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Incorporation of ^{32}P and ^{14}C into Photosynthetic Products of Ankistrodesmus braunii as Affected by X-Rays

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Summary. The incorporation of ^{32}P and ^{14}C into organic compounds by *Ankistrodesmus* is strongly inhibited by X-rays. In the same phosphorylated compounds ^{32}P-incorporation apparently is more severely inhibited by X-rays than the ^{14}C-labelling. The ^{32}P-incorporation into organic compounds is more strongly inhibited than ^{32}P-labelling of inorganic phosphate in the cell. The inhibition of ^{32}P-incorporation into a number of compounds is strikingly uniform. It is concluded that the inhibition of ^{32}P-incorporation and of ^{14}C-incorporation into phosphorylated compounds in vivo is due to an uncoupling by X-rays of photophosphorylation as in vitro. The difference in X-ray sensitivity of ^{14}C- and ^{32}P-incorporation into one organic phosphorous compound is attributed to a dual action of X-rays on ^{32}P-incorporation in organic compounds (both via the uncoupling of photophosphorylation) and only a single effect on ^{14}C-incorporation and ^{32}P-labelling of inorganic phosphate. The effect of X-rays on ^{14}C-incorporation into organic compounds included inhibition in most cases but also stimulation as in the case of glycolic acid. These differences may be due to interference in the intercellular regulations following the application of X-rays. The inhibition of ^{14}C-incorporation in many cases exhibits different behaviour at low (<200 krad) and high doses. These changes are discussed on the assumption that at the lower doses X-rays cause uncoupling of photophosphorylation and at the higher doses an additional inhibition of electron transport.

Investigators of the action of ionizing radiation on photosynthetic processes in isolated chloroplasts have observed that the phosphorylation reactions are more sensitive than the electron transport and the O_2-evolving system (4, 7, 13). Simonis and Urbach (19) obtained similar results in vivo using *Ankistrodesmus*. Additionally, experiments on total $^{14}CO_2$-fixation by *Ankistrodesmus* revealed a lesser X-ray sensitivity compared to phosphorylation but a somewhat higher sensitivity than of O_2-evolution (19). Zill and Tolbert (21), however, have found a considerably higher sensitivity of $^{14}CO_2$-fixation than of O_2-evolution. Because of the discrepancy in radiosensitivity of $^{14}CO_2$-fixation and of ^{32}P-labelling of soluble organic products, it was proposed that X-ray action on transport phenomena rather than on phosphorylation itself causes the inactivation of ^{32}P-labelling of organic products in vivo (19). Simonis and Fuchtbauer (16) have shown that X-rays inactivate in vitro both cyclic and noncyclic photophosphorylation, so that it seemed possible to use X-rays like a specific inhibitor of phosphorylation. It seemed desirable to investigate the differences in radiosensitivity of $^{14}CO_2$-fixation and ^{32}P-labelling in the light and especially to study the effects of X-rays on ^{32}P- and ^{14}C-labelling of chemically defined products rather than of larger fractions. The use of both ^{14}C and ^{32}P might give some more insights into the meaning of ^{32}P-labelling in vivo.

Methods

Ankistrodesmus braunii (Naegeli) was grown synchronously (11) in a culture medium according to Pirson and Ruppel (15). The cells were depleted of phosphate by growth for 3 hours in phosphate-free solution in the light. Then they were X-irradiated in the dark in a concentrated suspension (100 μg chlorophyll/ml) in a phosphate-free buffered solution at 25° at a dose rate of 3000 rad/min (140 kv, 12 ma). For details of the irradiation apparatus see references (5, 6). Control samples were kept in the apparatus behind a lead shield. After the irradiation the algae were adjusted to a chlorophyll content of 20 to 30 μg/ml and kept for 30 minutes at 25° in the dark with aeration.

The incorporation of ^{32}P was carried out with aeration in "lollipops" at 25° and 20,000 lux. The reaction mixture contained in a final volume of 13.3 ml: algae corresponding to 50 μg of chloro-

phyll; 50 mM tris buffer (pH 8.0); and 10 ml of phosphate-free culture medium. After 10 minutes of preillumination the algae were incubated with 0.2 mc of carrier-free ^{32}P for 5 minutes. The phosphate concentration on the culture medium of the algae after phosphate-depletion was about 5×10^{-7} mole/l. Thus the specific activity of "carrier-free" ^{32}P in this case was about 0.04 $\mu c/\mu$mole. The reaction was terminated by separating the algae from the reaction mixture by filtration. The filter with the algae was washed 2 times and dropped quickly into a mixture of methanol/chloroform/0.7 M formic acid (12/5/3, v/v/v/) held at −70° (1).

The incorporation of ^{14}C was carried out in Warburg vessels at 25° and 13,500 lux. The reaction mixture contained in a final volume of 5 ml: algae corresponding to about 0.1 mg of chlorophyll, 50 mM tris buffer (pH 8.0), and 4 ml phosphate-free culture medium. After 10 minutes of preillumination the reaction was started by adding 1, .68 μmole HCO_3^- containing 0.05 mc ^{14}C and was stopped after 5 minutes as described above.

The algae were extracted for 4 hours at −20° with the methanol / chloroform / formic acid medium and twice for 12 hours with water at 5°. The extract was freed of volatile solvents by evaporation in a stream of air at 0° and subsequently freeze dried. Aliquots of 0.01 to 0.025 of the algal extracts were analysed by 2-dimensional cellulose thin layer chromatography (17) with I) isobutyric acid / 1 N NH_4OH / 0.1 M EDTA (100/60/1.6 : v/v/v) and II) *n*-butanol/*n*-propanol/propionic acid/water (77/546/600/804) as solvents. For the separation of ^{14}C-labelled products "semistench" (3) (17 N NH_4OH/H_2O/*n*-propanol/isopropanol/*n*-butanol/isobutyric acid/EDTA (100/950/350/75/75/2500/1.2 g) proved to be a better solvent in the first direction. The chromatograms were radioautographed and counted directly on the plates with a methane flow counter.

Results

Before considering the action of X-rays upon ^{32}P- and ^{14}C-incorporation in the light it seems desirable to consider the photosynthetic performance of the unirradiated algae (table I). The rate of photosynthesis agrees with reported values for *Chlorella* (14). The labelling of organic soluble products with ^{32}P, however, is considerably smaller, compared on a molar basis, than the labelling with ^{14}C as revealed, for instance, by the ratio $^{14}C/^{32}P$ in P-glycerate (table I). Even if we suppose that all 3 C-atoms in P-glycerate are ^{14}C-labelled only about 7.5 % of the ^{14}C-labelled P-glycerate is also ^{32}P-labelled. This heavier labelling of the same compounds with ^{14}C than with ^{32}P suggests a similarity hindered access of labelled phosphate ions to the chloroplast because of their passage through the plasma and chloroplast membranes in *Ankistrodesmus* as in *Elodea* (9). Heber et al. (9) reports that only 1 mole ^{32}P incorporated into soluble organic products corresponds to 25,000 moles $^{14}CO_2$ fixed in *Elodea*. Almost the same ratio is found for *Ankistrodesmus* (table I), but the ratio should depend upon the experimental conditions.

Table I. *Photosynthetic Performance of Unirradiated* Ankistrodesmus

Comparison of the molar amounts of CO_2 and P_i incorporated, as calculated from the specific activities of ^{14}C and ^{32}P. In the case of ^{32}P 5×10^{-7} mole/l was taken as the phosphate concentration in the surrounding medium, as measured in a parallel experiment.

Process	Rate μ mole · hr^{-1} · mg $chlorophyll^{-1}$
Total $^{14}CO_2$-fixation (photosynthesis)	135
^{14}C-Incorporation into P-glycerate	0.95
^{32}P-Incorporation into P-Glycerate	0.024
^{32}P-Incorporation into acid-soluble organic compounds	0.056
$^{14}C/^{32}P$ in P-glycerate ^{14}C-fixation	40
^{32}P-incorporation in soluble organic compounds	24,000

The effect of X-rays on the ^{32}P-incorporation (curve A) and ^{14}C-incorporation (curve B) into soluble organic phosphorous compounds in *Ankistrodesmus* is seen in figure 1. The ^{32}P-incorporation is considerably more radiosensitive than the ^{14}C-incorporation. As distinguished from the results of the total $^{14}CO_2$-fixation (19) the ^{14}C-incorporation into the soluble compounds (curve B or C, fig 1) is definitely inhibited at 100 krad. A similar inhibition by X-rays of ^{14}C-incorporation into soluble products in *Chlorella* and wheat has been reported (21).

Because we do not have sufficient information on the X-ray action at low doses, the inhibition curves are drawn to the origin (fig 1–5), although they possibly are sigmoid, with no inhibition below 5 to 6 krad (4, 19). It may even be possible that at low doses the ^{32}P-incorporation into organic compounds is stimulated by X-rays (Nalborczyk, D. Urbach, Kovacs, unpublished).

Figures 2 and 3 and table II show the effect of X-rays on ^{32}P-incorporation into a number of organic phosphorous compounds. All of these exhibited a very similar behaviour, being inhibited strongly on an almost linear curve in the semilogarithmic plot. The ^{32}P-labelling of inorganic phosphate is inhibited to a far smaller degree (fig 2, table II). Only the ^{32}P-labelling of ATP has a sensitivity about as small as of inorganic phosphate (table II, fig 2).

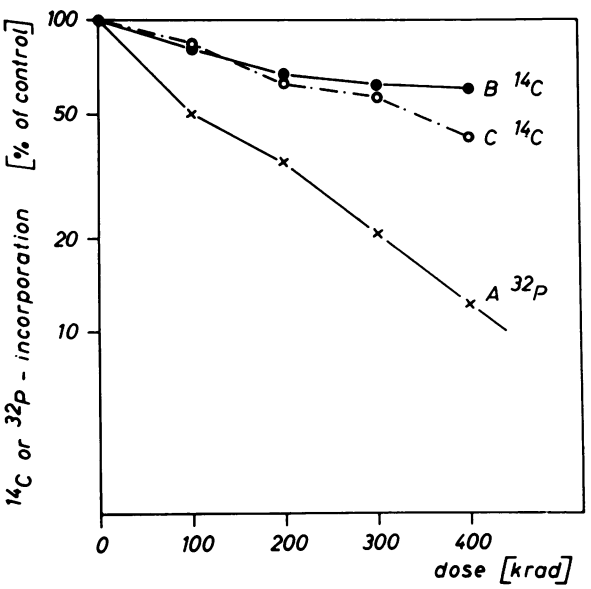

FIG. 1. Effect of X-rays on the ^{32}P- and ^{14}C-incorporation. Curve A shows the inhibition by X-rays of the incorporation of ^{32}P, and curve B that of ^{14}C into the sum of organic acid-soluble phosphorylated compounds. Curve C presents the X-ray inhibition of ^{14}C-incorporation into the sum of acid-soluble organic compounds (phosphorylated + non-phosphorylated). Each point represents the average of 4 independent experiments with 2 parallel determinations in each experiment.

Table II. *Doses of Half Inhibition by X-Rays of the ^{32}P-Incorporation into Various Compounds*

Compound	Dose causing 50 % inhibition of ^{32}P-incorporation
	krad
P_i in the cell	180
ATP	150
ADP	115
P-glycerate	120
P-enolpyruvate	90
glucose-P	100
fructose-P	110
sugar diphosphates	125
UDP-glucose	120

The inhibition by X-rays of the ^{14}C-incorporation into the same phosphorylated compounds is also illustrated in figure 3. As with the sum of all compounds (fig 1) we find a great difference of the radiosensitivity of ^{14}C- and ^{32}P-incorporation. The ^{14}C-incorporation into P-glyceric and P-enolpyruvic acids is inhibited only up to about 200 krad. At higher doses it stays at a level of about 50 % of the control up to 400 krad (fig 3).

The influence of X-rays on the ^{14}C-incorporation into some nonphosphorylated compounds and the sugar diphosphates is shown in figures 4 and 5. Comparison of the figures 3, 4 and 5 with the figures 2 and 3 reveals a great variety of inhibition patterns of ^{14}C-incorporation, which is almost absent in the ^{32}P-incorporation. Most remarkably, the ^{14}C-incorporation into glycine, serine, alanine, and sucrose is decreased only to a slight degree at lower doses, but considerably at high doses (fig 4 and 5). The ^{14}C-incorporation into aspartic acid as well as glucose and fructose phosphates is inhibited according to a logarithmic dose response (fig 3 and 4). The ^{14}C-incorporation into glycolic acid is not inhibited, but was enhanced by X-irradiation in all experiments. The differences in the inactivation curves of various compounds were repeatedly found in all experiments.

Discussion

^{32}P-incorporation. Three major results shall be discussed: A) X-ray inhibition of ^{32}P-incorporation into organic compounds is strikingly uniform (fig 2 and 3). B) ^{32}P-labelling of inorganic phosphate in the cells is considerably less sensitive to X-rays than the ^{32}P-incorporation into organic compounds (fig 2 and table II). C) ^{14}C-incorporation into the same compounds apparently is less sensitive to X-rays than the ^{32}P-incorporation (fig 1 and 3).

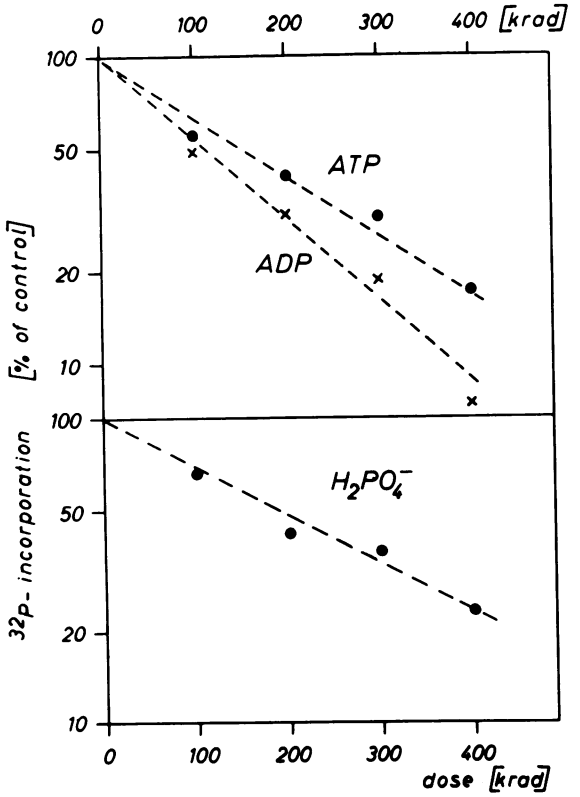

FIG. 2. Effect of X-rays on the ^{32}P-incorporation into ATP, ADP, and P_i in the cell. The experimental points represent the average of 2 independent experiments with 2 parallels each.

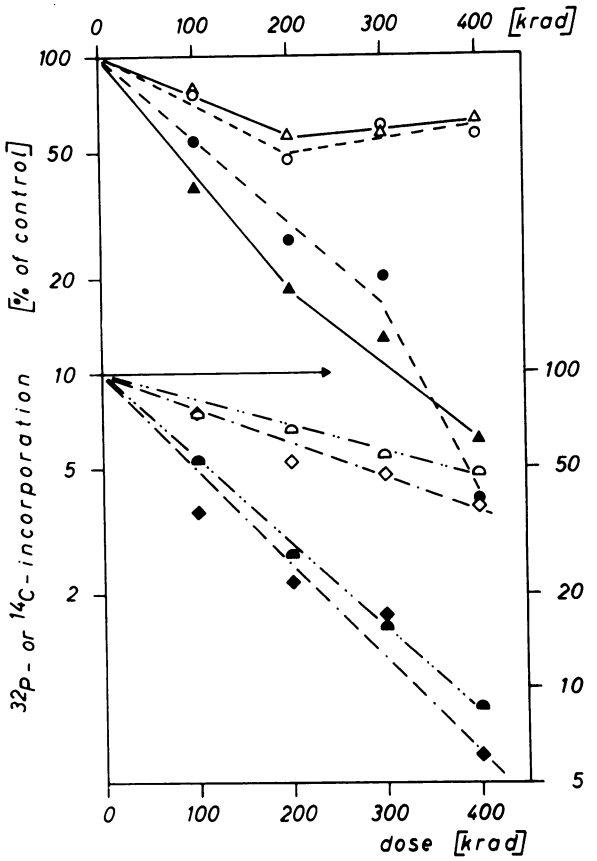

FIG. 3. Inhibition by X-rays of the ^{32}P-incorporation or the ^{14}C-incorporation respectively into P-glycerate (○ - - - - ○), P-enolpyruvate (△———△), fructose-P (○-..-.-..-○) and glucose-P (△--.--.-△); ^{14}C: open symbols, ^{32}P: filled symbols. Each points represents the average of 2 experiments with 2 parallels each.

From the uniformity of inhibition of ^{32}P-incorporation into almost all compounds it may be inferred, that X-rays act primarily on 1 (or 2) reactions that are common to the ^{32}P-incorporation into all compounds. In the light 3 processes can be considered as primary reactions: 1) uptake of phosphate ions, 2) transport of phosphate ions to the chloroplasts, and 3) photophosphorylation.

The first possibility, direct X-ray action on phosphate uptake, was excluded by Simonis and Urbach (19) on the basis of different X-ray inhibition in light and darkness. This conclusion is verified by the result that the ^{32}P-labelling of inorganic phosphate is not so severely inhibited (fig 2) as should be anticipated if phosphate uptake alone were primarily affected by X-rays. The second possibility, X-ray action on phosphate and ATP transport into the chloroplasts in the light, was proposed by Simonis and Urbach (19) as a working hypothesis to explain the differences of the radiation effects on ^{32}P- and ^{14}C-incorporation. From our results of X-ray action on different products of ^{32}P-incorporation, it now seems improbable that X-rays primarily act on transport into the chloroplasts. Decreased transport of phosphate ions or

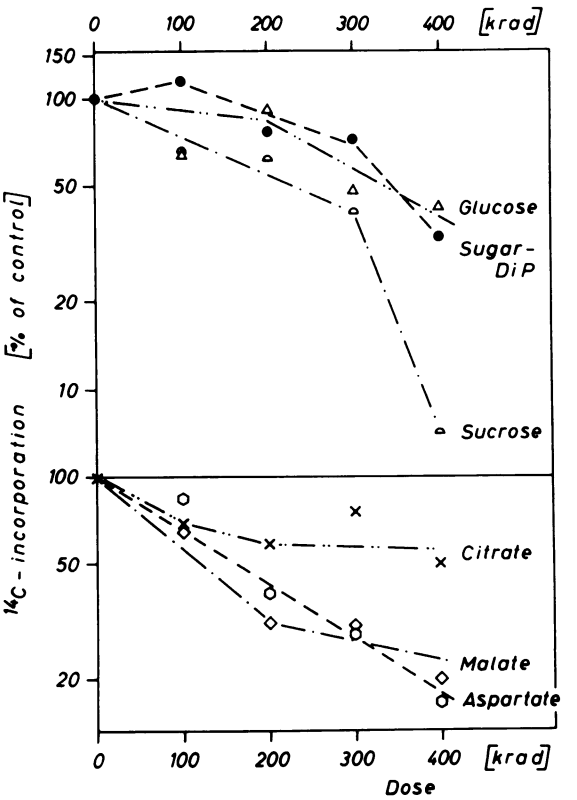

FIG. 4. Effect of X-rays on the ^{14}C-incorporation into glucose (△-..-..△), sugar diphosphates (● - - -●), sucrose (○-.-.-.-○), citric acid (x-..-..-..x), malic acid (△-.-.△), and aspartic acid (⬠ - - -⬠) Averages of 2 experiments with 2 parallels each.

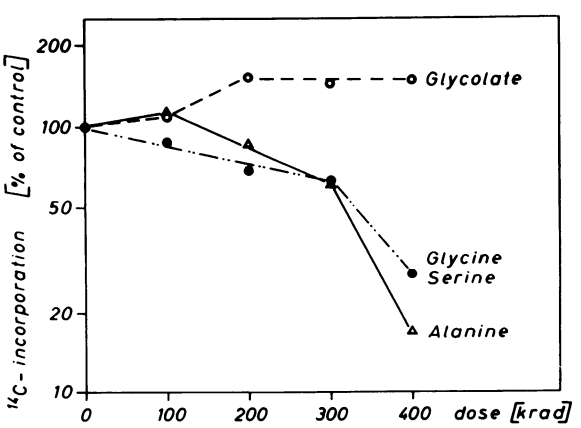

FIG. 5. Effect of X-rays on the ^{14}C-incorporation into glycolic acid (○ - - - - ○), glycine and serine (●-..-..●), and alanine (△———△). Averages of 2 independent experiments with 2 parallel determinations each.

ATP into the chloroplasts should not affect the ^{32}P-incorporation into sugar phosphates to the same degree as into P-glyceric acid, since in vivo sugar phosphates are reported to be ^{32}P-labelled in the cytoplasm rather than in the chloroplasts where ^{32}P-glycerate is formed (9). Thus they should not be affected by a transport barrier as much as P-glycerate provided that in the 5-minutes-experiments the steady state of ^{32}P-labelling has not yet been reached in the inhibited samples.

The present results suggest that X-ray inhibition of ^{32}P-incorporation in vivo is due only to an uncoupling of photophosphorylation (third possibility). This conclusion is consistent with X-ray action on chloroplasts in vitro (16). It applies at least to lower doses (up to 200 krad) which do not inhibit the O_2-production (19). At higher doses additional inactivation may be expected from deterioration of electron transport. This conclusion that X-rays uncouple photophosphorylation is supported by the following argument. If photophosphorylation is inhibited, then uptake of P_i will be lowered, too, because presumably it is dependent on ATP formation (10, 12, 18). As a consequence of lowered phosphate uptake the specific activity of $H_2{}^{32}PO_4^-$ inside the irradiated cell will be decreased. Thus in the cell, phosphorylation of a compound at lowered rate and with $H_2{}^{32}PO_4^-$ of lower specific activity will result in even less ^{32}P-labelling of the compound. Hence, ^{32}P-labelling of organic compounds will be affected in 2 ways by the action of X-rays and this will yield the pattern of inhibition as experimentally found (fig 2 and 3). It should be possible to estimate the theoretical decrease in ^{32}P-labelling of a compound if we knew the decrease of its actual phosphorylation and the decrease of the specific activity of $H_2{}^{32}PO_4^-$. As for the specific activity, we have established that X-irradiation at least up to 200 krad does not notably affect the internal concentration of phosphate in *Ankistrodesmus*. Thus a decrease in ^{32}P-labelling of phosphate is indicative and a measure of its lowered specific activity. Further, if only the phosphorylation is decreased whereas the O_2-production (TPN-reduction) is almost unaffected by X-rays, we can assume that the relative degree (with respect to the unirradiated control) of ^{14}C-labelling of a phosphorylated compound should be a measure of its lowered phosphorylation. With these assumptions we have calculated the theoretical inhibition of ^{32}P-labelling of a few substances by forming the product of the relative degree of their ^{14}C-labelling and of ^{32}P-labelling of inorganic phosphate (table III). The reasonable coincidence of experimental and calculated values for ^{32}P-labelling in the range of up to 200 krad supports the conclusion that in *Ankistrodesmus* in vivo uncoupling of photophosphorylation is the only major effect of X-rays in this range of doses (table III). The differences in X-ray sensitivity of the incorporation of ^{14}C and of ^{32}P into identical phosphorylated compounds then only would be apparent differences. They probably are due to the dual action (both via photophosphorylation) on ^{32}P-incorporation and only a single action of X-rays on the ^{14}C-incorporation.

ATP does not seem to fit into our picture of X-ray action on phosphorylation only as it is almost as little affected by irradiation as is $^{32}P_i$ (table II). However, ATP is one of the first substances to come to an isotopic equilibrium with $^{32}P_i$ in the cell. Hence, after the rather long incubation time of 5 minutes the decrease in ^{32}P-labelling of ATP may partly reflect the smaller decrease in labelling of P_i.

Table III. *Comparison of the Effect of X-Rays on ^{14}C- and ^{32}P-Incorporation into Phosphorylated Compounds, and of Experimental and Calculated Values for ^{32}P-Incorporation at Various Doses of X-Rays*

Values of ^{32}P-incorporation are calculated as the product of the relative ^{14}C-incorporation into the compound and the relative ^{32}P-labelling of P_i at each X-ray dose. The values are listed as % of the unirradiated control.

Dose	^{14}C- or ^{32}P Incorporation	Products				
		P-Glycerate	Fructose-P	Glucose-P	P-Enol pyruvate	UDP-Glucose
krad		% Of unirradiated control				
100	^{14}C-Experimental	76	74	75	77	91
	^{32}P-Experimental	54	54	37	38	51
	^{32}P-Calculated	51	49	50	51	60
200	^{14}C-Experimental	47	66	52	56	37
	^{32}P-Experimental	27	27	22	18	33
	^{32}P-Calculated	20	28	22	23	16
300	^{14}C-Experimental	61	54	48	57	36
	^{32}P-Experimental	20	16	17	13	14
	^{32}P-Calculated	23	20	18	21	13
400	^{14}C-Experimental	56	49	39	62	26
	^{32}P-Experimental	4	9	6	4	8
	^{32}P-Calculated	13	14	9	15	6

The uptake of phosphate by *Elodea* at low external phosphate concentrations is limited by diffusion of phosphate in the unstirred layer of liquid around the leaf (film kinetics) (10). This certainly applies even more to *Ankistrodesmus* since film kinetics are favored by small dimensions. Thus it would not seem likely that phosphate uptake at low phosphate concentrations should be inhibited by X-rays, when active uptake is not rate limiting. However, diffusion in the unstirred layer only sets an upper limit to the rate of uptake (depending on concentration). Yet if active uptake is inhibited, then less phosphate will be taken up than is available by diffusion in the unstirred layer around the cell.

The action of X-rays up to about 200 krad can be regarded as a type of uncoupling of photophosphorylation (16). Thus our considerations should also have some bearing on investigations on the action of uncouplers on ^{32}P-incorporation in vivo. It can be expected that uncouplers of photophosphorylation would affect the ^{32}P-labelling of P_i to a lesser degree than of organic compounds.

^{14}C-Incorporation into Acid-Soluble Products. The inhibition of ^{14}C-labelling of organic phosphorous compounds by lower doses of X-rays (below 200 krad) has been partly discussed in the preceding paragraph. The most striking features of X-ray action on ^{14}C-labelling are the diversity of inhibition patterns for different compounds and the change in degree of inhibition for some compounds at about 200 krad (see fig 3, 4 and 5). Although the decrease of ^{14}C-labelling at lower doses possibly is induced solely by an uncoupling of photophosphorylation (see the preceding paragraph), the inhibition is not equal in all compounds as is generally true with ^{32}P-labelling (table II). These specific effects of X-irradiation on ^{14}C-labelling (see also Zill and Tolbert, 21) seem to stem from intracellular regulations after X-irradiation. The drop or rise of the inhibition curves at higher doses probably may coincide with the onset of an inhibition of electron transport by X-irradiation. The latter inhibition has less affect upon ^{32}P-labelling since non-cyclic phosphorylation already is reduced at lower doses (16).

For the discussion 3 groups of ^{14}C-labelled substances may be distinguished: compounds of primary sugar metabolism (phosphorylated compounds, glucose, sucrose, alanine); compounds related to glycolic acid metabolism (glycolic acid, glycine, and serine) (2) and compounds related to the Krebs cycle (carboxylic acids, glutamic and aspartic acids). It must be kept in mind, however, that only 1 incubation time has been studied.

At lower doses the ^{14}C-incorporation into compounds of sugar metabolism is inhibited similarly to P-glycerate (^{14}C), which may reflect the inhibition of photophosphorylation. Exceptions are only the sugar diphosphates, alanine and possibly glucose (fig 4 and 5). An increase in sugar diphosphates upon the action of the inhibitor of photophosphorylation hexylresorcinol has been reported by Gould and Bassham (8) and may correspond to our finding with X-rays. The increase of alanine at lower doses may be due to a relative abundance of TPNH because of unrestricted electron flow and consequently increased reductive amination of P-enolpyruvate.

At higher doses we find a relative increase of ^{14}C-incorporation into P-glycerate and P-enolpyruvate (fig 3). This may be due to decreased reduction of P-glycerate and of reductive amination of P-enolpyruvate as a consequence of inhibited electron transport. The corresponding decrease in the labelling of the products of reduction can be found in the sugar diphosphates, sucrose, and alanine (fig 4 and 5).

Surprisingly, X-irradiation enhances the ^{14}C-labelling of glycolate and relatively enhances (compared to P-glycerate) the labelling of glycine and serine at lower doses. The latter is comparable to the results of Zill and Tolbert (21). It seems that the photosynthetic metabolism is shifted toward glycolate and its derivatives by the uncoupling action of X-irradiation. A similar shift of the metabolism toward glycolate by the action of an uncoupler of photophosphorylation has been reported by Tolbert (20). The Krebs cycle products, however, were more severely inhibited (20). In our experiments ^{14}C-labelling of citric, malic, and aspartic acids correspondingly is decreased more severely by low doses of X-rays (fig 4). On the other hand, Tolbert (20) reports a predominant inhibition of the products of glycolate metabolism (glycine and serine) as well as of sucrose as a consequence of inhibited electron transport, while under the same conditions the products of the Krebs cycle are less inhibited. Our experiments (fig 4 and 5) also show that high X-ray doses, which inhibit the electron transport (16, 19), decrease considerably the ^{14}C-incorporation into glycine, serine, and sucrose while that into aspartate and malate is not additionally decreased and the ^{14}C-labelling of citrate is even somewhat less inhibited.

Hence, with all 3 groups of substances the results of ^{14}C-incorporation can be explained at this stage with the assumption that also in vivo lower X-ray doses act primarily on photophosphorylation while higher doses additionally begin to suppress electron transport. Thus our results are in agreement with the findings on isolated chloroplasts (16).

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