The Effect of Glucosone on the Proliferation and Energy Metabolism of *in vitro* Grown Ehrlich Ascites Tumor Cells

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Dedicated to Prof. F. Zilliken *on the Occasion of His 60th Birthday*

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Ehrlich Ascites Tumor Cells, Proliferation, Energy Metabolism, Glucosone

1) Proliferation and energy metabolism of *in vitro* grown Ehrlich ascites tumor (EAT) cells in the presence of glucosone, (D-arabino-3.4.5.6-tetrahydroxy-2-oxo-hexanal) a competitive inhibitor of hexokinase, were studied.
2) Proliferation of the cells was completely inhibited by 2 mM glucosone without severely affecting viability (dye exclusion test). No phase specific arrest of cell growth was observed.
3) Incorporation of [^{14}C]thymidine into an acid insoluble fraction of the cells decreases to 5% of the controls within 8–10 h. Incorporation of [^{14}C]leucine begins to slow down immediately after treatment with glucosone.
4) The inhibitor (2 mM) reduces the lactate production of the cells by 60%, respiration by about 20%; the ATP/ADP ratio slows down from 4.75 to 3.5.
5) The total inhibition of cell proliferation by 2 mM glucosone cannot be explained exclusively by inhibition of hexokinase activity and impairment of energy metabolism. Because of a lack of specificity, glucosone is not a suitable inhibitor for studies on the relationship between hexokinase activity and cell proliferation of tumor cells.

Of all the glycolytic enzymes it may be that hexokinase is the most important in amplifying the lactic acid producing capacity of rapidly growing tumors [1]. The relationship that hexokinase may have to the enhanced glucose utilization capacity of these tumors, however, needs further study.

Previous investigations by Yushok [2] have shown that glucosone (D-arabino-3,4,5,6-tetrahydroxy-2-oxo-hexanal) is a potent inhibitor of glycolysis of intact Ehrlich ascites tumor cells. Of any sugar analogues tested so far [2], this compound has the highest affinity for tumor hexokinase.

Considering this property glucosone should be a suitable tool for the study of relations between glucose metabolism and cell proliferation of tumor cells.

In the present communication we report the results of experiments on the effect of glucosone on the proliferation, energy metabolism and energy dependent biosynthetic processes of *in vitro* grown Ehrlich ascites tumor cells. These results demonstrate that the impairment of cell proliferation and macro molecular syntheses by glucosone cannot be explained exclusively by inhibition of hexokinase in the cells. Rather additional effects of glucosone on cellular metabolism must be taken into consideration.

Reprint requests to Prof. Dr. Fr. Schneider.

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Materials and Methods

All chemicals, buffers and media components were of the purest grade available from Merck (Darmstadt), Serva (Heidelberg), Sigma (München) and Boehringer (Mannheim). Glucosone (D-arabino-3.4.5.6-tetrahydroxy-2-oxo-hexanal) was synthesised according to Bayne [3]. [U-^{14}C]leucine, spec. act. 342 mCi/mmol, [2-^{14}C]thymidine, spec. act. 58 mCi/mmol and [2-^{14}C]uridine, spec. act. 57.4 mCi/mmol were purchased from Amersham Buchler (Braunschweig) whilst "Cocktails" for scintillation counting were from Roth (Karlsruhe). Horse serum was a gift from Behringwerke (Marburg).

Cells and growth techniques

Hyperdiploid Ehrlich ascites tumor cells were serially grown in the peritoneal cavity of female NMRI mice. Cells for explantation to cultures *in vitro* were usually withdrawn from mice, inoculated 6 days previously, and transferred to modified Eagle's medium, supplemented with 15% horse serum, 30 mg/l streptomycin, 575 mg/l Microcillin, to obtain a suspension of $6-7\times10^5$ cells/ml of culture medium

in 600 ml non-siliconized glass flasks. For this line of Ehrlich ascites tumor cells, the nutrients of the medium were sufficient for a 24 h culture period; for details see Karzel and Schmidt [4]. Growth was estimated by the counting of cells with a haemcytometer. Viability of cells was assessed by dye exclusion test with 0.1% nigrosin.

After 16 h cultivation at 37° under standard conditions (first passage *in vitro*) the cells were separated by centrifugation ($500 \times g$, 2 min) and transferred to fresh culture medium. The inoculum density in the second and subsequent passages was $6-7 \times 10^5$ cells/ml.

For studies with the inhibitor *in vitro* the cells were cultured in the presence of 2 mM glucosone.

Measurement of metabolic parameters

L-Lactate production of the cells was assayed as described by Hohorst [5], glucose consumption was measured with the glucose oxidase/perid test from Boehringer (Mannheim) and respiration was determined amperometrically with a Clark electrode [6].

Protein- and DNA synthesis

The protein content of cell cultures was estimated in aliquots of 1 ml using the Folin method [7] with bovine serum albumin as standard, after washing the cells twice with Hank's solution. The relative rates of protein- and DNA synthesis were measured by incorporation of [L-U^{14}C]leucine and [2-^{14}C]thymidine into acid insoluble material. Aliquots of cells (1 ml) were withdrawn at intervals from the cultures and incubated for 15 min at 37° with 0.1 µC leucine and 0.1 µCi thymidine. After being washed twice with ice cold Hank's balanced salt solution, the cells were suspended in 5% trichloroacetic acid, collected on a membrane filter (Gelman 0.45 µm), dried and counted using Rotiszint 11.

Determination of ATP and ADP

ATP and ADP concentrations of the cells were assayed by the luciferin/luciferase system [8] using a Biolumat 9500 (Bertholt). At each incubation interval, 0.1 ml cell suspension was added to 0.9 ml of redistilled water at 95° and heated for 5 min in a boiling water bath. ATP was measured in 20 µl aliquots. ADP, was estimated by difference, after pretreatment of 200 µl aliquots of each sample with pyruvate kinase/phosphoenol pyruvate to convert ADP to ATP. ATP and ADP determinations should be performed on the same day with the same luciferase preparation.

Results and Discussion

Viability and cell proliferation in the presence of glucosone

Since no information on the effect of glucosone on the viability of *in vitro* grown Ehrlich ascites tumor cells was available, we studied the relationship between concentration of the inhibitor and the number of dead cells after an incubation time of 24 h. The

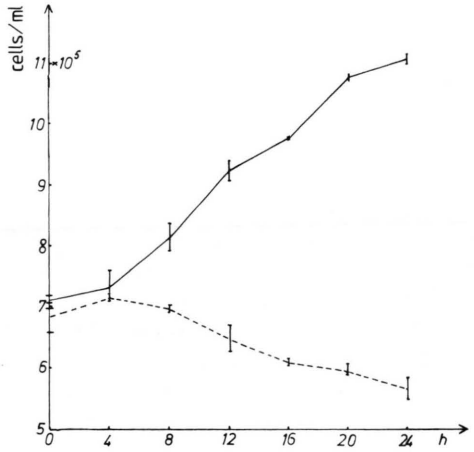

Fig. 1 A. Proliferation of Ehrlich ascites tumor cells.

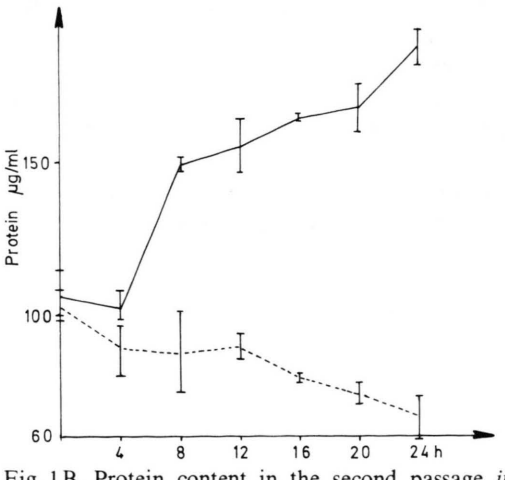

Fig. 1 B. Protein content in the second passage *in vitro*. Controls cells ——, + 2 mM glucosone - - - -. Values represent the mean of 16 separate cultures.

results of a typical experiment are summarized in Table I. In the presence of 3 mM glucosone or more the viability of the cells is severely affected; a drastic increase of nigrosin positive cells in the dye exclusion test is observed in the second part of the passage, about 12 h after beginning the incubation. In the later experiments we chose a concentration of 2 mM glucosone, which was well tolerated, *i. e.* no morphological changes could be observed in the light and electron microscope after 24 h. However, a significant depression of the lactic acid producing capacity of the cells was achieved by this concentration (see below).

The effect of 2 mM glucosone on the proliferation of Ehrlich ascites tumor cells is illustrated in Fig. 1. An increase of cell number of $48 \pm 11\%$ was observed in the control cultures within 24 h, while in the presence of the inhibitor proliferation stops beyond 4 h and the number of cells decreases by $12 \pm 7\%$ at the end of the passage. No cell cycle specific arrest of growth could be revealed by flow cytophotometric analysis. Starting with an inoculum density of 6×10^5 cells/ml the mean protein content of the cultures increased from 110 ± 9 µg/ml to 180 ± 6 µg/ml, an increase of 70%. In accordance with the growth rates, the protein content of the glucosone containing cultures was reduced by 22%. Further information on the protein turnover of the cells was obtained from incorporation experiments with [^{14}C]leucine.

DNA- and protein synthesis

The incorporation of [2-^{14}C]thymidine into acid insoluble material for control cells and cells treated with glucosone is shown in Fig. 2A. A typical incorporation pattern with a maximum at 10–12 h was obtained under normal conditions. The uptake of thymidine, the activation of thymidine kinase and the incorporation of the nucleotide into DNA are closely coupled processes and take place only during the synthesis of DNA [9]. The thymidine nucleotide pool during the S-phase is nearly constant [10] and the radioactivity of the DNA is therefore a fairly reliable measure of the rate of DNA synthesis.

Table I. The effect of increasing concentrations of glucosone on the viability of *in vitro* grown Ehrlich ascites tumor cells as determined by dye exclusion. Incubation time 24 h.

Glucosone [mM]	0	1	2	3	4	5
Number of stained cells [%]	2	2	4	15	22	33

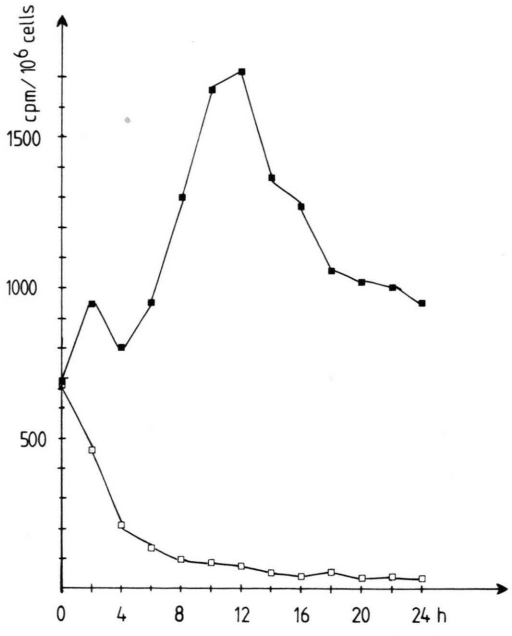

Fig. 2A. Incorporation of [2-^{14}C]thymidine into acid insoluble material of Ehrlich ascites tumor cells. 15 min pulse of 0.1 µCi/ml cell suspension, standard deviation never exceeded 10% of the mean. ■–■ control cells; □–□ in the presence of 2 mM glucosone.

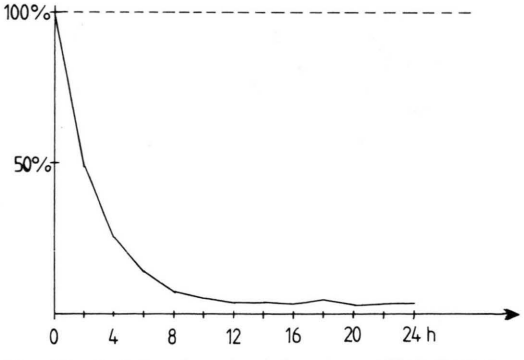

Fig. 2B. Relative incorporation rates of labelled thymidine into cells in the presence of glucosone as a percent of the controls.

As is illustrated in Figs. 2A and B, in the presence of 2 mM glucosone the incorporation of [^{14}C]thymidine into DNA decreased to about 5% of the controls within 8–10 h. This severe impairment of the DNA synthesis of the cells by glucosone is in good agreement with the observed growth rates.

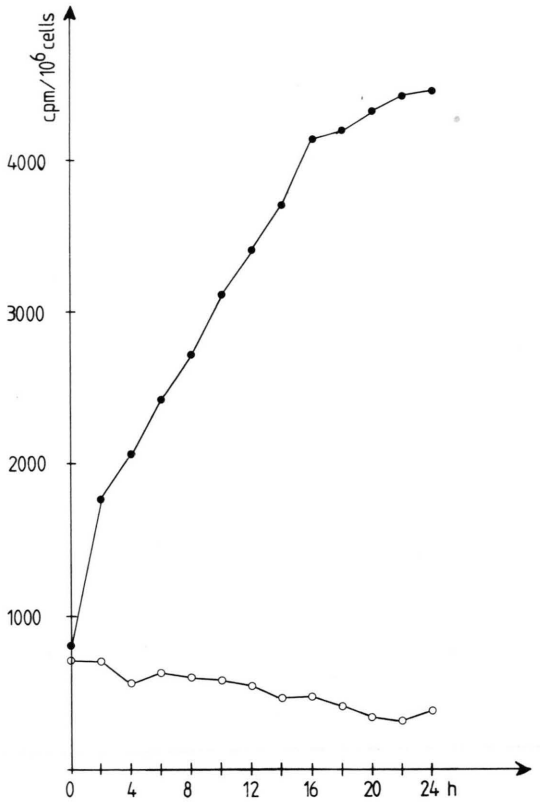

Fig. 3 A. Incorporation of [^{14}C]leucine into acid insoluble material of Ehrlich ascites cells. 15 min pulse of [U^{14}C]leucine (0.1 µCi/ml cell suspension). Standard deviation never exceeded 10% of the mean. ●–● control cells, ○–○ + 2 mM glucosone.

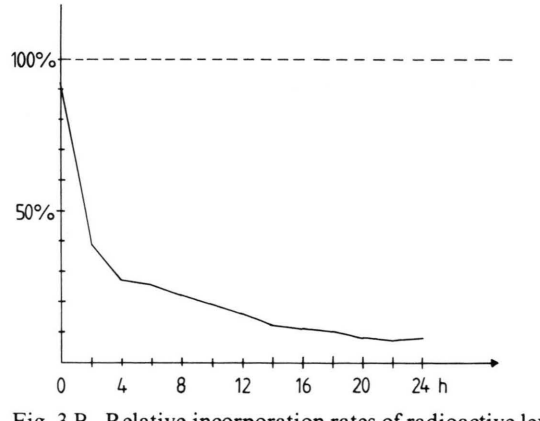

Fig. 3 B. Relative incorporation rates of radioactive leucine into the cells in the presence of glucosone as percent of the controls.

The results of the incorporation studies with [^{14}C]leucine are depicted in Figs. 3 A and B. A close correlation between growth rates and protein synthesis was found. Incorporation of radioactivity from leucine into the acid insoluble precipitate began to slow down immediately after treatment of the cells with glucosone.

From these experiments we may conclude that glucosone in a concentration of 2 mM is a strong inhibitor of growth, protein- and DNA-synthesis of *in vitro* cultured Ehrlich ascites cells.

Energy metabolism

When glucosone was added to the suspension of cells an increased inhibition of lactate production was observed with increased concentrations of this inhibitor of hexokinase. The quantitative relations are given in Table II.

The lactate production of controls and of cultures containing 2 mM glucosone over a passage of 24 h is depicted in Fig. 4.

In the presence of 2 mM glucosone only 18% of the lactate of the controls was produced in the first 12 h of a passage, in the second half it was 33%. Over a culture period of 24 h a mean lactate production, about 40% of the controls, was observed. The uptake of glucose by the cells in the absence or presence of glucosone corresponded to the lactate production, as is depicted in Fig. 4 A. The data of Table III illustrate that the glucose uptake in the presence of 2 mM of the inhibitor was found to be about 18% of the controls within the first 12 h and about 44% between 12 and 24 h. The glucose consumption of the controls between 12 and 24 h was 30% lower than at 0–12 h, whilst in the presence of glucosone it was 60% higher between 12 and 24 h than between 0 and 12 h.

Table II. Lactate production in µM/10^6 cells per 12 h of *in vitro* grown Ehrlich ascites tumor cells in the presence of increased concentrations of glucosone.

Concentration of glucosone [mM]	Time of incubation	
	0–12 h	12–24 h
controls	0.824 ± 0.098	0.547 ± 0.082
1.00	0.389 ± 0.138	0.313 ± 0.099
1.75	0.237 ± 0.029	0.199 ± 0.050
2.00	0.152 ± 0.046	0.179 ± 0.051
2.25	0.144 ± 0.001	0.234 ± 0.011
5.50	0.055 ± 0.021	0.090 ± 0.029

Table III. Consumption of glucose by EAT cells in μM/10^6 cells.

	0–12 h	12–24 h
Controls	0.49 ± 0.06	0.34 ± 0.06
2.0 mM glucosone	0.09 ± 0.04	0.15 ± 0.01

The oxygen uptake of the controls and the cultures in the presence of the inhibitor over a period of 24 h is summarized in Table IV. Somewhat unexpected is the decrease of oxygen consumption because inhibition of the lactate production should stimulate respiration (Pasteur effect).

Changes of the ATP and ADP level in control cultures and in the presence of glucosone over a period of 24 h are depicted in Fig. 5. The most striking effect of glucosone on the adenine nucleotide concentration of the EAT cells was an increase in the ATP- and ADP content of the cells. At the end of the incubation period the ATP concentration of the glucosone treated cells is 250% that of the controls; at the same time the ADP-level was three times higher than the control values. Since chemical energy is always a matter of concentration ratios, a more reliable parameter of the state of the energy metabolism of the cells is the ATP/ADP ratio, which is illustrated in Fig. 6. This diagram reveals that the

Table IV. Oxygen uptake of the EAT cells in μM/10^8 cells × h.

Time [h]	Controls	+ 2 mM glucosone
0	18.0 ± 3.3	18.2 ± 3.2
4	20.4 ± 5.3	20.3 ± 3.0
8	21.6 ± 5.0	18.0 ± 1.1
12	23.7 ± 5.0	18.0 ± 1.8
16	21.5 ± 1.3	17.6 ± 2.1
20	20.8 ± 3.6	15.5 ± 3.2
24	16.3 ± 3.5	12.0 ± 3.0

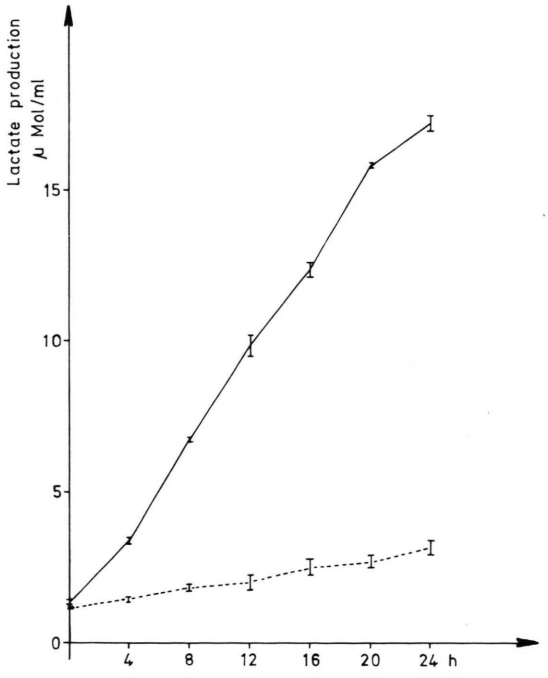

Fig. 4A. Lactate production of Ehrlich ascites tumor cells during the second passage *in vitro* in the presence or absence of 2 mM glucosone. Inoculum density 6×10^5 cells/ml.

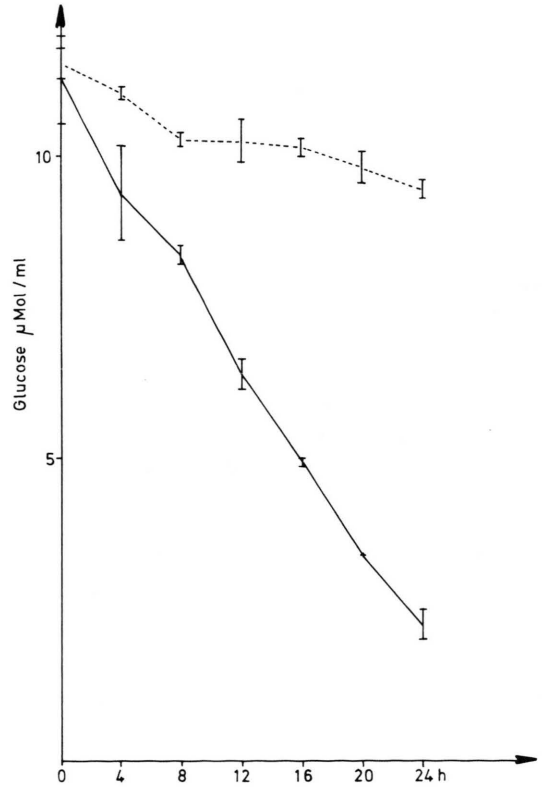

Fig. 4B. Glucose consumption of EAT cells in the second passage *in vitro*. Controls — + 2 mM glucosone - - - -.

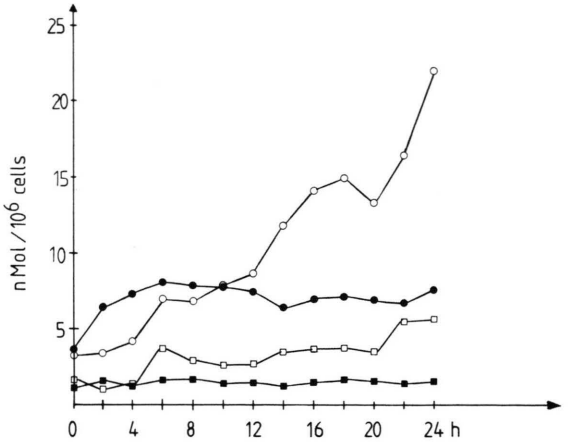

Fig. 5. ATP and ADP content of EAT cells over a culture period of 24 h. Controls ●–● ATP, ■–■ ADP; + 2 mM glucosone ○–○ ATP, □–□ ADP.

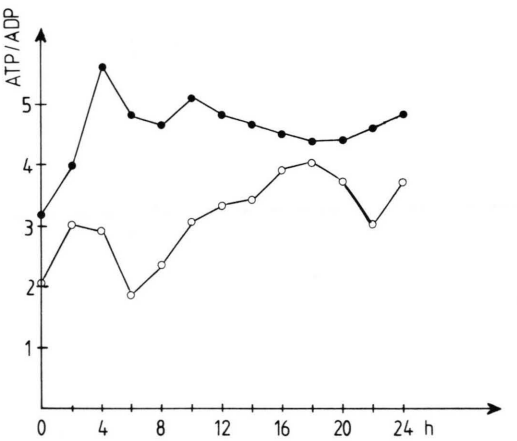

Fig. 6. ATP/ADP ratio of EAT cells. Controls ●–●, + 2 mM glucosone ○–○.

phosphorylation potential of the cells is really reduced by the inhibitor; but this reduction is smaller than it would be expected considering the impairment of cell proliferation.

Discussion

Glucosone, which has a high affinity for hexokinase but is only slowly phosphorylated, inhibits the phosphorylation of other sugars as was shown by Yushok *et al.* [2] with Krebs ascites tumor- and particulate brain hexokinase. Evidence was further obtained by these authors which suggest that the phosphorylated product of glucosone, presumably glucosone-6-phosphate, also inhibits tumor hexokinase. This is in agreement with the fact that glucosone-6-phosphat in its enol form meets the configurational requirement proposed by Sols and Crane [11] for product inhibitors of hexokinase.

Of particular interest is the strong inhibition by glucosone of glycolysis in intact ascites tumor cells, as studied by Yushok [2] and Poole [12] which could be confirmed by our experiments with Ehrlich ascites tumor cells grown *in vitro*. As was already pointed out by Yushok *et al.* [2], the K_M of glucosone for tumor hexokinase (8×10^{-6} M) is, however, not low enough to explain completely its potent inhibition of glycolysis in intact cells. Furthermore, it is impossible to explain the total inhibition of cell proliferation by the impairment of energy metabolism by 2 mM glucosone, a concentration which inhibits glycolysis of the cells by only about 60% and reduces the mean value of the ATP/ADP ratio from 4.75 ± 0.35 to 3.5 ± 0.5. Consequently, there is no direct correlation between the effects of glucosone on energy metabolism and the inhibition of cell proliferation.

The high cell proliferation inhibiting capacity of glucosone seems possibly be due to its open chain ketoaldehyde structure which is in equilibrium with the pyranose form and is more reactive than the ring form. It is well known from studies on cell proliferation, protein- and RNA-synthesis in the presence of methylglyoxale that ketoaldehydes interfere with different cellular processes [13, 14]. It is postulated that such compounds exert their effects by combining to form hemimercaptals with SH groups, so inactivating vital SH-dependent enzymes [15] or by modifying essential arginine residues [16, 17]. Preliminary studies have shown that glucosone reacts fairly rapidly with glutathione [18].

Further new findings come from experiments by Keppler *et al.* [19] who have observed the formation of UDP-glucosone and a drastic fall of the UTP-concentration of TA3 mammary tumor cells in the presence of glucosone. These effects are comparable with those obtained after treatment of mammalian cells with other sugars substitued in position 2-(D-glucosamine, D-galactosamine, 2-deoxy-D-galactose). The consequences for the cell of these severe perturbations of uridine nucleotide metabolism are extensively discussed by these authors [20].

In conclusion, glucosone is a highly potent inhibitor of cell proliferation with multifarious intracellular points of attack; it is not a suitable tool for studying relations between hexokinase activity and cell proliferation because of a lack of specificity.

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