Arh. hig. rada, *26* (1975) 3.

AUTORADIOGRAPHIC STUDIES OF ^{3}H-URIDINE UPTAKE BY HUMAN CELLS IN TISSUE CULTURE TREATED WITH PUROMYCIN (PURO AND PAN) AND X-RADIATION.

YVETTE ŠKREB, LJERKA RADEŠIĆ and JADRANKA RAČIĆ

Institute for Medical Research and Occupational Health, Yugoslav Academy of Sciences and Arts, Zagreb, Yugoslavia

(Received for publication February 5, 1975)

The effect of X rays, puromycin dihydrochloride (Puro) and puromycin aminonucleoside (Pan) was studied on HeLa cells growing asynchronously. In one group, the cells were submitted to a single treatment: they were irradiated with 300 rads X rays or incubated for 2 hours in a nutrient medium supplemented with Puro (50 μg/ml) or Pan (10 μg/ml). In the second group the irradiated cells were submitted to a post or pretreatment with Puro or Pan in the same conditions.

To evaluate the effect, the survival and RNA synthesis were used as parameters. Besides examination of colony forming ability, autoradiographic experiments related to the ^{3}H-uridine uptake were performed.

Regarding the survival, it can be seen that when the agents are applied separately, they produce different effects, those of X rays being more drastic and of Pan less so. In post or pretreatment, Puro enhances the effect of X rays while Pan diminishes it.

When applied separately or successively, the three agents produced a similar inhibition of ^{3}H-uridine uptake in the nucleoli. In the nucleoplasm and the cytoplasm, the three agents applied separately produced the same kind of effect, the depression caused by Puro being slightly higher. In the case of double treatments, the inhibitory effect of Puro did not add to that of radiation which was only enhanced. Pan which also reduces the ^{3}H-uridine uptake in the nucleoplasm and cytoplasm when applied alone, given after or before irradiation prevents to a large extent the X-ray inhibition so that the final labelling is very high.

It can be concluded that applied before or after X irradiation Puro does not affect significantly the survival and RNA synthesis, Pan significantly improves both the survival and RNA synthesis (apart from the nucleoli) in the same conditions. This differential effect seems to be due to a particular effect on the synthesis of proteins especially those involved in the repair mechanisms.

Appropriate treatments with various metabolic inhibitors modify the radiation response of mammalian cells in culture. Most investigations are focussed on the modification of the survival of the treated cells. *Elkind* (1) showing some of the effects of drugs on the recovery pattern suggested that the synthesis of new proteins is probably not required for the repair of damage. *Phillips* and *Tolmach* (2) found in some instances that postirradiation treatment with specific inhibitors of DNA or protein synthesis alter the survival of HeLa cells. In the same way *Kim, Eidinoff* and *Laughlin* (3) in HeLa cells and later *Elkind, Moses* and *Sutton-Gilbert* (4) in C. Hamster cells showed that low doses of puromycin, a strong inhibitor of protein synthesis, did not alter the pattern of repair. *Sinclair* (5) reported that when C. Hamster cells are treated with similar inhibitors before irradiation, the survival depends upon the stage reached by the cells in the cycle and not whether active DNA or protein synthesis are in progress just prior to irradiation. The data obtained by *Đorđević, Kim* and *Kim* (6) showed that puromycin aminonucleoside markedly reduces the colony-forming ability of HeLa cells especially when irradiated at the end of the S phase. *Arlett* (7) underlined the complexity of the interpretation of the modification of post-irradiation effects by metabolic inhibitors and concluded that the radiation response found by *Phillips* and *Tolmach* cannot be largely extended. Among the various inhibitors assayed, *Berry* (8) mentioned that the possibilities offered by puromycin to modify the radiation effect are not exhausted. Puromycin dihydrochloride (Puro) is structurally similar to the 3' — terminus of aminoacyl-t RNA. Puro blocks protein synthesis by replacing an aminoacyl-t RNA and reacting with the nascent polypeptide on the peptidyl-t RNA site of the ribosome (9). Puro acts by prematurely releasing incompleted polypeptide chains and produces a rapid breakdown of polysomes. It also inhibits RNA synthesis (10). Puromycin aminonucleoside (Pan) obtained by hydrolytic cleavage of puromycin dihydrochloride is often used. Pan in high concentrations is not a specific inhibitor of protein synthesis but interferes with phosphorylation mechanisms and inhibits the synthesis of all types of nucleic acids and proteins. In lower concentrations it produces a selective inhibition of RNA synthesis especially that of r RNA (11) which is similar to the one produced by actinomycin D.

In this work we intended to make a contribution to the numerous and heterogenous data mostly obtained on the modification of cell survival, focussing our attention on RNA synthesis in the treated cells. With this aim, we used HeLa cells in asynchronous culture as experimental material and either puromycin dihydrochloride (Puro) or puromycin aminonucleoside (Pan) as modifiers after or before X irradiation.

MATERIALS AND METHODS

HeLa cells were grown asynchronously in Eagle 's minimum essential medium supplemented with 10% calf serum, without CO_2. In the exponential phase of growth, one aliquot of the cells was submitted to irradiation or to the action of metabolic inhibitors, the second part was incubated with inhibitors after or before irradiation.

The schedule of the treatments was the following:

1. control group with no treatment
2. cells treated with X rays
3. cells treated with Puro
4. cells treated with Pan
5. cells treated with X rays and Puro (2+3)
6. cells treated with Puro and X rays (3+2)
7. cells treated with X rays and Pan (2+4)
8. cells treated with Pan and X rays (4+2)

To evaluate the effect of irradiation and metabolic inhibitors on the cell viability, the number of colonies formed from treated cells was determined. The cells were trypsinized, counted and diluted. An inoculum was chosen which yields from 50 to 200 colonies per culture vessel after a 14-day incubation.

To measure the rate of RNA synthesis, the uptake of ^{3}H-uridine in the cells was followed. The cells were labelled by addition of the precursor to the regular nutrient medium at a final concentration of 0,1 μCi/ml. After two hours, the radioactive medium was removed, the cells fixed in acetic acid: ethanol (1:3) and then treated with TCA 5% at 4°C.

The specificity of the incorporation was controlled enzymaticaly with deoxyribonuclease from Fluka (Switzerland). The cells were submitted to the usual autoradiographic procedure with the stripping film Kodak AR 10 (12).

After development, the radioactive cells were stained by the classical Unna-Brachet method to localize the labelling of the RNA.

For each experiment the rate of RNA synthesis was evaluated by counting the number of reduced silver grains in the whole cell and separately in the nucleoli, the nucleoplasm and the cytoplasm. The mean per one cell, standard deviation and »chi-square« test were performed to estimate our experimental data.

The X-ray dose and the concentration of inhibitors were selected after examination of the survival and the kinetics of ^{3}H-uridine uptake. Finally 300 rads X rays, 10μg/ml Pan or 50 μg/ml Puro which applied separately reduced the labelling of the nucleoli of about 50% were chosen. A two-hour application of the agents was the shortest possible to produce both reproducible results and a sufficient labelling in the whole cell.

The X rays were produced from a Philips X-ray source (220 kV, 20 mA, h. v. l. 0,5 mm Cu) at a dose rate of 100 rads/m. The distance from the source was 40 cm and irradiation was performed at room temperature (Rudjer Bošković Institute, Zagreb).

Puromycin dihydrochloride was obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio, USA. Puromycin aminonucleoside came from Sigma Chemicals, St. Louis, Missouri, USA. Deoxyribonuclease was from Fluka, Switzerland. ^{3}H-uridine spec. act. 1.74 Ci/mM came from the Radiochemical Centre, Amersham, England.

RESULTS

The results can be divided in three parts according to the survival, the ^{3}H-uridine uptake and the comparison beween the survival and the labelling of the treated cells. All experiments were done in triplicate. The results of a typical experiment are illustrated by one table and three groups of curves.

Table 1

Effect of X rays, Puro and Pan on the survival and the ^{3}H-uridine uptake in HeLa cells shown in a typical experiment

Treatments	Survival	Residual incorporation								Ratios	
		Whole cells		Nucleoli		Nucleoplasm		Cytoplasm		a/c	$\frac{d+f}{h}$
	% (a)	No grains (b)	% (c)	No grains (d)	% (e)	No grains (f)	% (g)	No grains (h)	% (i)	(j)	(k)
1. Controls	100	1922	100	710	100	803	100	403	100	1	3.7
2. 300 rads	32	1211	63	424	60	553	69	234	57	0.5	4.2
3. Puro	52	924	48	350	49	418	52	156	38	1	4.9
4. Pan	89	1137	59	341	48	541	67	255	62	1.5	3.4
5. X irr.+Puro	26	1139	59	373	53	531	66	233	57	0.45	3.9
6. Puro+X irr.	30	1178	61	390	55	538	67	250	61	0.5	3.7
7. X irr.+Pan	52	1416	74	377	53	700	87	339	83	0.7	3.1
8. Pan+X irr.	48	1409	73	392	55	702	87	315	77	0.65	3.4

(a) percent of survival when controls are taken as 100%
(b) total number of reduced silver grains in random 30 cells in every group
(c) percent of residual incorporation when controls are taken as 100%
(d), (f), (h) number of grain counted in the nucleoli, the nucleoplasm and the cytoplasm. — (e), (g), (i) percent of incorporation
(j) ratio between the percent of survival and incorporation
(k) ratio between incorporation in the whole nucleus and the cytoplasm

Table 1 gives a general view of all results. First it can be seen that apart from Pan, the colony forming ability is strongly affected by each treatment. When treatments are combined, puromycin does not affect significantly the survival of irradiated cells, but Pan improves their survival by about 20%. The time of addition of inhibitors does not appear to be critical.

The labelling is expressed either as the total number of grains recorded in 30 cells in every group and as the nucleolar, nuclear and cytoplasmic counts separately. The percentage of the labelling in the treated

cells in relation to the control ones considered as 100% is also mentioned. The incorporation in the whole cell does not reflect exactly what happens in every compartment as shown in columns (e), (g) and (i).

The reduction of the ^{3}H-uridine uptake can be characterized as follows:

1. The inhibition is strongest in the nucleoli whatever the treatment.
2. In the nucleoplasm and the cytoplasm the level of inhibition is very similar.
3. The most pronounced inhibitory effect is due to Puro alone, the weakest to Pan with irradiation.
4. The inhibitory effect of X rays alone, X rays and Puro and Puro alone is similar.
5. The combined effect of X rays with Puro or Pan is not additive and Pan improves the uridine uptake after X irradiation.

The reduction of ^{3}H-uridine uptake affects temporarily all the three cell compartments and suggests an inhibition of RNA synthesis rather than of nucleocytoplasmic transfer. This is reflected in Table 1 (k) where it is shown that the ratio of overall nuclear to cytoplasmic labelling is of the same size range.

The curves obtained after calculation of »chi-square« test permit to examine with more details what happens in every part of the treated cells.

Fig. 1 which relates to the nucleoli is very clear. It is obvious that each treatment with Puro and Pan applied either alone or after X irradiation temporarily reduces the ^{3}H-uridine uptake. The four groups treated with antibiotics closely resemble one another regarding the level of the incorporation as well as the distribution of the labelling. There is no significant difference between the treatments with antibiotics and X rays alone.

Although the curves in Fig. 2 relative to the incorporation in the nucleoplasm are different, they are not easy to characterize. Puro alone produces the strongest inhibition which is greater than the one obtained for X rays alone or even with X rays followed by Puro. The depression produced by Pan alone is of the same order of magnitude as for X rays alone. However, the labelling of the nucleoplasm after X rays is significantly greater than with either of these agents alone. X rays seem to increase the nucleoplasm labelling applied before Pan. The distribution of the labelling in the nucleoplasm does not seem much affected by the respective treatments. Although the curves are not in the same position, they have a similar shape.

In the cytoplasm (Fig. 3) the differences between the groups are more pronounced, Puro alone is always the strongest inhibitor. However, applied after irradiation it does not modify the level of the precursor incorporation. The effect of Pan alone is of the same order as that of

X rays but the distribution of the grains per cell is different and very homogenous. Applied after irradiation, Pan seems to stimulate the ^{3}H-uridine incorporation in the cytoplasm.

When the combined treatments for cell survival and RNA synthesis are considered, returning to Table 1 column (j) the analysis of the ratios shows that

1. Applied before or after X rays Puro does not affect significantly the survival and RNA synthesis,
2. Pan significantly improves both survival and RNA synthesis (except in the nucleoli).

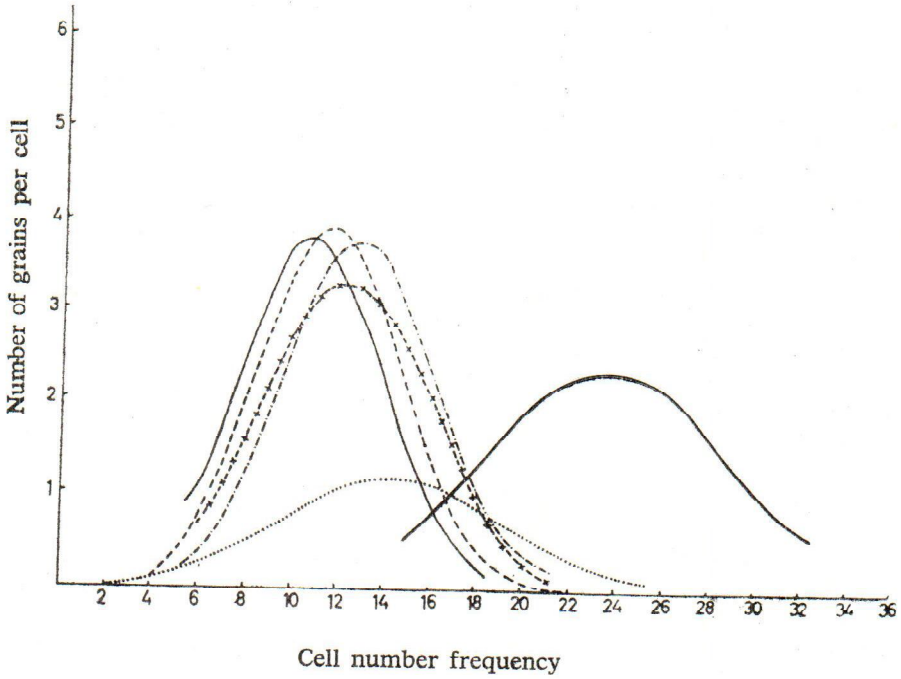

Fig. 1. — *Comparison of treatments in terms of their effect on the ^{3}H-uridine uptake in the nucleoli of individual cells. The reduced silver grains were counted in 30 random cells from each sample. Assays were made in triplicate. The means and the standard error of the means were calculated. The »chi-square« test was performed in a typical experiment illustrated with 6 curves shown here. Each curve is drawn on the basis of the frequency of the cells or compartments of cells classified according to the number of grains per cell.*
——controls; ———Pan; — — — —Puro; .....X rays, 300 rads; —.—.—300 rads +Pan; — — x — — x — — x 300 rads+Puro

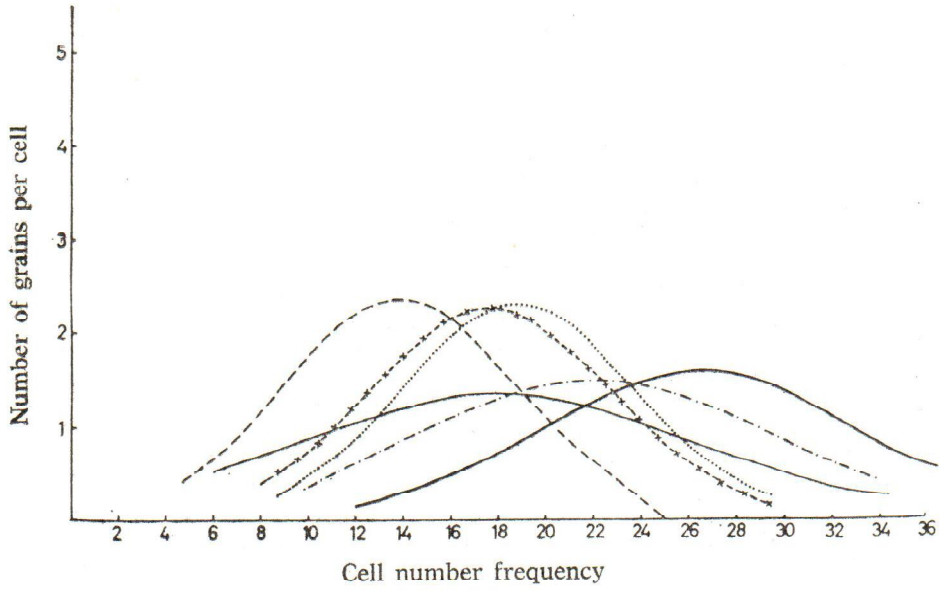

Fig. 2 — *Comparison of treatments in terms of their effect on the ^{3}H-uridine uptake in the nucleoplasm of individual cells. The rest of the legend is the same as in Fig. 1*

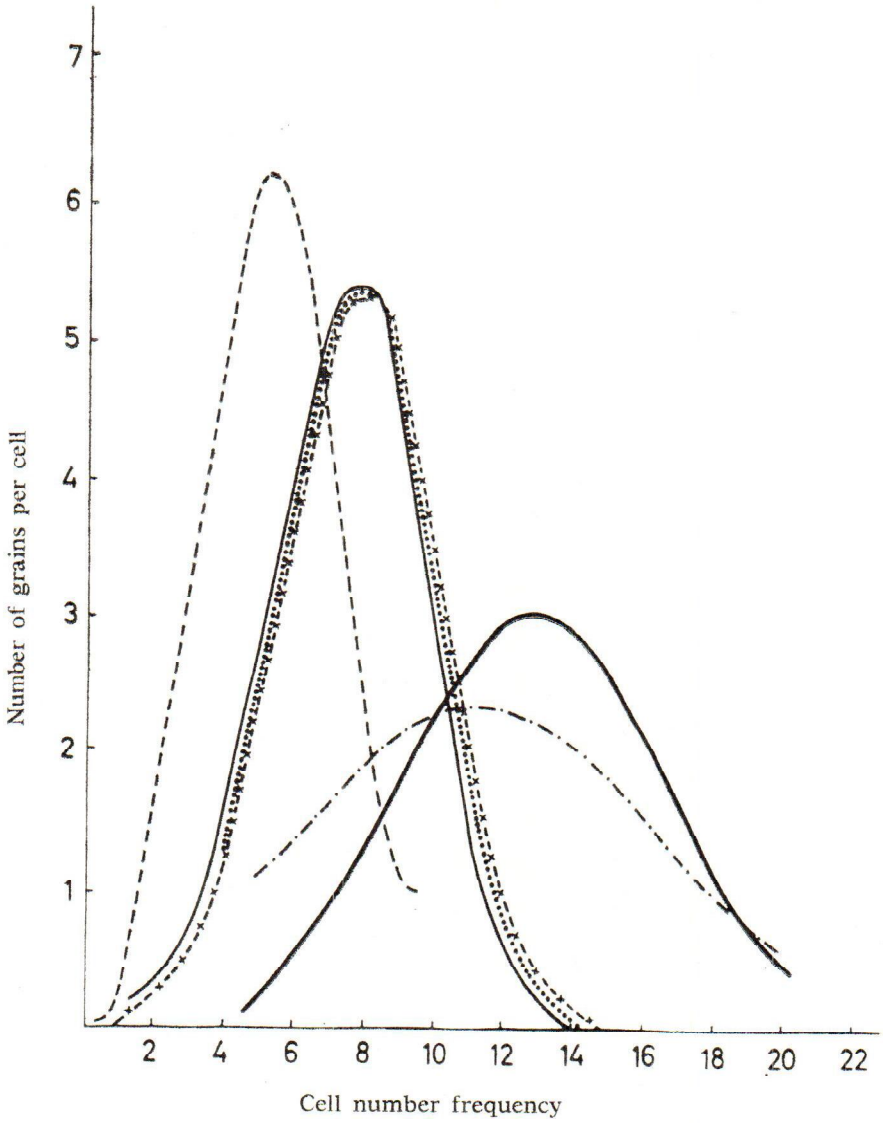

Fig. 3. — *Comparison of treatments in terms of their effect on the ^{3}H-uridine uptake in the cytoplasm of individual cells. The rest of the legend is the same as in Fig. 1.*

DISCUSSION

Is it possible to find an explanation for the differences observed in the ^{3}H-uridine uptake as well as in the distribution of grains per cell compartment? How can the differences in the level of survival be explained? How do these results fit with those obtained in other laboratories?

Focussing our attention on RNA synthesis one could assume that the three agents applied separately inhibit the same step but in this case, one should expect their effects to be additive. It seems therefore more probable that the agents act in a manner where the lesions produced by the first begin to repair while the second is still acting. So the final effect is never additive.

The differences in the mode of action of the agents could also be explained by the fact that the cells grow asynchronously and that the treatments affect the cell cycle in a different manner which could account for the lack of correlation. It is well known that inhibitory treatments can retain the cells in a single phase of the cycle. There is only one case with Puro applied alone when correlation between parameters is really good. With X rays alone or in combination with Puro the survival and the synthesis are reduced but the survival is even more so. With X rays and Pan, the survival is higher than RNA synthesis. With Pan alone, it is the contrary.

As regards other data, *Painter* (13) underlined the difficulties in finding good reproducibility between experiments. Ten years later, *Enger* and al. (14) report that divergences always exist between the X-ray effects upon RNA synthesis from one laboratory to another. In our case, the reduction of ^{3}H-uridine incorporation is very pronounced.

As to Puro, our data do not fit entirely with those of *Jackson* and *Studzinski* (11). We observed a similar level of inhibition in every compartment of the cell. The results of combined treatments are largely in agreement with those of *Arlett* (15) and *Đorđević* and *Kim* (16) although experiments were performed under different conditions. However, they offer some discrepancy with those obtained after a combination of actinomycin D (17) or lucanthone with X rays (18).

Our results with Pan alone confirm the high depression of RNA synthesis in the nucleoli shown by *Studzinski* and *Ellem* (19), in a somewhat different way. They also corroborate the lack of correlation between proliferation of cells and ^{3}H-uridine uptake (20).

Applying Pan after 300 rads X rays, Đorđević and al. (6) found that there is no significant inhibitory effect of Pan on protein and DNA synthesis, only on RNA synthesis.

As recently mentioned by *Bleehen* (21) confusion remains about the role of combined drugs and radiation. For the time being we can only conclude that the ^{3}H-uridine uptake quickly responds to external factors such as irradiation or metabolic inhibitors but not as quickly as the survival. The role of RNA especially r RNA in the survival remains to be ex-

plained. It can be only suggested that the differences in response to the action of radiation and inhibitors regarding the two parameters used may be due to an intricate action on the protein synthesis especially on enzymes involved in the repair mechanisms.

ACKNOWLEDGEMENTS

The authors are greatly indebted to Prof. M. Errera for critical comments on the manuscript and to Mr. D. Bečarević for help in statistical evaluation of the results.

This investigation was supported by a grant from the Council for Scientific research of Croatia.

References

1. *Elkind, M. M.:* Jap. J. Genet., *40* (Suppl) (1964) 176.
2. *Phillips, R. A., Tolmach, L. J.:* Radiat. Res., *29* (1964) 413.
3. *Kim, J .H., Eidinoff, M. L., Laughlin, J. S.:* Int. J. Radiat. Biol., *11* (1966) 509.
4. *Elkind, M. M., Moses, W. B., Sutton-Gilbert, H.:* Radiat. Res., *31* (1967) 156.
5. *Sinclair, W. K.:* Proc. Natl. Acad. Sci. U. S., *58* (1967) 115.
6. *Đorđević, B., Kim, J. H., Kim, S. H.:* Int. J. Radiat. Biol., *14* (1968) 1.
7. *Arlett, C. F.:* Int. J. Radiat. Biol., *17* (1970) 515.
8. *Berry, R. J.:* Radiol. Clin. N. Amer., *3* (1965) 249.
9. *Nathans, D.:* Antibiotics (ed. D. Gottlieb & PD. Shaw) vol. 1, p. 259 Springer-Verlag, Berlin (1967).
10. *Holland, J. J.:* Proc. Natl. Acad. Sci. U. S., *50* (1963) 436.
11. *Jackson, L. G., Studzinski, G. P.:* Exptl. Cell Res., *52* (1968) 408.
12. *Pelc, J. R.:* Int. J. Appl. Radiat. Isotop., *1* (1956) 1972.
13. *Painter, R. B.:* Radiat. Res., *16* (1962) 846.
14. *Enger, M. D., Campbell, E. W., Walters, R. A.:* Biochim. Biophys. acta, *324* (1973) 120.
15. *Arlett, C. F.:* Int. J. Radiat. Biol., *13* (1968) 369.
16. *Đorđević, B., Kim, J. H.:* Radiat. Res., *37* (1969) 435.
17. *Horvat, D., Škreb, Y.:* Stud. Biophys., *18* (1969) *119.*
18. *Bases, R.:* Cancer Res., *30* (1970) 2007.
19. *Studzinski, G. P., Ellem, K. A. O.:* J. Cell Biol., *29* (1966) 411.
20. *Studzinski, G. P., Gierthy, J. F.:* J. Cell Physiol., *81* (1973) 71.
21. *Bleehen, N. M.:* Brit. Med. Bull., *29* (1973) 54.

Sažetak

AUTORADIOGRAFSKI STUDIJ INKORPORACIJE ^{3}H-URIDINA U LJUDSKIM STANICAMA U KULTURI KADA SU TRETIRANE PUROMICINOM (PURO i PAN) PRIJE ILI POSLIJE RENDGENSKOG ZRAČENJA

Cilj rada bio je proučavanje posljedica jednokratnog djelovanja puromicina na HeLa stanice u asinkronoj kulturi, prije ili poslije rendgenskog zračenja. Za zračenje stanica upotrijebili smo dozu od 300 rada. Koncentracije puromicina kojima su stanice bile izvrgnute tijekom 2 sata bile su za dihidroklorid puromicina (Puro) 50 μg/ml a za aminonukleozid puromicina (Pan) 10 μg/ml.

Kao parametri za evaluaciju djelovanja nabrojenih agensa poslužili su nam preživljavanje stanica i sinteza RNA. Pored sposobnosti formiranja kolonija,

autoradiografskom smo metodom pratili sintezu RNA ugrađivanjem specifično obilježenog prekursora ^{3}H-uridina. Radioaktivni tragovi su brojeni ne samo u cijelim stanicama svake skupine, nego posebno u nukleolima, nukleoplazmi i citoplazmi.

Što se tiče preživljavanja, može se uočiti da agensi, apliciran odvojeno, imaju različit učinak: rendgensko zračenje djeluje najjače, a Pan najslabije. U nukleolima svaki od postupaka uzrokuje sličan inhibitorni učinak na inkorporaciju ^{3}H-uridina (50%). Dok Puro izaziva isti stupanj inhibicije (50%) u cijeloj stanici, Pan pospješuje inkorporaciju ^{3}H-uridina u nukleoplazmi i citoplazmi ozračene stanice u takvoj mjeri da je sinteza RNA gotovo normalna.

Iz dobivenih rezultata može se zaključiti da Puro apliciran prije ili poslije zračenja ne mijenja bitno preživljavanje stanica niti RNA sintezu. Pod istim uvjetima Pan povećava preživljavanje i RNA sintezu gotovo do normale (osim u nukleolima). Čini se da razlike između djelovanja Puro i Pan leže u diferencijalnom efektu na jednu od funkcija DNA, što indirektno utječe na enzime potrebne za uključivanje reparatornih mehanizama.

Institut za medicinska istraživanja i medicinu rada JAZU u Zagrebu

Primljeno 5. II 1975.