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Feedback Inhibition and Ionizing Radiation. Mechanism of Inactivation of Allosteric Properties of Aspartate Transcarbamylase by X-Rays*

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ABSTRACT: The effect of X-rays on aspartate transcarbamylase from *Escherichia coli* in solution has been studied. The feedback inhibition properties of native aspartate transcarbamylase are three to four times more readily destroyed by X-rays than the catalytic activity. The catalytic subunit aspartate transcarbamylase is inactivated two to three times more easily by X-rays than the native enzyme. X-Rays cause native aspartate transcarbamylase to dissociate into subunits and this accounts for the major part of the loss of allosteric properties. Substrates, activators, and inhibitors were found to have a profound effect on the ease of destruction of various properties. L-Aspartate plus a competitive inhibitor, such as PP_i or P_i, fully protected the active site against destruction by X-rays, but not the allosteric properties.

Carbamyl phosphate and nucleotides were less effective as protectors. The destruction of allosteric properties of aspartate transcarbamylase was found to be irreversible.

The feedback inhibition concept, first proposed by Novick and Szilard (1954), has become of great importance for the understanding of cellular control mechanisms, particularly in bacteria where many biosynthetic pathways are known to possess feedback inhibition. In higher organisms metabolic control by end-product inhibition is more uncertain. A recent theory by Monod and co-workers (1963, 1965) on the nature of allosteric enzymes provides an insight into the molecular mechanisms of feedback inhibition.

In the pyrimidine metabolism in *Escherichia coli* the work of Gerhart *et al.* (1962, 1965) has demonstrated that the first enzyme in this pathway, aspartate transcarbamylase (EC 2.1.3.2), is inhibited by the end product, CTP.[1] These workers also showed that this enzyme consists of two catalytic subunits and several regulatory subunits. The regulatory subunits bind CTP but have no catalytic activity. Feedback inhibition by CTP can be abolished by treating the enzyme with such agents as *p*-mercuribenzoate, Hg^{2+}, and urea or by heating the enzyme. Desensitization leads to dissociation of the enzyme into active catalytic subunits and noncatalytic regulatory subunits.

In the present work we have examined the effect of X-rays on aspartate transcarbamylase from *E. coli* in solution. A preliminary note has appeared (Kleppe *et al.*, 1966).

Materials

Enzyme. Aspartate transcarbamylase was prepared from a mutant of *E. coli*, R-185-482, kindly provided by Dr. A. B. Pardee, Princeton University. The enzyme was purified according to a procedure slightly modified from that of Gerhart and Pardee (1962). A column of Sephadex G-200 (1.5 × 90 cm) was used to separate native aspartate transcarbamylase from subunit aspartate transcarbamylase in the final purification step. Subunit aspartate transcarbamylase accounted for approximately one-third of the protein and activity before passing it through the column. The subunit enzyme obtained from this column was kinetically identical with that obtained by heating or treating native aspartate transcarbamylase with *p*-mercuribenzoate. Both native and subunit aspartate transcarbamylase gave a single protein band on sucrose density

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[1] Abbreviations used: CTP, cytidine triphosphate; ATP, adenosine triphosphate; UTP, uridine triphosphate.

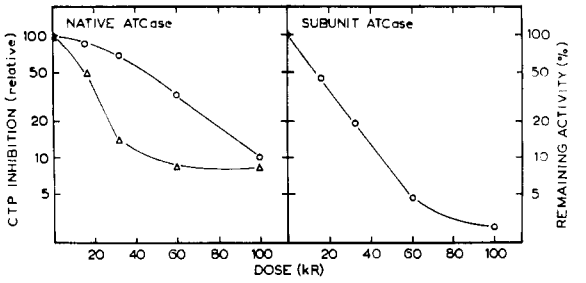

FIGURE 1: Effect of X-ray dose on the activity (○——○) and CTP inhibition (Δ——Δ) for native and subunit aspartate transcarbamylase. The concentration of protein was 0.1 mg/ml, dissolved in 0.02 M air-saturated phosphate buffer (pH 7.0). The concentration of L-aspartate used in the activity measurement was 6 mM.

centrifugation. The specific activity was approximately 8000 units/mg of protein. The enzyme was usually stored in 0.02 M phosphate (pH 7.0) buffer in the presence of 2 mM mercaptoethanol and 0.1 mM EDTA. All irradiation experiments were, however, carried out in 0.02 M phosphate buffer (pH 7.0). Mercaptoethanol and EDTA were removed by passing the enzyme through the same column of Sephadex G-200 as described above immediately prior to the experiments.

Chemicals. Nucleotides and carbamyl phosphate were from Sigma Chemical Corp. Carbamyl phosphate was recrystallized before use.

Methods

Activity and CTP Inhibition Assay. The enzymatic activity was measured at 25° by determining the amount of carbamyl-L-aspartic acid formed according to published procedure (Gerhart and Pardee, 1962). The assay mixture consisted of 15 mM L-aspartate, if not otherwise stated, 3.2 mM carbamyl phosphate, and 0.1 μg of protein in a total volume of 0.5 ml. The buffer used was 0.05 M imidazole, 0.03 M acetate, and 0.1 mM EDTA (pH 7.0). In certain experiments where CTP inhibition and activity were measured simultaneously a concentration of L-aspartate of 6 mM was used, whereas the concentration of CTP was 1 mM. Other conditions were as described above. The reason for employing a smaller concentration of L-aspartate in these experiments was that the CTP inhibition is abolished by high concentrations of L-aspartate, and further since subunit aspartate transcarbamylase has a higher affinity for L-aspartate than the native enzyme, the shapes of the dose–effect curves measured at 6 mM L-aspartate might give some indications of whether or not dissociation of the native enzyme into subunits had taken place.

The CTP inhibition of native aspartate transcarbamylase varied for the different preparations from 70 to 50%. For subunit aspartate transcarbamylase the CTP inhibition in all cases was less than 5%. The reason for the variations in the CTP inhibition for the native enzyme is not clear. It is, however, not caused by dissociation of the native enzyme into subunits. Owing to these variations the CTP inhibition in this work is given in relative rather than in absolute units. The CTP inhibition of the unirradiated sample has been set to 100 and the observed inhibition after irradiation is expressed in per cent of this figure.

In the density gradient experiments the enzymatic activity was expressed in relative units. The highest activity in the unirradiated enzyme peak was set to 100 and the observed activity was then calculated as per cent of this figure.

Density Gradient Centrifugation. Density gradient centrifugations were carried out in sucrose gradient from 35 to 5%. The sucrose was dissolved in 0.02 M phosphate buffer (pH 7.0). The volume of the sucrose gradient in the lusteroid centrifuge tubes was 4.7 ml, and 0.2 ml of the sample was then layered on top of the tube and centrifugation was carried out for 18 hr at 35,000 rpm and 3°. Immediately after completion of the run the tubes were punctured with a needle in the bottom and fractions of 8 drops were collected. Usually 21–22 fractions were obtained in this way. The fractions were then analyzed for activity, CTP inhibition, and protein content.

Determination of Protein. Protein was determined by the method of Lowry *et al.* (1951) and occasionally by the method of Kalckar (1947). In some of the density gradient fractions the absorbance at 280 mμ was used as a measure of the protein content.

X-Ray irradiation was carried out using a dose rate of 2 kR/min in air-saturated 0.02 M phosphate buffer (pH 7.0). The protein content varied from 0.1 to 0.15 mg/ml. Most of the irradiation experiments were done at 2°. In some experiments room temperature was used, but there was no significant difference in the results. The dose rate was determined with the Fricke dosimeter (Baarli and Borge, 1957).

Results

Effect of Dose. Figure 1 shows the effect of increasing dose on activity and CTP inhibition for native and subunit aspartate transcarbamylase. The dose–response curve for the native enzyme has an initial shoulder and in the semilogarithmic plot becomes linear first after approximately 30 kR. The curve for subunit aspartate transcarbamylase is a normal dose–response curve and is linear from 0 to 60 kR, or until approximately 95% of the activity has been lost. It is apparent also that the CTP inhibition is abolished more readily than is the enzymatic activity of the native enzyme. Thus the dose needed to reduce the activity by 80% is three to four times larger than that required to reduce the CTP inhibition to the same level. However, the CTP inhibition was not completely abolished by the dose used, from 5 to 10% of the original inhibition always remained. The initial shoulder on the dose–response curve for native aspartate transcarbamylase suggests that catalytic active subunits are formed during irradiation.

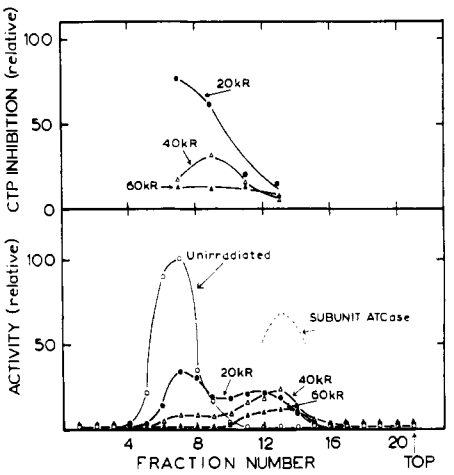

FIGURE 2: Sucrose density centrifugation of native aspartate transcarbamylase irradiated with various doses of X-rays; activity and CTP inhibition in the various fractions. The concentration of protein during irradiation was 0.13 mg/ml.

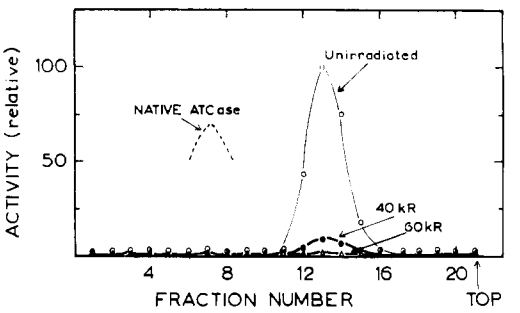

FIGURE 3: Sucrose density gradient centrifugation of irradiated subunit aspartate transcarbamylase; activity in the various fractions. The concentration of protein during irradiation was 0.15 mg/ml.

For subunit aspartate transcarbamylase a *G* value (*i.e.*, the number of enzyme molecules inactivated/100 ev of energy adsorbed) of 0.06 was obtained and the apparent *G* value for the inactivation of native enzyme was found to be 0.01 which corresponds to a *G* value of 0.02/catalytic subunit. It would, therefore, appear that subunit aspartate transcarbamylase has a higher radiosensitivity than the native enzyme.

Additions of small amounts of catalase to the irradiation mixtures prior to irradiation did not effect the rate of inactivation of neither allosteric nor catalytic properties, suggesting that the H_2O_2 which is formed during irradiation is not responsible for the inactivation. Further evidence for this view was obtained by incubating the native enzyme with 10^{-4} M H_2O_2 for different periods of time. No significant decrease in catalytic activity or loss of allosteric properties could, however, be detected.

Sucrose Density Gradient Centrifugation. Several possible hypothesis can be put forward to explain the preferential inactivation of the allosteric properties of native aspartate transcarbamylase. Thus it could be that X-rays cause the enzyme to dissociate into catalytic and regulatory subunits as is the case with other desensitizing agents. Another possibility is that the mechanism by which the allosteric effector exerts its influence on the kinetic parameters is affected by X-rays or possibly also that the binding site for the allosteric effector is destroyed. In order to distinguish between these possibilities sucrose density gradient centrifugation of irradiated enzyme samples were performed. Figure 2 shows the activity and CTP inhibition of various fractions obtained from density gradient centrifugation. The unirradiated native enzyme sample gave a single symmetrical peak. Irradiation gave rise to a new peak which initially sedimented slightly faster than the subunit enzyme. However, after a dose of 40 kR the sedimentation rate for this new peak was the same as for the subunit enzyme. It was also apparent that as the dose increased the major portion of the enzymatic activity was found in the tubes corresponding to the subunit aspartate transcarbamylase. The CTP inhibition was also lowest in this peak. When higher doses of X-rays were employed there was, however, little difference in the CTP inhibition between the enzyme sedimenting as the original native enzyme and the new enzyme peak. The data suggest that during irradiation dissociation of native aspartate transcarbamylase into subunits takes place and that this accounts for at least 70–80% of the loss of allosteric properties. The remaining part is due to desensitization with no dissociation, indicating that either the binding site for CTP or the allosteric transition mechanism has been affected by ionizing radiation for these enzyme species.

The mechanism of inactivation of subunit aspartate transcarbamylase by X-rays was also investigated by the sucrose density gradient technique. As shown in Figure 3 the activity after irradiation remains in the same fractions as the original unirradiated enzyme. Traces of activity were, however, found in fractions further down in the centrifuge tube, suggesting that associations had taken place.

Several attempts were made to correlate protein with activity in the different fractions, but this proved to be difficult due to the small amount of protein used in these experiments. However, in general these studies did confirm the above results with respect to dissociation of the native enzyme. Further, they indicated that with high doses of X-rays extensive degradation and association occurred both in the case of native and subunit aspartate transcarbamylase. Attempts were also made to identify possible regulatory subunits. These subunits have a small molecular weight and would be expected to be in fractions 16–18. Small amounts of protein, as judged by absorbance at 280 mμ, were indeed found to be present in these fractions from the native enzyme. This protein was not present in the unirradiated control. However, it was not clear

whether this protein represented degradation products of aspartate transcarbamylase or true regulatory subunits, since all attempts to reconstitute the native enzyme from partially inactivated enzyme samples failed. A sample of the native enzyme, desensitized in the presence of L-aspartate and which thus still retained the full enzymatic activity (see following section), was incubated with 2 mM mercaptoethanol for 10 hr. There was, however, no increase in the CTP inhibition during this period. It must, therefore, be concluded that the preferential inactivation of the allosteric properties of native aspartate transcarbamylase by X-rays is an irreversible process.

Effect of Substrates, Inhibitors, and Activators. Substrates, inhibitors, and allosteric effectors might have a profound influence on the ease of destruction of various properties. The effect of such compounds on the destruction of aspartate transcarbamylase by X-rays was therefore studied in some detail and the results for the native enzyme are shown in Table I. The concentrations of substrates, inhibitors, and activators used in this study were usually the same as those employed in assay systems. As expected all compounds added reduced the loss of activity caused by X-rays. In most cases the CTP inhibition also was less reduced when the native enzyme was irradiated in the presence of these compounds. The protection is probably in part due to radical scavenger effect of the compounds added and to the fact that some of the compounds become bound to the active and regulatory sites and thus may protect these from radical attack.

TABLE 1: Effect of Substrates, Inhibitors, and Activators on the Inactivation of Native Aspartate Transcarbamylase by X-Rays.

Compounds Added (mM)	% Loss of Activity	% Loss of CTP Inhibn
Control (no additions, unirradiated)	0	0
Control (no additions, irradiated)	65	87
Carbamyl phosphate (3.2)	38	26
L-Aspartate (15)	−5[a]	74
PP_i (2)	17	57
CTP (1)	35	34
ATP (1)	19	31
UTP (1)	58	67
L-Aspartate (15) + carbamyl phosphate (3.2)	32	45
L-Aspartate (15) + PP_i (2)	−15[a]	88
L-Aspartate (15) + PP_i (2) + CTP (1)	−4[a]	84

[a] The (−) sign denotes activation. The enzyme (0.1 mg/ml) was irradiated with a dose of 50 kR. PP_i was adjusted to pH 7.0 using acetic acid. All other compounds were neutralized with NaOH.

Carbamyl phosphate appears to protect both the active site and allosteric properties of the native enzyme. With L-aspartate, on the other hand, a slight increase is observed in the activity and very little protection of CTP inhibition. PP_i, which is an inhibitor competitive with carbamyl phosphate (Kleppe, 1966), is a better protector of the activity than carbamyl phosphate. The allosteric effectors CTP and ATP afford some protection both of the activity and CTP inhibition properties. UTP, which does not become bound to the enzyme, is less effective in this respect. Some caution must, however, be used when interpreting the data for the different compounds, particularly the nucleotides, since proper controls are very difficult to obtain.

The activation observed in the case of L-aspartate was not found when L-aspartate plus carbamyl phosphate were added. In the presence of L-aspartate plus the competitive inhibitor PP_i the activation and loss of CTP inhibition is even more pronounced than when L-aspartate was added alone. When the enzyme was irradiated in the presence of L-aspartate, PP_i, and CTP there was very little protection of allosteric properties whereas the activity was higher than in the unirradiated control.

The effect of substrates, inhibitors, and nucleotides on the X-ray-induced inactivation of the catalytic subunit was also investigated. With the exception of the nucleotides the results proved similar to those obtained for the native enzyme. The nucleotides protected the enzyme somewhat and they all gave equal protection. This protection was probably due to radical scavenger effect of the nucleotides, since they do not bind to the catalytic subunit.

The dose–effect curve for the native enzyme irradiated in the presence of L-aspartate and PP_i is shown in Figure 4. The activity increases gradually and reaches a plateau after approximately 60 kR and at the same time the CTP inhibition also reaches a minimum level. The fact that activity and CTP inhibition reach a plateau level at the same dose suggests that dissociation of the enzyme takes place also when it is irradiated in the presence of L-aspartate and PP_i. Further evidence for this view was obtained from density gradient experiments. A sample of the enzyme was irradiated with a dose of 80 kR in the presence of L-aspartate and PP_i and subjected to sucrose density gradient centrifugation. Figure 5 shows the distribution of activity from such an experiment. It is evident that at least 80% of the activity is present in the form of the subunit enzyme. The remaining activity is situated in a peak which moves slightly more slowly than the native enzyme.

The enzyme which was exposed to X-rays in the presence of L-aspartate and PP_i was found to have normal Michaelis–Menten kinetics and the kinetic parameters K_m and V_{max} were the same as for subunit aspartate transcarbamylase. Native aspartate trans-

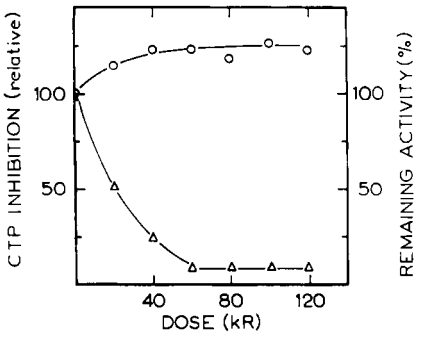

FIGURE 4: Effect of X-ray dose on activity (o——o) and CTP inhibition (Δ——Δ) on native aspartate transcarbamylase irradiated in the presence of 15 mM L-aspartate and 2 mM PP_i. The enzyme (0.13 mg/ml) was dissolved in 0.02 M phosphate buffer (pH 7.0). The concentration of L-aspartate used in the activity assay was 6 mM.

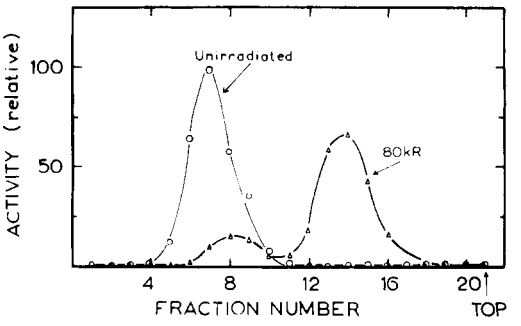

FIGURE 5: Sucrose density gradient centrifugation of native aspartate transcarbamylase (0.13 mg/ml) irradiated with a dose of 80 kR in the presence of 15 mM L-aspartate and 2 mM PP_i. The unirradiated control sample was also incubated with the same concentrations of L-aspartate and PP_i for the same period of time prior to centrifugation.

carbamylase is known to give sigmoid saturation curve with L-aspartate, whereas the active catalytic subunit gives normal saturation curves (Gerhart and Pardee, 1962; Kleppe, 1966). Thus the increase in activity observed, when native aspartate transcarbamylase is irradiated in the presence of L-aspartate and PP_i, is due to the fact that dissociation takes place and since the subunit enzyme has a higher affinity for L-aspartate than the native enzyme, the subunit aspartate transcarbamylase will be more saturated with L-aspartate at the concentration of L-aspartate used in these experiments.

Discussion

The present work is an attempt to study the effect of ionizing radiation on feedback inhibition in a model system, using the allosteric enzyme aspartate transcarbamylase from *E. coli*. The results from this investigation suggest that the feedback inhibition properties of this enzyme are several times more sensitive to destruction by X-rays than is the catalytic activity.

In analogy with other desensitizing agents of aspartate transcarbamylase X-rays also cause dissociation of the enzyme into active catalytic subunits and regulatory subunits. The latter subunits could not be identified as such due to the fact that the desensitization process was found to be irreversible. The evidence from the sucrose density centrifugation suggests, however, that the molecular weight of the catalytic subunit produced by high doses of X-rays is the same as that of the true subunit aspartate transcarbamylase. With low doses the molecular weight of the degradation products is somewhat higher than that of the true subunit indicating that the dissociation of the native enzyme may proceed *via* a number of intermediate molecular weight species.

Studies on other proteins have shown that a number of amino acid residues may be modified by ionizing radiation (Augenstine, 1962). SH groups are, however, particularly sensitive to modification by radiation (Damjanovich *et al.*, 1967). These groups are easily oxidized to disulfide bonds. A more drastic modification involves splitting off the SH group to give H_2S and thus leaving an alanine residue (Kuzin, 1962). In the case of aspartate transcarbamylase no attempt was made to investigate the exact changes which occurred in the various residues during irradiation. However, in view of the large number of SH groups found in the native enzyme, a total of 34 half-cysteine residues calculated on the basis of the data from Gerhart and Holoubek (1967) and at least 24–26 of these are SH groups easily titrated with *p*-mercuribenzoate (Gerhart and Schachman, 1965), the possibility must be considered that the X-ray-induced dissociation into subunits is caused by modification by some of these groups. This would in some respect be analogous to the well-known dissociation of aspartate transcarbamylase by mercurials (Gerhart and Pardee, 1962). The four regulatory subunits reportedly contain most of the SH groups of native aspartate transcarbamylase. It would therefore seem likely that the major modification occurs on these subunits. The increased radiosensitivity of the catalytic subunit may in part be explained by the fact that SH groups are known to exert protective effects (Augenstine, 1962). The SH-rich regulatory subunits appear to be tightly bound to the catalytic subunits in the native enzyme and they may therefore shield these subunits from radical attack.

The X-ray-induced inactivation of the allosteric properties was not restored by additions of such reducing agents as mercaptoethanol, suggesting that perhaps H_2S has been split off and that none or very few disulfide bonds are formed. An alternative possibility is of course that other amino acid residues may have been modified as well. This possibility seems, however, more probable concerning the destruction of the catalytic activity. In this case it should be noted that

mercurials do not significantly affect the activity. During ionizing radiation small amounts of H_2O_2 are produced (Kuzin, 1962). However, experiments in this work have clearly demonstrated that the X-ray-induced modifications of aspartate transcarbamylase are not caused by H_2O_2. Therefore, the changes in the protein structure must be brought about possibly both by direct action of the X-rays and by radiation-induced radicals.

An interesting finding is the fact that L-aspartate or L-aspartate plus the competitive inhibitor PP_i protects the active site but not the allosteric properties of the enzyme. In a previous paper (Kleppe, 1966) it was suggested that competitive inhibitors with carbamyl phosphate, such as PP_i and P_i, form ternary complexes with L-aspartate at the active site at high concentrations of L-aspartate. The results from the present study give further support for this idea. Such a complex at the active site will protect it from radical attack. Since all irradiation experiments were performed in 0.02 M P_i buffer it is likely that an L-aspartate–P_i complex is formed at the active sites when L-aspartate was added alone, which may explain the large protection given by this compound. The observation that carbamyl phosphate plus L-aspartate do not show any significant protection is surprising. In this case an enzymatic reaction takes place initially and it might be that intermediates in this reaction are particularly sensitive to irradiation.

The increase in desensitization observed in the presence of L-aspartate plus a competitive inhibitor and the fact that CTP does not protect the allosteric site in the presence of these compounds lend support to the allosteric enzyme theory recently proposed by Monod *et al.* (1965). According to this theory the enzyme exists in two different conformational states, $R \rightleftharpoons T$, which are in equilibrium with each other and which possess different affinities for the substrates and the allosteric effector. In the present case the T state might have a high affinity for L-aspartate and a low affinity for CTP, whereas the R state has a low affinity for L-aspartate and a high affinity for CTP. Thus in the presence of high concentrations of L-aspartate, CTP would be expected to have little protective effect.

The observation that substrates and inhibitors can protect aspartate transcarbamylase from destruction by X-rays may be of importance from a biological point of view. Several earlier studies with other enzymes (Dale, 1940; Okada, 1957; Sutton, 1956; Yost *et al.*, 1958) have shown that the different substrates give a marked decrease in radiosensitivity for these enzymes. It is thus tempting to suggest that as a rule the substrates will protect an enzyme from inactivation by ionizing radiation. It would, therefore, appear that *in vivo* the inactivation of the different enzymes by X-rays depends on the concentrations of substrates and inhibitors present, or in other words, to some extent on the metabolic state of the cell.

A number of enzymes are now known to be allosteric. The possibility therefore exists that these might be affected by X-rays in a similar way to that observed for aspartate transcarbamylase. Recently it has been found (Damjanovich *et al.*, 1967) that the allosteric site on phosphorylase *b* is more sensitive to destruction by X-rays than is the catalytic site. The destruction of cellular control mechanisms, such as feedback inhibition, may be an important aspect of the action of ionizing radiation on living cells. Thus, in the present case the dose needed for 50% inactivation of the allosteric properties of a 0.1% solution of aspartate transcarbamylase is approximately 20 kR and this of the same order of magnitude as the LD_{50}, *i.e.*, the dose needed to kill 50% of the cells, for *E. coli* (Casey, 1962).

Present knowledge on the ability of ionizing radiation to cause dissociation and association of proteins is scarce. It would appear, however, that such radiation-induced changes in the protein structure may have wide implications for the understanding of the mechanism of ionizing radiation on biological materials.

Acknowledgments

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