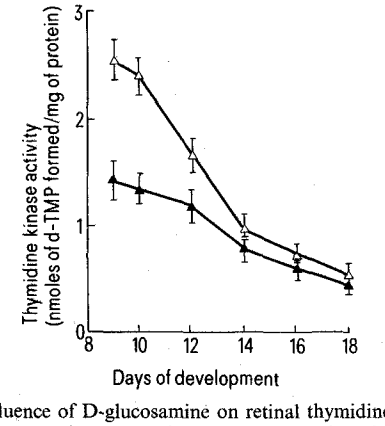

Figure 3. Influence of D-glucosamine on retinal thymidine kinase from chick embryos at various ages of development. Retinas from chick embryos at various ages were incubated at 37 °C for 5 h under the conditions reported in the text in the presence of 20 mM D-glucosamine (▲—▲) or in its absence (△—△). Values, expressed as nmoles of d-TMP formed/mg of protein, are the mean ± SE of 4 separate experiments.

Results and discussion. As shown in figure 1, in isolated retinas from chick embryos D-glucosamine causes a decrease of thymidine incorporation into the TCA(trichloroacetic acid)-insoluble fraction, which was smaller the older the embryo was. After 10-h exposure to 20 mM D-glucosamine, TCA-precipitable thymidine was inhibited in 9-, 14- and 18-day retinas by respectively 40, 20 and 10% as compared to non-glucosamine-treated controls. This effect was much lower than that observed by Friedman[9] in rat C6 glioma cells, where approximately 3 h of incubation with D-glucosamine inhibited the incorporation by 65–90%. That the amino sugar reduces thymidine incorporation into the TCA-insoluble fraction markedly in tumor cells was confirmed by us in HeLa cells. As shown in figure 1, an exposure of intact HeLa cells for 3 h to 20 mM D-glucosamine was sufficient to reduce TCA-precipitable thymidine by 60%.

D-Glucosamine also inhibits thymidine kinase in isolated retinas. This effect was clearly age-dependent. In fact, the younger the chick embryo was, the shorter the exposure to D-glucosamine that was necessary to reveal the inhibitory effect (fig. 2). Further, the magnitude of the inhibition decreased with increasing age of the embryos. This is clearly shown in figure 3, where 5 h of exposure to D-glucosamine inhibited retinal thymidine kinase from 9-day embryos by 46%, and the inhibition fell to 18% on the 14th day.

With HeLa cells the inhibition of thymidine kinase was clear after 1 h and 5 h of treatment with D-glucosamine reduced the activity to only 15% of the control value (fig. 2). This result confirms that the amino sugar markedly inhibits thymidine kinase in tumor cells.

Data reported in the table indicate that in cell-free extracts from 14-day retinas thymidine kinase was not inhibited by D-glucosamine. The same result was obtained with retinas on the 18th day. D-glucosamine was able to inhibit thymidine kinase in cell-free extracts from 9-day retinas or HeLa cells, but the effect, according to Friedman[9], was much lower than that produced with intact cells. Glucosamine-6-phosphate was able to inhibit thymidine kinase, but with retinal enzyme a clear effect was observed only at 20 mM. In the 3 conditions reported in the table both N-acetylglucosamine and UDP-N-acetylglucosamine were ineffective.

The results demonstrate that D-glucosamine inhibits thymidine utilization, not only in tumor cells, but also in embryonal cells, particularly in the early stages of development. Data reported in the table exclude the possibility that the inhibition of retinal thymidine kinase is dependent on a direct interaction between D-glucosamine or its metabolites and the enzyme. As postulated by Friedman[9] for glial tumor cells, in chick embryo retina also the effect of D-glucosamine may be a reduction of the enzyme synthesis or a modification of newly-synthesized enzyme. Thymidine kinase in chick embryo retinas reaches its highest values between the 8th and 10th days of incubation. After day 10 it declines, at first rapidly until day 16, and then more slowly, reaching its lowest value at the time of hatching[15]. This observation agrees with the general statement that thymidine kinase is correlated with DNA production. The fact that a shorter time of exposure to D-glucosamine was required in young retinas to reveal the inhibitory effect suggests that at least 2 different thymidine kinase, both sensitive to glucosamine, are produced during development: one, which turns over rapidly, contributes in a major way to the total cellular thymidine kinase activity in young retinas, where higher amounts of enzyme are synthesized; another, which turns over slowly, prevails during the final period of embryonic development, when the synthesis of thymidine kinase is reduced. The decrease of the magnitude of the inhibition with the age of chick embryos suggests that these different forms of thymidine kinase may be inhibited to a different degree by D-glucosamine. A new study is in progress to test the validity of this hypothesis.

1 Quastel, J.H., and Cantero, A., Nature *171* (1953) 252.
2 Bekesi, J.G., Molnar, Z., and Winzler, R.J., Cancer Res. *29* (1969) 353.
3 Bekesi, J.G., Bekesi, E., and Winzler, R.J., J. biol. Chem. *244* (1969) 3766.
4 Bosmann, H.B., Biochim. biophys. Acta *240* (1971) 74.
5 Bekesi, J.G., and Winzler, R.J., J. biol. Chem. *244* (1969) 5663.
6 Plagemann, P.G.W., and Erbe, J., Cancer Res. *33* (1973) 482.
7 Sukeno, T., Kikuchi, H., Saeki, H., and Tsuiki, S., Biochim. biophys. Acta *244* (1971) 19.
8 Decker, K., Keppler, D., Rudigier, J., and Domschke, W., Physiol. Chem. *352* (1971) 412.
9 Friedman, S.J., Trotter, C.D., Kimball, T., and Skehan, P.J., Cancer Res. *37* (1977) 1141.
10 Friedman, S.J., Kimball, T., Trotter, C.D., and Skehan, P.J., Cancer Res. *37* (1977) 1068.
11 Tesoriere, G., Vento, R., and Calvaruso, G., Exp. Eye Res. *24* (1977) 537.
12 Tesoriere, G., Vento, R., Calvaruso, G., and Taibi, G., Exp. Eye Res. *29* (1979) 213.
13 Tesoriere, G., Vento, R., Calvaruso, G., and Taibi, G., Molec. cell. Biochem. *25* (1979) 171.
14 Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J., J. biol. Chem. *193* (1951) 265.
15 Tesoriere, G., Tesoriere, L., Giuliano, M., and Vento, R., FEBS Lett., in press.

0014-4754/84/070705-03$1.50 + 0.20/0

Volatile constituents of wolf *(Canis lupus)* urine as related to gender and season[1]

J. Raymer, D.Wiesler, M. Novotny[2], C. Asa, U.S. Seal and L.D. Mech

Department of Chemistry, Indiana University, Bloomington (Indiana 47405, USA); Veterans Administration Medical Center, Minneapolis (Minnesota 55417, USA), and Patuxent Wildlife Research Center, US Fish and Wildlife Service, Laurel (Maryland 20708, USA), 29 September 1983

Summary. The volatile constituents of wolf urine were examined via capillary gas chromatography and compared among male, female, and castrate male. Several compounds including methyl isopentyl sulfide, 3,5-dimethyl-2-octanone, and acetophenone were clearly associated with the gender of the animal and many displayed a seasonal dependence. In addition, 2 long-chain aldehydes isolated from urine samples by an HPLC procedure also correlated with the endrocrine status of the animal.

Communication in the canidae is quite complex[3]. This complexity varies directly with the degree of sociability of the species such that a gregarious animal like the wolf *(Canis lupus)* appears to have a more complex communication system than the more solitary red fox *(Vulpes vulpes)*. The wolf utilizes visual, vocal, and tactile as well as chemo-olfactory modes of communication[3]. A major mode of chemo-olfactory communication (scent-marking) in the wolf is urination, and the way urine is deposited relates to behavior displayed during marking[4,5]; urination, and the chemical scent constituents of urine, are intimately involved in communication in general. Following our previous characterization and behavioral testing of the chemical scent constituents in red fox urine[6-8], the present study deals with the urinary scent chemistry of the wolf as related to sex, endocrine status and seasonality. Capillary gas chromatography (GC), gas chromatography/mass spectrometry (GC/MS), and gas chromatography/Fourier-transform infrared spectroscopy (GC/FTIR) were used to identify and quantitate typical wolf urinary components. For comparison, urinary scent profiles of arctic foxes *(Alopex lagopus)* and common dogs *(Canis familiaris)* were also investigated.

Urine samples were collected from several captive wolves maintained near Minneapolis, Minnesota. The collection procedure involved anesthetizing the animals with ketamine hydrochloride and promazine hydrochloride to provide adequate relaxation for urine withdrawal by catheter. Aliquots of about 10 ml were discharged into acid-washed vials, which were immediately closed with a Teflon-lined cap. The samples were shipped on dry ice to our laboratory at Indiana University where they were stored frozen until analysis. Similar procedures were employed for collections from dogs and foxes.

Wolf urine samples from every 2 or 3 weeks, from 31 October 1979 to 30 October 1980, were chosen. To minimize individual variations, aliquots of 2–7 (mean = 4.4) samples were combined when both date and class (i.e., male, female, or castrate male) were alike.

The profiles of urinary volatiles resulting from the GC separation of the samples were obtained through a headspace sampling procedure estabished in our laboratory[9,10]. Following thermal desorption of volatiles from a porous polymer (Tenax GC, Applied Science Laboratories, State College, Pennsylvania) onto a 60 m × 0.25 mm, inner diameter, glass capillary column coated with a polypropylene glycol fluid, UCON 50-HB-2000, the separations were followed with the flame ionization detector (FID). Compound identifications were initially aided by the nitrogen-sensitive (thermionic) and the sulfur-sensitive (flame-photometric) GC detectors. Data obtained from combined capillary GC/MS provided the major information leading to a positive identification of the urinary constituents. GC/MS data were obtained with a Hewlett-Packard 5982A dodecapole instrument. For several component identifications, a combination of capillary GC with FTIR was essential[11]. The compound identifications discussed below were verified through both retention and mass-spectral data of synthesized compounds.

Representative FID chromatograms are shown in figure 1 for normal male, normal female and castrate male urine volatiles. The numbered peaks and their corresponding identities in the table were selected for discussion in this paper; the components showing no clear relationship to sex or season are excluded. The sulfur-containing compounds were examined with great interest, because similar ones (or the same in the case of Δ^3-isopentenyl methyl sulfide) were seen in red fox urine[6] and later shown to induce scent-marking by foxes in the wild[7,8].

The ketones are discussed here for 2 reasons: a) these compounds appear to be unique to the wolf, or closely related canids[12]; and, b) this series of compounds is closely associated with the male. Some of these ketones were seen (in lesser amounts, however) in the dog, while there was no evidence of this series in the arctic fox. Acetophenone was of particular interest because of its obvious association with the sex of the animals. Ketones **7**, **9**, **11**, and **12**, which have never been reported to occur naturally, were synthesized by conventional methods as mixtures of diastereoisomers. One can do no more than speculate regarding stereochemistry, but it is of special interest that in each case the earliest of the synthetic diastereo-