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CHANGES IN ENERGY METABOLISM FOLLOWING ROENTGEN IRRADIATION OF IN VIVO GROWING EHRLICH ASCITES TUMOUR CELLS STUDIED BY ^{31}P MAGNETIC RESONANCE SPECTROSCOPY

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Abstract

The energy metabolism in Ehrlich ascites tumour cells following in vivo irradiation of a dose of 5.0 Gy was studied in vitro in their ascites fluid up to 48 hours using ^{31}P magnetic resonance spectroscopy measuring ATP, ADP and inorganic phosphate (P_i). The results are also related to radiation induced changes in cell cycle composition. ATP was reduced by more than 50 per cent 20 to 24 hours after irradiation but normalized at 48 hours. ADP was reduced to about half the normal level 24 to 48 hours after irradiation. When the ATP and ADP had reduced levels, the inorganic phosphate increased correspondingly. Addition of glucose to the ascites cell suspension at the time of minimum ATP level immediately raised the ATP : P_i ratio. Since the glucose concentrations in blood and in ascites fluid following irradiation were also reduced, lack of glucose for energy production might have been a major contributing factor for the reduced ATP production.

Key words: Radiation biology, cell studies; Ehrlich ascites cells, irradiation, energy metabolism, magnetic resonance spectoscopy.

In recent experiments the ATP utilization during the cell cycle of non-irradiated and roentgen irradiated in vivo growing Ehrlich ascites tumour cells has been investigated (12, 13) by conventional biochemical methods. In these studies the ATP utilization was determined from the ATP content and the ATP production, the latter calculated from the O_2 uptake and the lactate production of the cells incubated in vitro. The ATP content doubled during the cell cycle but in a non-linear way. The ATP turnover time reached its maximum value at the G_1/S-phase borderline and remained constant during the remainder of the cell cycle. In irradiated cells the ATP content was reduced at 5 hours and reached a maximum reduction at 24 hours; at this time the accumulation of cells in G_2 showed its maximum value. Since O_2 uptake and lactate production were measured in vitro, deviations from the energy metabolism of in vivo growing cells due to external factors acting in vivo could not be excluded. It was therefore uncertain whether the ATP utilization following irradiation was really reduced or not. Alternative mechanisms leading to the decline of the ATP content have been discussed, but since only the ATP was studied no clear-cut alternatives have been established.

The aim of the present investigation was further to elucidate the mechanism of the radiation induced ATP reduction by measuring the ATP : ADP : P_i phosphate using ^{31}P magnetic resonance (MR) spectroscopy. This is a non-invasive technique which makes it possible to study cells under conditions comparable to the in vivo growth. Possible disturbances of the glycolysis were also studied by adding glucose. In this connection we also determined the transport rate of glucose into the tumour cells and from the hosts to the tumour.

To exclude the effects of changes in the cell cycle composition on the results, the cell cycle distribution of the cell material was determined by means of flow-cytometric DNA measurements.

Material and Methods

Experimental procedure. The experimental procedures have been described in detail in a recent report (14).

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Briefly, hyperdiploid Ehrlich ascites tumour cells growing in 3-month-old female NMRI mice were used. The animals were whole-body irradiated with a dose of 5.0 Gy at day 4 after transplantation.

^{31}P MR measurements. At different times following irradiation, the animals were killed by cervical dislocation and the ascites cell suspension was removed by pre-cooled pipettes and put into pre-cooled MR tubes. Two to 3 min after killing the mouse MR measurement started and ran for 8.5 min at a temperature of +7°C. The MR spectrometer used was a Varian XL-300 operating in the Fourier transform mode at a phosphorus frequency of 121.4 MHz. No internal or external field-frequency lock was used. A typical spectrum was a result of a number of 512 45° pulses with a repetition time of 1 s. In the glucose feeding experiments a concentrated glucose solution was added to the ascites cell suspension (0.1 ml glucose: 3.0 ml cell suspension, giving a final glucose concentration of 30 mmol/l) just before starting the measurements.

In each spectrum peak-area ratios for different sets of components were used to describe the metabolic state of the cell suspension. These area ratios reflect concentration ratios only if saturation effects in the MR experiments are corrected (4, 8, 11). In a preceding investigation (13) we found that only the inorganic phosphate (P_i) peak needed this correction (saturation factor 2.0). In the present study the same experimental conditions were applied and thus we used the same correction factor.

In order to select the most appropriate method to determine the changes in the amount of the phosphorus compounds, PCA-acid extracts of cells and of cells frozen in liquid nitrogen were studied. The PCA extract was obtained by a mixture of PCA with unwashed ascites cell suspension to a final concentration of 0.2 mol/l PCA and frozen at −20°C. Before measuring in the spectrometer 0.5 mmol/l EDTA was added in order to bind divalent cations and thereby reduce line-broadening effects (3). The cell concentration of the acid extracts was comparable to the suspension of intact cells. We found that either almost all adenosine nucleotides were transferred to the nucleoside with a dramatical rise in the amount of inorganic phosphate, or that the proportion of inorganic phosphate: ADP: ATP was more or less unchanged but that the total amounts of these compounds had decreased markedly.

When freezing the cell suspension at −70°C by putting the tubes into liquid nitrogen, most of the signals from the adenosine nucleotides and inorganic phosphate were lost combined with an elevated signal in the sugarphosphate region.

Since acid-extracted and nitrogen-freezing procedures give rise to drastic changes in the composition of the phosphorus compounds, only intact cell suspensions were used in all the experiments.

Glucose concentration and ^{14}C-deoxyglucose uptake. The glucose concentration in the blood plasma and in the ascites fluid was determined using a test-combination Gluco-quant kit (Boehringer Mannheim GmbH Diagnostica). Briefly, the animals were killed by cervical dislocation, 0.1 to 0.2 ml blood was put into 0.9 to 1.0 ml 0.33 mol/l PCA and 0.4 ml of the ascites fluid into 0.7 ml 0.33 mol/l PCA. The ascites fluid was collected by centrifuging the ascites cell suspension at 3000 rpm for 10 min. 0.1 ml from the blood sample and 0.25 ml from the ascites fluid sample of the PCA soluble fractions were mixed with 2.0 ml and 1.85 ml phosphate buffer (phosphate 70 mmol/l, Mg^{++} 4 mmol/l, NADP 1.3 mmol/l, ATP 1.3 mmol/l, pH 7.7). After reading at 340 nm, 0.02 ml of an enzyme reagent (glucose-6-phosphate dehydrogenase ≥180 U/ml and hexokinase ≥100 U/ml) were added and again read at 340 nm.

The transport of the glucose from the hosts to the ascites fluid and from the ascites fluid into the cells was also studied by measuring the cellular uptake of ^{14}C-deoxyglucose. Thirty-seven kBq (1.85 GBq/mmol) was injected into the vein of the tail or intraperitoneally with an isotope time up to 20 min. The animals were killed by cervical dislocation and the ascites cell suspension was removed. The cells were washed twice in TRIS-EDTA buffer accompanied by centrifuging at 500×g for 5 min at +4°C. After precipitation with 0.2 mol/l PCA, the cells were washed twice in 0.2 mol/l PCA. 0.5 ml of the PCA-soluble fraction was mixed with 5.0 ml emulsifier scintillator 299 (Packard), and the activity was measured in a Packard Tri-Carb 300 CD.

Results

Cell growth. At the time of irradiation four days after inoculation, the tumour cells were exponentially growing with a proportion of G_1, S-phase and G_2+M cells of 37, 48 and 15 per cent, respectively (Fig. 1 a). The irradiation dose of 5.0 Gy completely inhibited the increase in the number of cells up to 35 hours. After 35 hours the proliferation rate of the cells was again comparable to non-irradiated cells. During the irradiation-induced growth-inhibited phase (0 to 35 hours) the proportion of cells in the cell cycle changed (Fig. 1 b–d). The cell flow was completely stopped in G_2 up to about 15 hours with an elevated proportion of cells in G_2 even at 24 hours (Fig. 1 d). At 48 hours after irradiation the cell cycle distribution was more or less normal (Fig. 1 e) and comparable to 6-day-old non-irradiated cells (Fig. 1 f).

^{31}P MR spectra of non-irradiated and irradiated cells. Fig. 2 shows examples of ^{31}P MR spectra of non-irradiated cell and cells at different times after irradiation. The various peaks were identified as described earlier (15). Briefly, standards of ATP, ADP, AMP and NAD, adjusted to a pH of 6.9, were used and compared with available data (11). The shift scale (in ppm) corresponds to relative peak positions within each spectrum. The peak to the left at a shift of 28.5 to 30.5 ppm mainly represents sugar

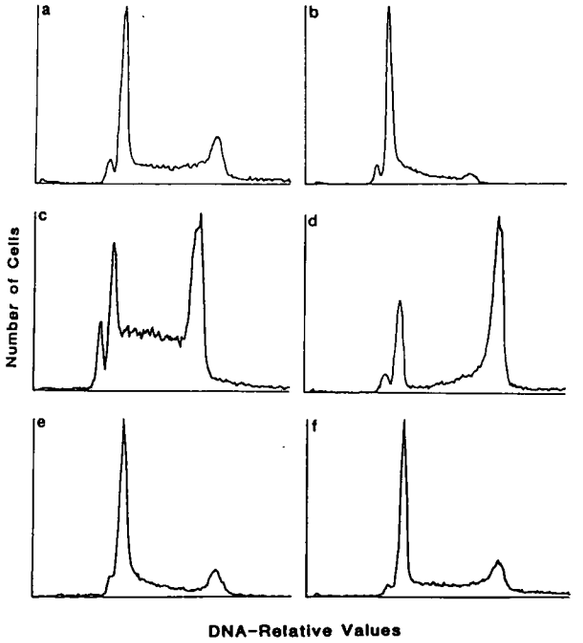

Fig. 1. The proportions of G_1, S-phase and G_2+M cells of non-irradiated cells 4 (a) and 6 (f) days after transplantation and of cells 6 (b), 15 (c), 24 (d) and 48 hours (e) after irradiation. The peak to the left represents normal mouse cells. The next peak and the peak to the right represent G_1 and G_2+M cells, respectively. The area between the G_1 and G_2+M peaks represents S-phase cells.

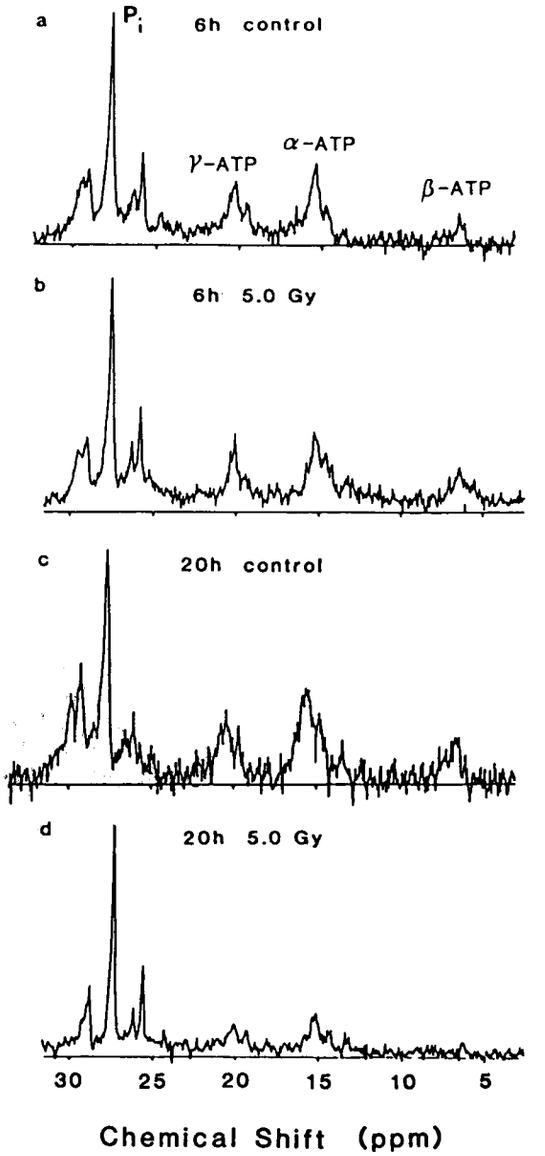

Fig. 2. ^{31}P MR spectra of non-irradiated tumour cells at 4 days (a) and 5 days (c) and of cells at 6 (b) and 20 (d) hours after irradiation. The accumulation time was 8.5 min (512 45° pulses with 1 delay).

phosphates but even phosphorylcholine and AMP. The peak at 27.2 ppm represents inorganic phosphates. The peaks at 20.3 and 6.7 ppm are γ and β ATP, respectively. The peaks at 19.6 ppm contain β-ADP, and the peaks at 15.4 ppm contain both α-ATP and α-ADP. The α-ATP, γ-ATP, α-ADP, and β-ADP-peaks also include some other nucleotides. However, the amounts of these compounds are negligible.

Changes in amounts of ATP, ADP and inorganic phosphate following irradiation. The results, shown in Fig. 3 and Table 1, were obtained from two series of experiments, one from 0 to 20 hours and the other from 24 to 48 hours after irradiation. In both series, corresponding controls were run in parallel. Since the level of the amounts of the phosphate compounds was different in the two series, the various relative concentrations following irradiation were taken as percentages of the corresponding values for the controls (Fig. 3).

The relative content of ATP up to 15 hours after irradiation was unchanged. At 20 to 24 hours both ATP and ADP were reduced to about half the normal values. ADP was still reduced up to 48 hours but at that time the ATP had returned to the ATP level of the control group or even to an elevated level. The level of inorganic phosphate increased around 24 hours by 15 to 20 per cent. The decrease in the ATP and ADP contents corresponded to the increase in the inorganic phosphate (Table 1). The amount of ADP was also significantly increased between 6 and 15 hours after irradiation.

These results were calculated regardless of changes in the composition of cells in the cell cycle. It is known that the ATP content of cells doubles during the cell cycle (12). As further shown in Fig. 1, considerable changes in the composition of cells in the cell cycle occur following irradiation. Thus, taking these changes in the cell cycle composition into consideration and recalculating the changes in the amount of ATP, the ATP content at 24 hours following irradiation was really reduced by 70 per cent and by 15 per cent already 15 hours after irradiation.

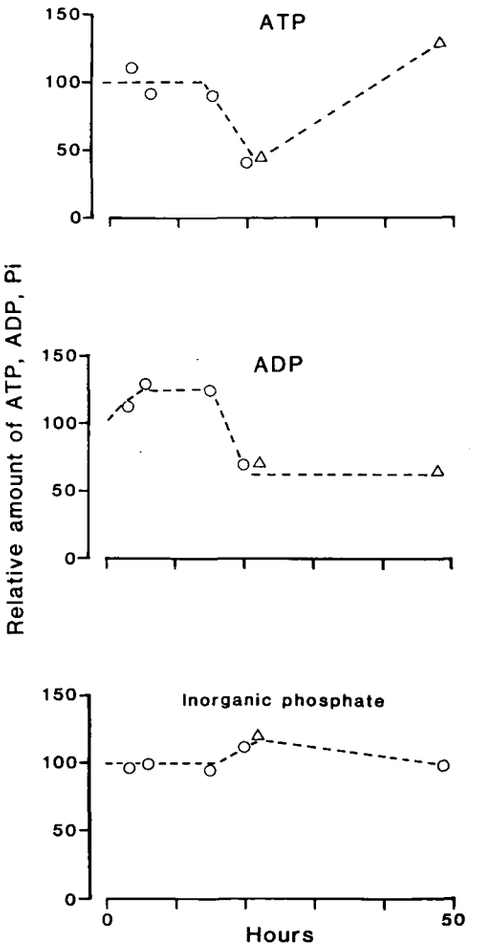

Fig. 3. The relative amounts of ATP, ADP and inorganic phosphate at various times after irradiation. The values are expressed as percentages of the control values (see Table 1). The values in the figure are based on two experimental series (○, △). The data are mean values of 4 to 8 animals, where each single value contains cells from 2 animals.

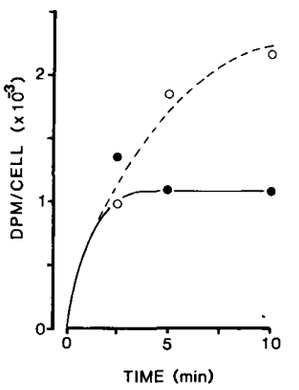

Fig. 4. Uptake of ^{14}C-deoxyglucose into non-irradiated cells (●) and into cells 24 hours following a dose of 5.0 Gy (○). The ^{14}C-deoxyglucose was injected intraperitoneally and the incorporated activity was expressed per cell and average DNA content. Mean values of 2 animals.

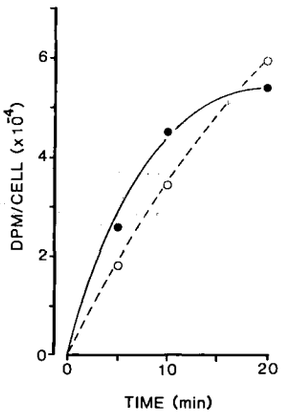

Fig. 5. Uptake of ^{14}C-deoxyglucose into non-irradiated cells (●) and cells 24 hours following a dose of 5.0 Gy (○). The ^{14}C-deoxyglucose was injected intravenously and the incorporated activity was expressed per cell and average DNA content. Mean values of 2 to 3 animals.

Addition of glucose. In order to study the capability of the glycolysis to produce ATP we added glycose to the cells at the time of the lowest ATP concentration, i.e. 24 hours after irradiation (Table 2). The ratio ATP : P_i increased from 0.11 : 1 to 0.73 : 1. Addition of glucose to non-irradiated cells increased the ratio ATP : P_i about four times to the same level as was found in the irradiated cells. The level of ADP did not change significantly, neither in the non-irradiated nor in the irradiated cells.

Uptake of ^{14}C-deoxyglucose. The results from the study in which glycose was added demonstrated that the reduction of ATP could be a result of inhibited glycolysis due to lack of glucose. To further elucidate these possibilities we determined the uptake rate of ^{14}C-deoxyglucose into the tumour cells by injecting the isotope intraperitoneally. We also studied the transport of ^{14}C-deoxyglucose from the blood plasma of the hosts to the tumour cells by injecting the ^{14}C-deoxyglucose into the veins of the tail (Figs 4, 5). Since the cell cycle composition changed following irradiation leading to an accumulation of G_2 cells (Fig. 1), and the glucose uptake per cell increased during the cell cycle (10, 13), the incorporated activity values of ^{14}C-deoxyglucose were related to the average cellular DNA content as obtained from the centroid of the DNA histograms.

The uptake rate of ^{14}C-deoxyglucose into the tumour cells following irradiation was unchanged but reached a higher intracellular level in comparison with the controls (Fig. 4), while the transport from the blood to the tumour cells was reduced by about 40 per cent (Fig. 5). To evaluate possible radiation induced changes in the concentration of glucose in blood and ascites fluid, the blood and ascites glucose concentrations were determined (Table 3). The concentrations of the glucose in both blood and as-

Table 1

Relative content of ATP, ADP and inorganic phosphate (P_i) of non-irradiated cells and of cells at various times after irradiation with a dose of 5.0 Gy. The data were obtained from the areas under the peaks of the various compounds in the MR spectrum corrected for different relaxation times. Mean values of 4 to 10 animals ± SE. In exp. II values from 2 controls at day 5 and 6. Each single value contains cells from two animals

	Control (days)			Times after irradiation (hours)					
	4	5	6	3	6	15	20	24	48
ATP									
Exp. I	12±1	14±1	–	13±1	11±2	13±2	6±1	–	–
Exp. II	–	19, 20	8, 10	–	–	–	–	9±1	12±1
ADP									
Exp. I	4±1	4±1	–	4±1	5±1	5±1	2±1	–	–
Exp. II	–	8, 9	7, 7	–	–	–	–	6±1	5±1
P_i									
Exp. I	85±1	82±1	–	83±1	85±2	82±2	92±1	–	–
Exp. II	–	72, 73	83, 85	–	–	–	–	85±2	83±1

Table 2

The relative concentration of the phosphorus compounds of non-irradiated cells and cells 24 hours after irradiation in the presence and absence of glucose (30 mmol/l). The data were obtained by measuring the area under the peaks in the MR spectrum and correcting for different relaxation times

	Relative concentration of		
	ATP	ADP	P_i
Controls	0.19	0.06	1
Controls + glucose	0.71	0.10	1
Irradiation	0.11	0.07	1
Irradiation + glucose	0.73	0.10	1

Table 3

The glucose concentration of the blood plasma of the mice and the ascites fluid of non-irradiated animals and animals 24 hours following a dose of 5.0 Gy. Mean values of 5 animals ± SE

	Glucose (mg/100 ml)	
	Blood	Ascites fluid
Controls	67±10	0.3±0.05
Irradiation	37±7	0.2±0.04

cites fluid were reduced by 30 to 50 per cent. After injection of ^{14}C-deoxyglucose the ^{14}C-deoxyglucose: glucose ratio was therefore higher in irradiated animals than in the controls. This higher ratio is reflected by an increased amount of radioactivity in the tumour cells after direct intraperitoneal injection. If the blood-peritoneal transport mechanisms were unchanged after irradiation an increased rate of cellular uptake would be expected as well. The reduced uptake of radioactivity in the tumour cells found after intravenous injection might be due to extraperitoneal factors such as a decrease in circulation.

Discussion

The use of MR spectroscopy on living cells and tissues during the last 10 years has provided experience which makes it possible to discuss the practicability but also the limitation of this technique. It is obvious that compounds bound to macromolecules cannot be detected by the MR spectroscopy. ADP is partly bound to macromolecules (4, 8, 11). In spite of this, the ratio ADP : inorganic phosphate in Ehrlich ascites tumour cells determined using conventional biochemical procedures (17, 18) was 0.1 : 1 which is in the same range as that found in the present study (0.07 : 1). On the other hand, the cellular amount of ATP found by MR spectroscopy corresponds well to data obtained by other techniques (4, 8, 11). Furthermore, the MR spectroscopy is non-invasive and permits working with cells growing in vitro and in vivo without changing the environment.

In the present study we have also investigated the possible use of cellular PCA extracts instead of living cells making it possible to prolong the measuring time in order to increase the signal to noise ratio, giving higher precision in the peak area determination. However, the relative concentrations of the various phosphorous compounds changed markedly. This has also been observed earlier (5). Also cells frozen in liquid nitrogen show dramatical changes and living cells were therefore used.

The tumour cells in our study were not measured in vivo. However, in order to keep the tumour environment as unchanged as possible during the MR measurement compared with the in vivo state, the unwashed ascites cell suspension was put directly into pre-cooled MR tubes and the suspension was measured during a relatively short time.

The relative cellular content of ATP following a dose of 5.0 Gy was unchanged up to 6 hours. In an earlier study (13) in which the ATP content was measured using the bioluminescence technique, a decrease of about 40 per

cent was obtained already at 6 hours after irradiation. In that study we also correlated the ATP content to the cell cycle and observed the ATP decrease in late S-phase and G_2+M cells only. In the present investigation the accumulation of cells in late S-phase and G_2 was delayed and this may explain the absence of the decreasing ATP value at that time.

The ATP content at 24 hours following irradiation was actually reduced by more than 50 per cent. This is true since the irradiated cells were compared with non-irradiated cells containing a lower ATP content than the cells which should have been used as controls, i.e. non-irradiated G_2 cells.

Based on an earlier irradiation experiment (13) in which the absolute values of ATP were studied, the decline in the relative ATP content 20 to 24 hours after irradiation in the present study also reflects an absolute decrease in the ATP. The comparably elevated value of inorganic phosphate at this time tends to indicate dephosphorylation of ATP either caused by an inhibition of the energy production or by increased utilization of ATP leading to a lower steady state of the ATP content. According to the earlier study (13), changes in the utilization of ATP following irradiation are less probable. Since the cells are growing under almost anaerobic conditions (1, 2), the inhibited energy production implies inhibited glycolysis. From the experiments with addition of glucose it is obvious that the function of glycolysis is not disturbed. This has also been concluded earlier (13). Furthermore, the presence of inhibitor(s) is not likely. Thus, lack of substrate for the glycolysis must be suggested. The stimulated ATP synthesis following the addition of glucose indicates lack of glucose. This is confirmed by the reduced transport rate of glucose from the blood plasma of the host to the tumour cells and the undetectable values of glucose in the ascites fluid.

In addition, depletion in the content of NAD^+/NADH as a reason for the decrease of ATP has also been discussed (6, 7, 16). Lack of NAD^+/NADH prevents the transport of H^+ in, for example, the glycolysis and thus induces inhibition. NAD^+/NADH can be identified by ^{31}PMR spectroscopy. However, the limited resolution in that part of the spectra together with the presence of other compounds with almost the same frequency makes the quantitative evaluation of these molecules more doubtful. In spite of this, we tried to quantify NAD^+/NADH and found no changes except at 20 to 24 hours after irradiation with a decrease of about 30 to 50 per cent (data not shown). This may indicate an involvement of NAD^+/NADH onto the mechanism of ATP production. However, since in the present study addition of glucose restorted the reduced ATP level, such an explanation for the radiation induced decline of ATP is less likely. Furthermore, the opposite relationship between NAD^+ and ATP following irradiation has been suggested (9), i.e. that depletion of ATP, which is involved in the biosynthesis of NAD^+, results in a limited generation of this compound. This hypothesis is in agreement with our observations since the addition of glucose normalized not only the ATP but also the NAD^+.

Forty-eight hours following irradiation, the relative ATP content was normalized and the ratio ATP: P_i was at the same level as the non-irradiated cells at that time, showing that the production of the ATP had been restored.

The significance of the slight increase in the ADP up to 15 hours is uncertain for the reasons discussed. If the elevated value of ADP up to 15 hours reflects dephosphorylation of ATP, an increase in inorganic phosphate should be found. This is not the case.

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