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Effects of Benzaldehyde on Survival and Cell-cycle Kinetics of Human Cells Cultivated *in Vitro**

ERIK O. PETTERSEN,†‡ OLE NOME,§ ØYSTEIN W. RØNNING†‖ and REIDAR OFTEBRO†

†*Department of Tissue Culture, Norsk Hydro's Institute for Cancer Research and* §*The General Department, The Norwegian Radium Hospital, Montebello, Oslo 3, Norway*

Abstract—*Synchronized cells of the human line NHIK 3025 were used to study inactivating and cell-cycle inhibitory effects induced by benzaldehyde. Inactivation was measured as loss of colony-forming ability after treatment of exponentially growing or synchronized cells. Cell-cycle inhibition was measured by flow cytometric recordings of DNA-histograms and microscopic recordings of cell division in synchronized cells. Treatment with benzaldehyde for* 4 *or* 24 hr *showed that a marked decrease in survival took place for concentrations above* 6.4 mM. *Cell-cycle inhibition was observed at concentrations as low as* 0.8 mM. *Synchronized cells were treated with* 3.2 *and* 6.4 mM *benzaldehyde for* 8 hr *starting at various stages of the cell-cycle. Both the colony-forming ability and the rate of cell-cycle traverse was measured. No difference in sensitivity was found whether the treatment was given in* G_1, *S or in* G_2. *Thus the results show that there is no specific part of interphase where the cells are particularly sensitive with respect to either the inactivating or the cell-cycle inhibitory effects of benzaldehyde in concentrations up to* 6.4 mM. *When benzaldehyde was present during mitosis both the inactivating and the cell-cycle inhibitory effects were markedly enhanced as compared to the corresponding effects during interphase. It is concluded that benzaldehyde must affect some process within the cell which represents a general requirement for cell-cycle progression. In addition, there are effects on processes that take place only during the last few minutes before and/or during mitosis.*

INTRODUCTION

CHEMOTHERAPEUTIC drugs usually exert their effect by inactivating cancer cells. In practical use the drugs are, however, not attacking the malignant cells exclusively, and serious side-effects are often seen due to damage of normal tissue. A few recent papers have suggested that benzaldehyde might induce an antitumour effect both in mice [1] and in humans [2] by mechanisms on the cellular level which are different from those of the more established anticancer drugs. Despite prolonged administration of benzaldehyde for more than 1 yr the treatment was found to be nontoxic to the patients and many of the human tumors were reported to change histologically towards higher differentiation [2].

In an effort to elucidate by which mechanism(s) benzaldehyde exerts its effect at the cellular level, we have initiated a series of experiments involving effects of the chemical on survival, growth and metabolism of human NHIK 3025 cells. In the present paper the effects of benzaldehyde on cell survival and cell-cycle kinetics have been studied.

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‡To whom requests for reprints should be addressed.
‖Fellow of the Norwegian Cancer Society.

MATERIALS AND METHODS

Cells and synchronization

Human cells, NHIK 3025, established from cervical carcinoma *in situ* [3, 4] were cultivated in medium E2a [5] supplemented with 20% human and 10% horse serum (Gibco). These cells have the ability to grow without attachment to a surface or to each other, but are not able to form tumours when implanted in athymic 'nude' mice (unpublished results). The cells were kept in continuous exponential growth by frequent reculturing, i.e. every 2nd or 3rd day. Populations of synchronized cells with a high degree of synchrony were obtained by selecting mitotic cells from populations in exponential growth [6]. These experiments took place in a walk-in incubator room at 37°C. During the selection procedure (shaking of the flasks [6]) the cells were

kept in medium E2a. The synchronization index as defined by Engelberg [7] and Bakke and Pettersen [8] was 95% for the first and 64% for the second division after selection [6]. Under growth conditions as used here the NHIK 3025 cells have a median cell-cycle time of ~18 hr, with median G_1 and S durations of ~7 and ~8 hr respectively. (The median durations are defined as the time until 50% of the cells have completed the cell cycle or the phase in question.) Mitosis lasts for 50 min.

Mitotic selection was usually repeated several times with 45-min intervals to provide enough populations for one experiment.

Cell survival

For measurement of cell survival an appropriate number of cells was seeded into 25-cm^2 tissue culture plastic flasks (Falcon). The number of cells was adjusted so that the number of surviving cells was similar in each flask (approximately 150). Synchronized cells were seeded immediately after selection while the cells were still in suspension. Asynchronous cells were trypsinized before seeding. Two hours after seeding, when the cells had attached to the bottom of the flask, the medium was removed and new medium containing the desired concentration of benzaldehyde was added. After the desired time of exposure the benzaldehyde-containing medium was removed and fresh medium added. Both on addition and removal of benzaldehyde, the flasks were rinsed once with the same medium as was to be added. Each time the flasks were opened for addition or removal of benzaldehyde each flask was separately degassed with 5% CO_2 in air and sealed. After 10–12 days at 37°C the cells were fixed in ethanol and stained with methylene blue, and then the colonies were counted. Cells giving rise to colonies containing more than 40 cells were scored as survivors [9].

Cell-cycle kinetics

For measurement of cell-cycle kinetics the cells from each separate selection ($4–8 \times 10^5$ cells in 240 ml medium) were seeded in 8 tissue culture plastic flasks (Falcon 75 cm^2) which were degassed with a mixture of CO_2 (5%) and air. At various times after mitotic selection cells were trypsinized and stained in suspension with mithramycin (Mithracin, Pfizer, Inc., U.S.A.) with simultaneous fixation [10] as described earlier [11]. Cell-cycle progression was analyzed from DNA histograms recorded on a laboratory-built flow cytometer using an Hg-lamp on a Leitz invertoscope with fluorescence optics for excitation and detection of the fluorescent light [12, 13]. The fraction of cells in G_1 was determined from the histograms [14].

To observe the time of division of individual cells, about 5000 cells were seeded into each one of 25-cm^2 plastic flasks immediately after selection. The NHIK 3025 cells do not move significantly during growth under the culture conditions used in these experiments, and by observation in an inverted microscope the time of cell division was recorded for approximately 100 cells in each flask.

RESULTS

Inactivating effects

The surviving fraction of cells treated with various concentrations of benzaldehyde is shown in Fig. 1. Exponentially (asynchronously) growing cells were cultured in medium containing benzaldehyde for 4 or 24 hr, while the treatment period for cells synchronized by mitotic selection was 24 hr, starting 2 hr after selection, i.e. when the cells were in early G_1. During the treatment with benzaldehyde the synchronized cells appeared as doublets. The recorded surviving fraction was therefore corrected according to the formula reported by Gillespie *et al.* [15], and the experimental points in Fig. 1 thus represent survival of single cells.

The curve representing 4-hr treatment is flat up to 6 mM. The two curves representing 24-hr

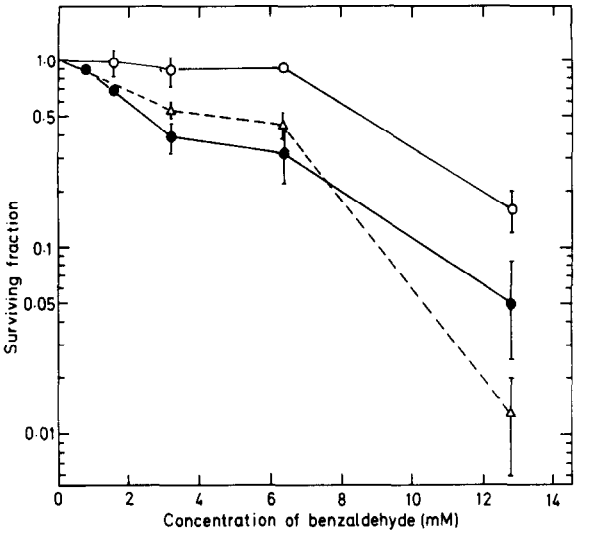

Fig. 1. Surviving fraction of asynchronous NHIK 3025 cells as a function of the concentration of benzaldehyde during a 4-hr (○) and a 24-hr (●) treatment. Single cells were plated in plastic flasks 2 hr before medium with the appropriate concentration of benzaldehyde was added. Also shown is the surviving fraction of synchronized cells (△) which were plated in the flasks immediately after mitotic selection, and 2 hr afterwards the cells were supplied with medium containing benzaldehyde. The medium containing benzaldehyde was replaced with fresh medium 24 hr after addition. The experimental points for synchronized cells represent values corrected for a multiplicity of 2 and are thus valid as single-cell survival. The experimental points represent the mean of observations in 2–4 different experiments. Standard errors are represented with vertical bars when exceeding the symbols.

treatment appear relatively flat in the concentration range between 3 and 6 mM, with somewhat steeper regions for both lower and higher concentrations.

The data in Fig. 1 indicate that the inactivating effect of benzaldehyde on NHIK 3025 cells must be cell-cycle-specific. This would explain the flat region of the curves between 3.2 and 6.4 mM provided that benzaldehyde at 6.4 mM, but not at 3.2 mM, inhibits cell-cycle progression to such an extent that a great proportion of the cells are unable to reach to the stage where the cells are highly sensitive to its inactivating effects.

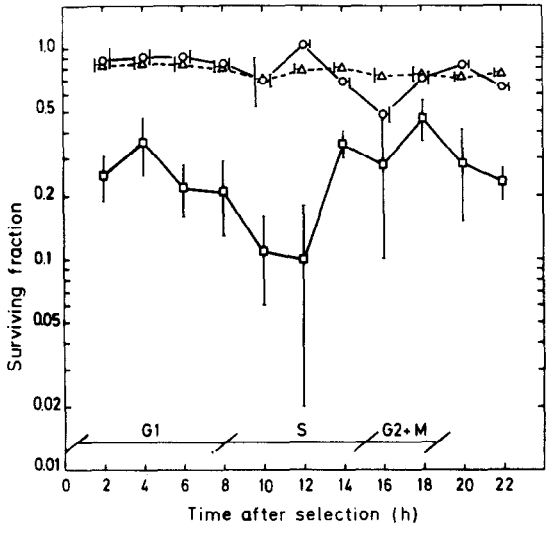

Fig. 2. Surviving fraction of synchronized NHIK 3025 cells treated for 8 hr *with enzaldehyde at concentrations of* 3.2 (○), 6.4 (△) *and* 12.8 (□) mM *as a function of the time after mitotic selection at which the chemical was added. The duration of the various cell-cycle phases for control cells are indicated separately. Data from one single experiment is shown. Each experimental point represents the mean of counts from 4 flasks. The vertical bars indicate standard error of mean.*

To study in more detail the cell-cycle specificity with respect to the inactivating effect of benzaldehyde, synchronized cells were treated for an 8-hr period starting at various times after selection. In Fig. 2 the surviving fraction is plotted as a function of the time after selection when the chemical was added. Three different concentrations, 3.2, 6.4 and 12.8 mM, were used. The data of Fig. 2 demonstrate that the cell-cycle variation of the survival is small after treatment with 3.2 or 6.4 mM. Only with the highest concentration, 12.8 mM, is there a marked variation, with highest sensitivity when the chemical was added at 10 or 12 hr after selection, i.e. when the majority of the cells was in S phase. Cells exposed to 3.2 and 6.4 mM responded similarly to the chemical in all parts of the cell cycle, except in the region around 16 hr after selection. In repeated experiments it was found that when benzaldehyde was added 16 hr after selection (i.e. just before the first cells enter mitosis) 3.2 mM of the chemical induced a greater effect than 6.4 mM. This indicates that the part of the cell cycle where the cells are most sensitive with respect to inactivating effects of 3.2 mM benzaldehyde must be in G_2 and/or mitosis.

By microscopic observation of the populations it was seen that the cells were delayed in mitosis in the presence of both 3.2 and 6.4 mM of benzaldehyde. The delay was, however, most severe with the highest concentration. To test the survival of cells delayed in mitosis the following experiment was performed: 3.2 and 6.4 mM of benzaldehyde was added to each one of two populations of synchronized cells 16 hr after selection. After 4 hr the flasks were shaken to detach all mitotic cells. The loosened cells were kept suspended in medium containing benzaldehyde for 4 hr more before the cell number was counted and an appropriate number of cells was seeded in fresh medium in plastic flasks for colony formation. The results, shown in Table 1, indicate that the colony number was 4–6 times higher when the cells were exposed to 6.4 as compared to 3.2 mM.

Table 1. Number of colonies formed by cells treated with benzaldehyde during mitosis

Concentration of benzaldehyde (mM)	No. of cells seeded	No. of colonies
3.2	200	9 ± 2
3.2	1000	44 ± 5
6.4	200	57 ± 3
6.4	1000	193 ± 8

Cell-cycle inhibitory effects in G_1, S and G_2

The effect of benzaldehyde on the cell-cycle traverse was studied by recording the delay of entry into S and the delay of entry into the subsequent G_1 after addition of the chemical to populations of synchronized cells. The cells were selected in mitosis at time zero, and benzaldehyde was added 2 hr thereafter, i.e. when the cells were in early G_1. In Fig. 3 the median durations of the part of G_1 and of interphase ($G_1 + S + G_2$) lasting from 2 hr after selection are plotted as a function of the concentrations of benzaldehyde. The values are expressed relative to the respective median durations of G_1 and interphase of control cells.

For concentrations of benzaldehyde greater than 0.8 mM, the duration of both G_1 alone and of interphase are increased, indicating a cell-cycle inhibitory effect of benzaldehyde. The duration in both cases is doubled for a drug concentration of about 2.5 mM.

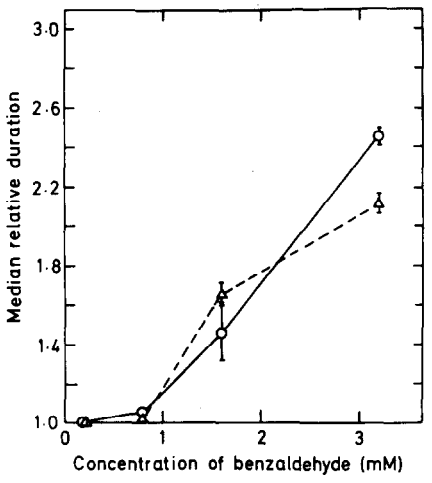

Fig. 3. The median relative duration of the part of G_1 (Δ) and of the part of the cell cycle (○) starting 2 hr after mitotic selection as a function of the concentration of benzaldehyde. Benzaldehyde was added 2 hr after selection and was not removed during the observation time (~35 hr). The entry of cells into S was recorded by flow-cytometric observation of the amount of DNA per cell and the entry of cells into the subsequent G_1 was recorded by microscopic observation. In both cases the duration of G_1 and of the cell cycle was calculated as a median value for the population. The experimental points represent the mean of such median value observations from 2–3 experiments. The values for treated cells are plotted relative to corresponding values for untreated cells. The vertical bars indicate standard error of the mean.

For concentrations lower than 1.6 mM the relative prolongation of G_1 alone is about the same as the relative prolongation of interphase (Fig. 3). At 3.2 mM interphase is prolonged more than G_1 alone, indicating a somewhat stronger inhibition in S and/or G_2 than in G_1 at this concentration.

To test this possibility more specifically we performed experiments where benzaldehyde (3.2 mM) was added for an 8-hr interval to two populations of synchronized cells: (A) the drug was added 2 hr after selection (early G_1) and removed 10 hr after selection; and (B) the drug was added 10 hr after selection (when almost 90% of the cells had entered S) and removed 18 hr after selection (when the first cells start to enter mitosis). Thus population A was treated for 8 hr during G_1 and population B was treated for 8 hr during S and G_2. The results are shown in Fig. 4, where the fraction of cells in G_1 and the fraction of cells that have not completed division are shown as a function of time after selection. For population A the delay of entry into S was about 4 hr, which was the same as the delay of cell division for population B. Thus benzaldehyde induced a similar inhibitory effect during an 8-hr treatment in G_1 as during an 8-hr treatment in S/G_2. However, from Fig. 4(A) it is seen that the cells were further delayed slightly in S and G_2 when treated during G_1 only. Thus the effect of benzaldehyde is not completely reversible. This finding may explain why the cell cycle is prolonged relatively more than G_1 during the continuous treatment with 3.2 mM benzaldehyde, as seen in Fig. 3: when benzaldehyde is present during the whole cell cycle, S and G_2 are both prolonged because benzaldehyde is present during these phases [the effect demonstrated in Fig. 4(B)], and because of the effect induced during G_1 [the additional delay expressed during S and G_2 in Fig. 4(A)].

Figure 4 also indicates that for both populations A and B the slopes of the curves representing benzaldehyde-treated cells are not much different from those of the control. This means that benzaldehyde has slowed the rate of cell-cycle traverse similarly for all cells, i.e. without significantly increasing the spread in individual cell-cycle durations.

One type of experiment was designed to test how rapidly after addition benzaldehyde acted to reduce the rate of DNA accumulation during S. Benzaldehyde (3.2 or 6.4 mM) was added to synchronized populations 8 hr after selection. At that stage about 50% of the cells had entered S. At intervals afterwards DNA histograms of the cells were registered by flow cytometry. Both the fraction of cells in G_1 and the median relative amount of DNA in cells in S [see insertion of Fig. 5(B)] were registered, and are plotted as a function of the time after selection in Fig. 5(A) and (B) respectively.

Figure 5(A) shows that during the time from 8 to 10 hr after selection the fraction of cells in G_1 is reduced from 50 to 20% in the control, i.e. 30% of the cells entered S during this time interval. Correspondingly, with 3.2 and 6.4 mM benzaldehyde 25 and 10% respectively entered S in the same period.

Figure 5(B) shows that the median relative amount of DNA in cells that have been able to enter S is reduced shortly after addition of benzaldehyde. During the first 2 hr after addition there is little or no increase in the amount of DNA for any of the benzaldehyde-treated populations. Later, the cells treated with 3.2 mM of the drug synthesized DNA at a rate similar to that of the control, while the cells treated with 6.4 mM had a negligible rate of DNA-synthesis.

Cell-cycle inhibitory effect during mitosis

During the experiments shown in Fig. 3 it was observed that mitosis was prolonged relatively more than interphase when benzaldehyde was present at a concentration of 3.2 mM. This effect was studied more closely by the following experiment. For each separate cell in populations

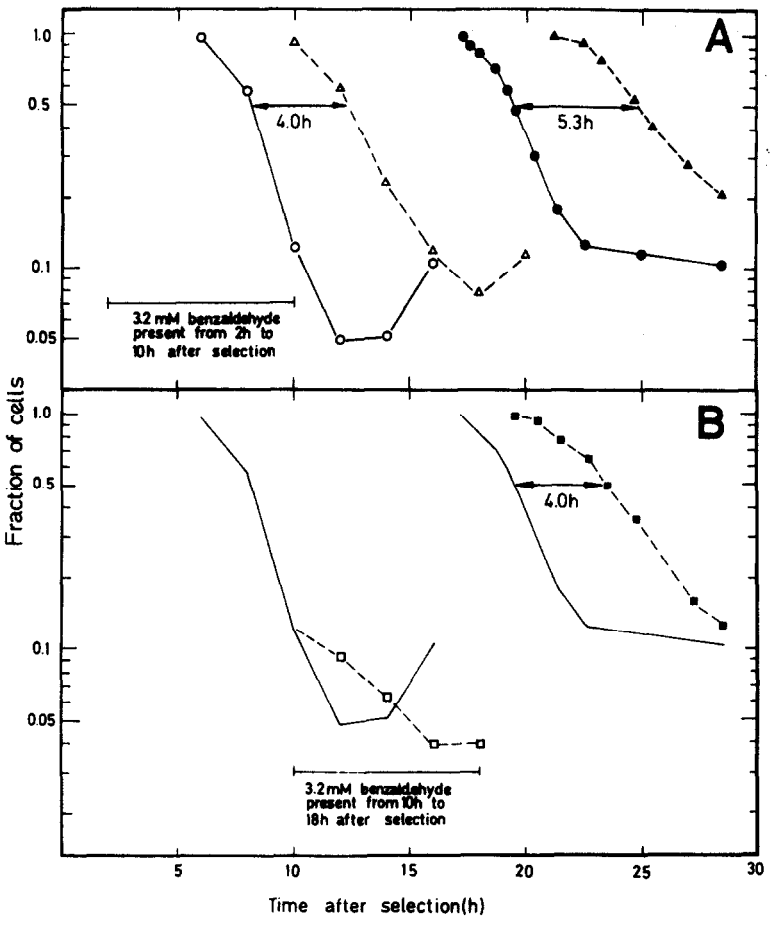

Fig. 4. The fraction of cells in G_1 (open symbols) and the fraction of cells that have not divided (closed symbols) as a function of time after mitotic selection. (A) Cells treated with 3.2 mM *benzaldehyde during the period from* 2 *to* 10 hr *after selection* (△,▲) *or untreated cells* (○,●); *(B) cells treated with* 3.2 mM *benzaldehyde during the period from* 10 *to* 18 hr *after selection* (□,■). *The curves for untreated cells shown in (A) are redrawn in (B).*

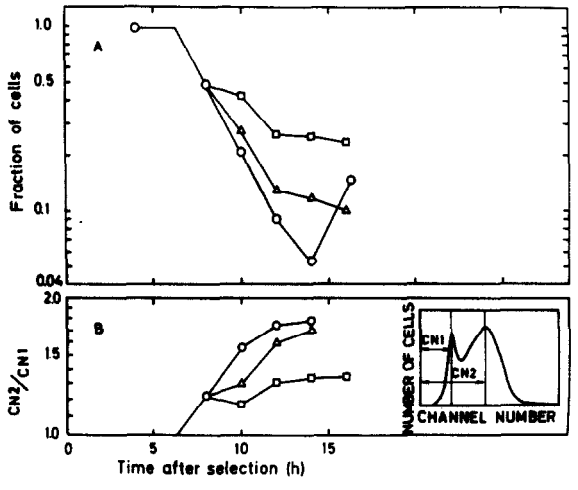

Fig. 5. (A) The fraction of NHIK 3025 *cells in G_1 as a function of time after selection of mitotic cells. Data represent an untreated control* (○) *and cells treated continuously with* 3.2 (△) *and* 6.4 mM (□) *benzaldehyde, added* 8 hr *after selection; (B) the median amount of DNA in S cells relative to the median amount of DNA in G_1 cells (CN_2/CN_1; see insertion) as a function of the time after mitotic selection. CN_1 and CN_2 are proportional to the median amount of DNA per cell in G_1 and in S respectively. The same DNA histograms as were analyzed to give the data in panel A were used. The symbols represent the same populations as described in panel A.*

of synchronized cells the duration of mitosis was recorded by microscopic observation. The results are shown in Fig. 6 as mitotic duration histograms of 4 different populations. The height of each column expresses the relative number of cells in the population which have a duration of mitosis between the limits noted on the base of the respective column.

From panel A, which represents untreated cells, one can see that 90% of the NHIK 3025 have a duration of mitosis shorter than 1 hr. Of the cells observed in this population, none had a duration of mitosis longer than 3 hr. The results presented in panel B, which represents cells treated with benzaldehyde for 8 hr during S and G_2 only, are identical with those presented in panel A. In this case benzaldehyde was removed immediately before the cells entered mitosis. Panels C and D represent cells treated with benzaldehyde from 14 or 16 hr after mitotic selection respectively. In these cases benzaldehyde was present during mitosis, and as demonstrated by the results, the duration of mitosis is extensively prolonged as compared with the untreated control. It was further observed that among the cells that

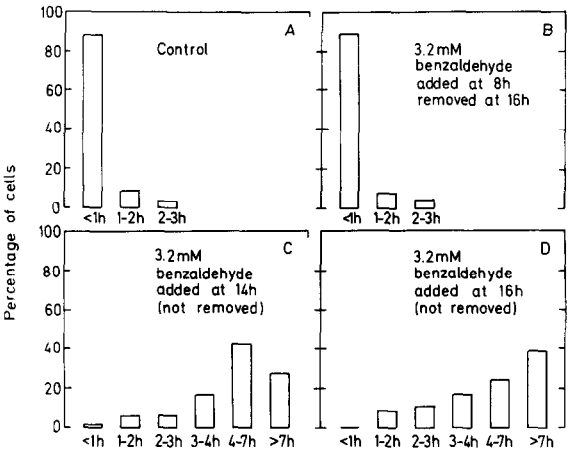

Fig. 6. Histograms showing the relative number of NHIK 3025 cells having a duration of mitosis within the discrete intervals as marked at the base of each column. The 4 populations were: (A) untreated cells (control); (B) cells treated with 3.2 mM benzaldehyde from 8 (early S) until 16 hr after mitotic selection (i.e. benzaldehyde was removed before the first cells entered mitosis); (C) and (D) cells treated with benzaldehyde from 14 and 16 hr after mitotic selection respectively (i.e. benzaldehyde was added either 2 hr before or immediately before the first cells entered mitosis). For each of the populations about 100 cells were observed repeatedly with less than 1-hr intervals in an inverted microscope placed within a walk-in incubator room. The time of entrance into, as well as the time of end of, mitosis was noted for each cell separately. Within the observation time used in these experiments about 95% of the observed cells in populations A and B and 50–60% of the observed cells in populations C and D entered mitosis and completed division. In populations C and D about 20% of the observed cells entered mitosis but were not able to divide during the observation time. Some of these cells entered mitosis shortly before the end of observation and could therefore not be included in the calculation.

completed division after being inhibited during mitosis, only about 25% gave rise to 2 daughter cells, while about 65% gave rise to 3 and 10% gave rise to 4 or more daughter cells. For untreated cells and for cells treated with benzaldehyde during interphase only (Fig. 6A and B), more than 99% of the cells produced 2 daughter cells.

DISCUSSION

Inactivation

Benzaldehyde has a low inactivating effect on cells in culture when compared with corresponding data for drugs in clinical use as cytostatics [16]. A 24-hr treatment with 6.4 mM (670 μg/ml) benzaldehyde was survived by 30% of the cells in the exponentially growing population and by 45% of the cells in the synchronized population receiving the drug in early G_1 (Fig. 2). For comparison, after 24-hr treatment with 0.3 μM vincristine 0.5% of the cells in an exponentially growing population of NHIK 3025 cells were able to form colonies [17].

The plateau in the concentration range between 3 and 6 mM as seen in Fig. 1 is well explained on the basis of the data in Table 1 and in Figs 2 and 5. Figure 5 clearly demonstrates that while the cells are able to traverse through the cell cycle when treated with 3.2 mM benzaldehyde, they are almost completely arrested when treated with 6.4 mM. Table 1 and Fig. 2 indicate that the cells are particularly sensitive during mitosis provided that they are able to progress through this phase, i.e. the cells are more sensitive during mitosis to 3.2 mM than to 6.4 mM. Also, the difference in sensitivity between the exponentially growing and the synchronized cells treated for 24 hr as shown in Fig. 1 may be a result of the cell-cycle inhibition and the specific cytotoxic effect induced in mitosis. Of the synchronized cells treated with 3.2 mM benzaldehyde from 2 hr after selection, only few cells reach to mitosis in 24 hr (see Fig. 3). However, among the exponentially growing cells many were in late S and G_2 when benzaldehyde was added. Thus more cells were able to reach to the sensitive stage among the exponentially growing than among the synchronized cells.

Inhibition of cell-cycle traverse

A significant division delay is observed for synchronized NHIK 3025 cells treated continuously with benzaldehyde at concentrations higher than 0.8 mM starting 2 hr after selection. Miyakawa *et al.* [18] showed that for rat 3Y1-B embryo fibroblasts the lowest concentration of benzaldehyde which induced a significant reduction in the rate of cell division was 50 μg/ml (~0.5 mM) and 10 μg/ml (~0.1 mM) for untransformed and transformed cells respectively. Thus the human NHIK 3025 cells show inhibitory effects at a slightly higher concentration than the untransformed rat 3Y1-B cells. The small difference could perhaps be due to technical differences in culture conditions etc. and is not judged as representing a significant difference in cell sensitivity.

The cell-cycle inhibition of NHIK 3025 cells is not related to any specific stage of interphase but, rather, the rate of cell-cycle traverse is slowed down about equally in G_1, S and G_2. This is most clearly shown by Fig. 4, where the delay induced during an 8-hr treatment with 3.2 mM benzaldehyde is shown to be equal (4 hr) whether the treatment was given in G_1 or in $S+G_2$. This finding suggests that DNA-synthesis is not a target process for the inhibitory effects of benzaldehyde. Nevertheless, the DNA-synthesis was inhibited completely by treatment with 6.4 mM benzaldehyde (Fig. 5) without causing any great degree of inactivation. Thus the process

or processes affected by benzaldehyde must influence the regulation of DNA synthesis without actually causing extensive lethal damage to DNA itself.

The mechanism by which benzaldehyde inhibits cell-cycle proliferation so efficiently without inducing lethal effects is unknown at the moment. Our recent studies on the regulation of cell-cycle traverse and cell growth [19–21] have, however, shown that the cell-cycle kinetics of the NHIK 3025 cells may be efficiently affected through a change in the rate of protein accumulation. Experiments with various aliphatic aldehydes have shown that these can depress protein synthesis [22–24]. Although these chemicals have a structure different from benzaldehyde they share the feature of having a terminal aldehyde group. Thus we have initiated experiments where the effect of benzaldehyde on protein metabolism (synthesis and degradation) will be studied and correlated with the inactivating and cell-cycle inhibitory effects as demonstrated in the present paper.

A specific inhibitory effect was observed during mitosis (Fig. 6). This effect appeared only if benzaldehyde was present during mitosis. Whether benzaldehyde was added immediately before the cells entered mitosis or 2 hr earlier, the effect on mitosis was the same. From these results we conclude that benzaldehyde affects some process taking place either during mitosis or a few minutes before the cells enter mitosis.

In conclusion, for concentrations below 6.4 mM, benzaldehyde was shown to induce inactivating effects which were particularly strong when the cells were treated in mitosis and with a concentration low enough to permit some cell-cycle progression. Furthermore, benzaldehyde was shown to induce cell-cycle inhibitory effects of similar strength in G_1, S and G_2. For a concentration of 3.2 mM the inhibition was relatively stronger in mitosis than in interphase.

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