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Factors Affecting The "In Vitro" Inactivation Of Aldolase By X-Rays

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FACTORS AFFECTING THE IN VITRO INACTIVATION OF ALDOLASE BY X-RAYS

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SUMMARY

The influence of urea and of various protective compounds on the "in vitro" inactivation of aldolase by x-rays has been studied.

Low concentrations of urea protect the enzyme from the inactivation, whereas high concentrations, able to induce an unfolding of the protein molecule, increase the degree inactivation by a given dose of radiation.

Cysteamine, cystamine, aminoethyl-isothio-uronium and glutathione, all protect the aldolase in solution from the inactivation by x-rays. Cystamine is as protective as cysteamine, in equimolecolar concentrations, when high inactivation levels are reached.

No protection can be demonstrated when the aldolase, after incubation with the tested compounds, is precipitated and redissolved in a new medium before irradiation. Nevertheless, with S^{35}-labelled cystamine, it can be demonstrated that at least seven residues of cysteamine are bound to each aldolase molecule.

The protective power of glutathione is reduced by a factor of about 0,2 in the presence of 4 M urea.

The possible implications of these findings are discussed.

Some experimental evidence has been accumulated showing that the sensitivity of a macromolecule to ionizing radiations can be influenced by the action of factors which cause physico-chemical alterations of the molecule itself (1, 2, 3).

In a previous investigation in this field, we have studied the influence of some factors on the process of inactivation by X-rays of a protein molecule, the enzyme aldolase from rabbit muscle (4). We have observed that the blocking, by suitable reagents, of a certain number of thiol groups not essential for enzymatic activity results in alterations in the radiosensitivity of aldolase under certain experimental conditions and that these alterations could be correlated in some way with modifications affecting the tertiary structure of the protein molecule.

In the present paper are reported the results of further studies on some factors which modify the sensitivity of aldolase irradiated "in vitro". Initially we have studied the influence of urea, extending the observations referred to in the previous paper (4). Subsequently, we have studied the influence on aldolase of some well known protective agents against radiation, i.e. glutathione, cysteamine, cystamine and S-(2 aminoethyl) isothiouronium bromide hydrobromide (A.E.T.).

The researches on the latter compounds were planned in order to investigate whether their radioprotective power "in vitro" could be correlated with their ability to compete with the target molecule for the free radicals formed from water, or to repair the site of damage induced by radiation in the same molecule, or rather to their interactions with the native protein molecule. The experimental data on the mechanism of chemical protection of protein molecules "in vitro" are not so numerous and in general this protection is explained on the basis of the scavenging ability of protective compounds.

MATERIAL AND METHODS

The experimental procedures for the preparation of the aldolase, for the aldolase catalytic activity assay and for the irradiation, have been reported in the preceeding paper (4).

Chemical products: Urea - analytical grade product recrystallized from 95° ethanol. Glutathione and cysteamine obtained respectively from the E. Merck AG., Darmstadt (Germany) and from Fluka AG., Buchs (Switzerland). Cystamine dihydrochloride - two different preparations have been used (both giving the same results). The first was prepared from cysteamine in our Laboratory according to the method of Natham and Bogert (5); the second was a generous gift of Prof. Z. Bacq. AET - prepared according to the method of Shapira et al. (6). Cystamine-S^{35}, dihydrochloride obtained from the Radiochemical Centre, Amersham, England, specific activity 109 mc/g.

Treatment of aldolase: All compounds tested for their influence on the radiosensitivity of aldolase were added to the enzyme solution just before irradiation, except when cystamine was tested. In the latter instance the solution was kept at room temperature 30 minutes before irradiation. When it was necessary to obtain the aldolase free from the added substances, it was precipitated by adding to the solution ammonium sulfate up to 65% of saturation. The enzyme was then separated by centrifugation, redissolved in water, reprecipitated with ammonium sulfate and finally dissolved in the buffered phosphate solution.

All solutions were buffered at pH 7.2 with 0,01 M phosphate. The reasons for selecting this medium have been discussed in our previous paper (4).

RESULTS

Urea: Urea exhibits a quite characteristic influence on the x ray effects on the aldolase in solution. In fact, it protects the enzyme at low concentrations, while at higher concentrations it shows a sensitizing effect (fig. 1-2).

Protective compounds: The data reported in table 1 and in fig. 3 show that all the substances which have been tested, very efficiently protect the aldolase against x rays. The data in fig. 3 have been plotted on linear scale, instead of the usual semi-log scale, in order to demonstrate more clearly the marked differences between the effects of the various substances on the inactivation curve of aldolase as a function of x rays dose. It can be seen, in fact, that only in the presence of cystamine the curve is exponential like that of control aldolase. In the presence of cysteamine or of AET the slopes of the curves tend to decrease with the increase of the dose of radiation.

In the presence of glutathione the curve is linear. A consequence of the different shapes of the inactivation curves is that it is rather difficult to compare the protective power of the various substances. This is due to the fact that it is not possible to use the "Competition Factor", which can be applied only to exponential curves. In addition comparisons using the DRF are complicated since in some cases, i.e. for cysteamine and AET the value of this factor shows very large variations at the different levels of inactivation. On the basis of the reasons considered in the discussion, we think that the values obtained at the level of ν_{90} may be more significant. With all tested substances, the protective effect completely disappears when the aldolase is removed from the solutions containing them, and irradiated in phosphate buffer as described in the experimental part.

Cystamine: From the data reported above it appears that cystamine has a very marked protective effect.

In fig. 4 are reported in semi-log scale the inactivation curves of aldolase in the presence of cysteamine and of cystamine. Fig. 5 shows graphically the relationship between the protective effect and the ratio between the molarities of cystamine and aldolase. From the inspection of the curve it can be realized that the "competition factor" is not directly proportional to the concentration of cystamine.

Cystamine - S^{35}: S^{35}-labelled cystamine has been used in order to ascertain whether this substance would form mixed disulphides with aldolase.

Aldolase incubated at room temperature with cystamine-S^{35}, after repeated precipitations with ammonium sulfate and paper electrophoretic separation retains an amount of radioactivity from the value of which is possible to calculate that about 7 residues of cysteamine are bounded to each enzyme molecule. Nevertheless the radiosensitivity of the modified enzyme is identical to that of the native enzyme.

Glutathione in the presence of urea: Considering the effects of urea at high concentrations and on the basis of the reasons which will be discussed later, the protective power of the glutathion has been tested in the presence of 3.75 M urea. As shown in fig. 6 the protective ability of the glutathione is markedly reduced in 3.75 M urea. The D.R.F. at D_{37} level falls from 13 to 3.

This kind of experiment has been possible only with glutathione, because with the other compounds, in the presence of 3.75 M urea, the aldolase is rapidly inactivated.

DISCUSSION

In our previous paper (4) we reported that the aldolase is much more radiosensitive in 3.75 M urea, and that in this medium an additional increase in radiosensitivity of the enzyme is detectable when some of its SH groups are blocked. On the contrary in 1,5 M urea the radiosensitivity of the aldolase is reduced.

In the present paper we report the influence of increasing concentrations of urea on the percentage of inactivation produced by a given x rays dose. The diagram where the percentage of protection is plotted as a function of the urea concentration, shows a curve increasing up to a certain level, where a plateau is reached. Then the curve falls down reaching negative values at the highest concentrations.

Swenson & Boyer (7) reported that urea induces on aldolase a loss of activity which is proportional to the urea concentration, being complete at 3.5 M. concentration . The inactivation is completely reversible by simple dilution with distilled water up to a concentration of 4M urea, whereas at higher concentrations it is irreversible.

It has been also reported by Rajagopalan et al. (8) that low concentrations of urea inhibit a certain number of enzymes by a competitive mechanism.

The curve in fig. 1 seems to indicate the existence of a competition phenomenon between a protective and a sensitizing effect. This latter predominates at the highest concentrations, that is when the aldolase molecule is considerably unfolded. Therefore it seems reasonable to assume that the sensitizing effect can be correlated with the unfolding of the protein molecule induced by the urea. The unfolding probably make accessible to the radicals formed from water some functional groups which were masked in the native molecule.

As far as the protective effect of low concentration of urea is concerned, we think rather improbable that it is due to the scavenging ability of this substance. In fact, several authors reported that urea shows a very poor affinity for the products of water radiolysis and therefore its protective power with many other systems is quite negligible (9). - The protective effect in the case of aldolase could be due to some kind of interaction with the protein molecule, different from that causing the unfolding.

Considering the effect of the other substances which have been tested, we would like to emphasize at first the different shape of the inactivation curves of the aldolase in their presence. In the case of cysteamine and of AET, the flexus which can be noted at high X-ray doses could be attributable to the fall of the concentration of these substances during irradiation, provided that their radiolysis proceeds with a rather high G. -

It is difficult on the other hand to explain why the inactivation curve in the presence of glutathione is linear. In any case the differences mentioned above may lead one to think that the protective effect of these substances cannot be explained with a common mechanism of action. -

The hypothesis that the protective effect may be due to an interaction with the protein molecule leading to the formation of stable bonds between protective and protected molecules, has been studied. The aldolase reprecipitated from solutions containing all tested compounds with the procedure described in the methods, has shown the same radiosensitivy as native aldolase. This result may be explained with one of the following hypothesis:

1) no interaction at all takes place between the protein molecule and the protective one;
2) labile complexes are formed
3) a stable combination occurs, but the products of this combination have the same radiosensitivity as the native molecule.

In relation to the second hypothesis some indications seem to be provided by the results obtained studying the protective effect of the glutathione in 3.75 M urea. It was possible to immagine that the urea at this concentration could compete with glutathione in the eventual formation of the addition compounds, reducing in this way the protective power of this substance. Effectively the DRF for the glutathione falls from 13 to 3 in 3.75 M urea.

Of course this result provides only an indirect evidence and several points remain to be clarified. In fact in 3.75 M urea, the aldolase is in particular conditions and shows a certain degree of unfolding . - On the other hand we do not know whether the respective affinities of the glutathione and of the aldolase for the products of water radiolysis are the same in the presence and in the absence of urea.

With regard to the third hypothesis we have been able to establish that it must be considered as verified, at least in the case of cystamine. In fact using S^{35}-labelled cystamine, it has been possible to establish that the aldolase incubated with this substance and reprecipitated several times with ammonium sulfate, after electrophoretic separation, retains an amount of radioactivity, from the value of which it can be calculated that 7 residues of cysteamine are bound to each protein molecule, probably in the form of mixed disulphides. Nevertheless the radiosensitivity of the modified aldolase is identical to that of the native aldolase.

The behavi...

The behaviour of cystamine requires some particular considerations. It may be noted at first that this substance shows a conspicuous protective effect, quite similar to that of the other tested substances. In particular this is true at level of the D_{37}, whereas at level of the D_{90} the cystamine appears to be much less efficient than cysteamine or A.E.T. - As we said formerly, it is not possible to correlate the protective effect of cystamine with the formation of mixed disulphides with the aldolase molecule. It may be considered therefore the possibility that cystamine displays its protective power through a scavenging mechanism. Shapiro et al. (10) and Cavallini et al. (11) demonstrated that the radiolysis of cystamine occurs with a very low yield. It would seem therefore rather difficult to assume that a compound so poorly reactive may actively compete with the target molecule for the products of water radiolysis. On the other hand, we have been able to demonstrate quite recently that cystamine irradiated in solution is reduced to cysteamine with a rather high yield, if the irradiation is carried out in the presence of substances able to block irreversibly the cysteamine formed (i.e. iodoacetic acid, N- ethylmaleimide). In these conditions a G of about 2 may be calculated, for the formation of cysteamine. When the blocking substances are absent no formation of cysteamine is detectable. This is due to the fact that cysteamine, in its turn, is reoxidized by radiation to cystamine and this reaction occurs with a yield much higher than the opposite reaction. According to the data reported by Shapiro et al. (10), the oxidation of the cysteamine to cystamine occurs with a G of about 11. The possibility exists therefore that the protective effect of the cystamine may be due to a mechanism of competition for the products of water radiolysis which cause the

reduction of the disulphide and the subsequent reoxidation of the sulphydryl groups.

It is now possible to point out that when a given system is irradiated "in vitro" in the presence of cysteamine, after a certain radiation dose, almost only cystamine will be present in the solution.

Therefore it would be expected, in our experimental conditions, that the inactivation curves of aldolase in the presence of each of the two substances (provided that the concentration of the sulphydryl compound is double of the concentration of the disulphide) would be parallel starting from a certain dose of radiation. In effect, looking at curves reported in fig. 4, it can be realized that, when the concentrations of the two compounds are those reported above, the protective power of both substances becomes rather similar with the increase of the radiation dose.

The results obtained with the urea seem to indicate that the modifications of the secondary and tertiary structure of a protein molecule may induce modifications of its radiosensitivity.

As far as the effects of thiol protective compounds are concerned, the results are less indicative and it is not possible from our data to exclude the preeminence of the scavenging mechanism. It appears to be demonstrated instead, that, at least in the case of aldolase, the formation of mixed disulphides between the protective and the protected molecules is not relevant for the protective effects observed.

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Table 1

Dose reduction factor (DRF) for various protective compounds on the x-rays inactivation of aldolase. Aldolase 0.7×10^{-5}M.

	Mol.protector: Mol.aldolase	D_{37} Kr	DRF at D_{37} level	D_{90} Kr	DRF at D_{90} level
Control	-	60	-	8	-
AET	250	850	14.2	340	42.5
Cysteamine	250	820	13.7	240	30.0
Glutathione	250	750	12.5	110	13.7
Cystamine	250	800	13.3	90	11.3
Cystamine	125	600	10.0	60	7.5

$$\text{Dose reduction factor} = \frac{\text{Radiation dose to produce a given effect without protector}}{\text{Radiation dose to produce a given effect in presence of protector}}$$

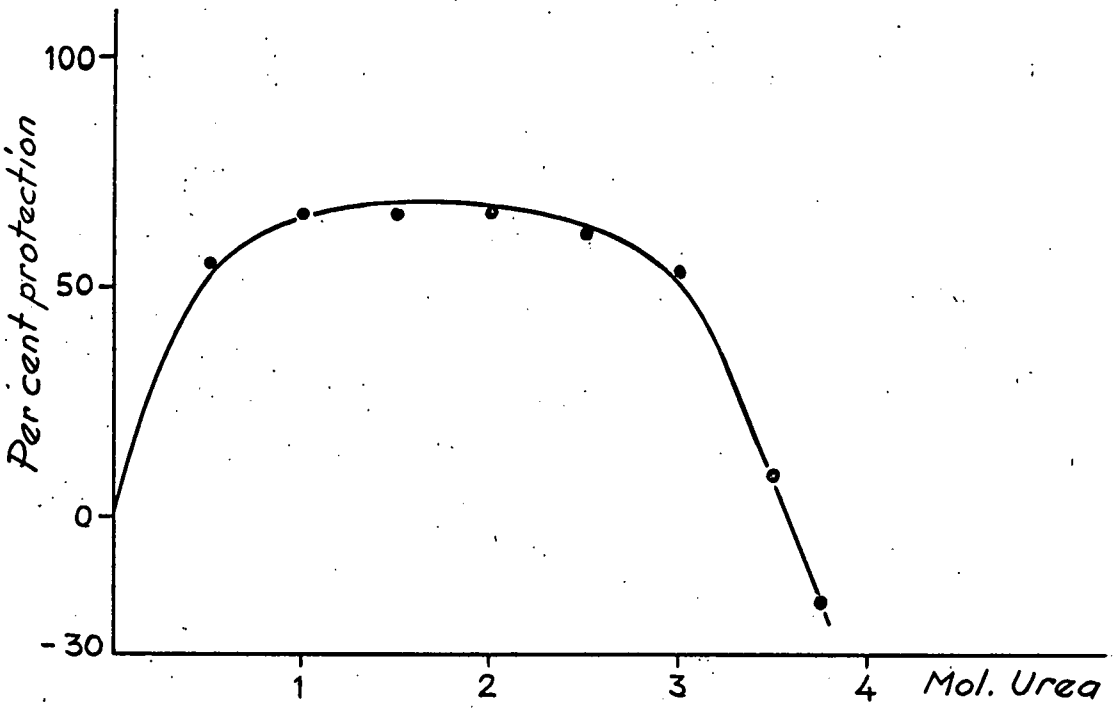

Fig. 1

Effects of various urea concentration on the radiosensitivity of aldolase. Aldolase 0.7×10^{-5}M. X-rays, 105.000 r.

$$\text{Per cent protection} = \frac{I_c - I_p}{I_c} \times 100$$

I_c = % enzyme activity lost in the absence of protector

I_p = % enzyme activity lost in the presence of protector

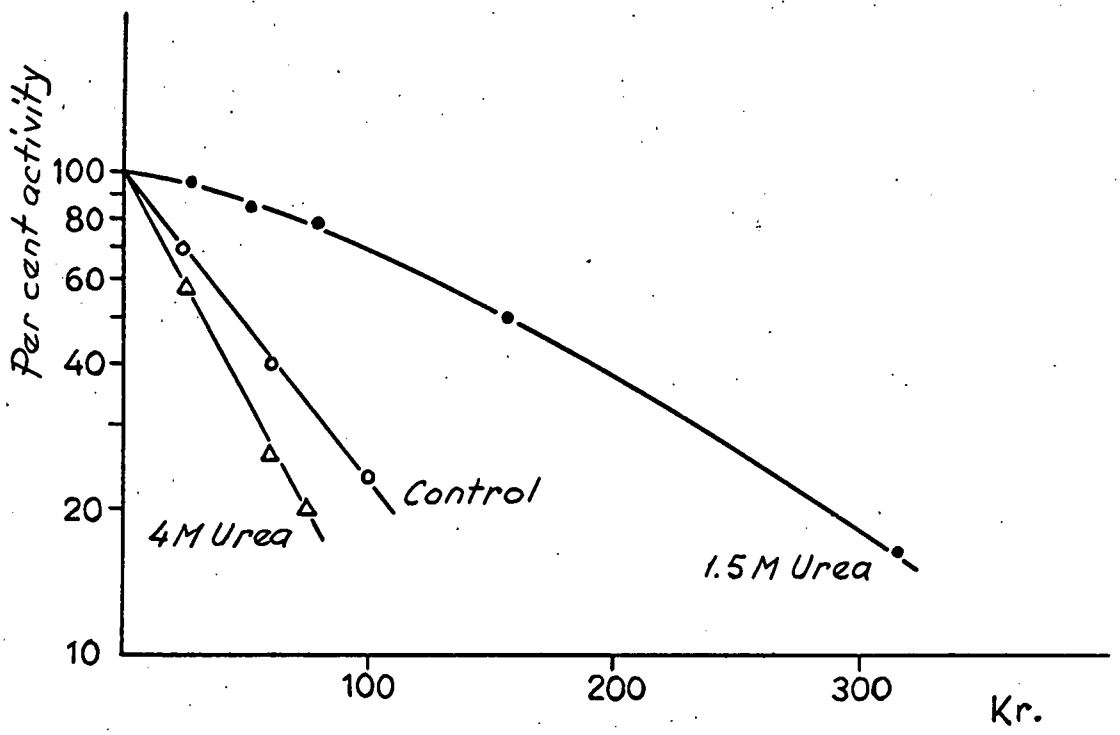

Fig. 2

Effects of the urea on the inactivation of aldolase by x-rays. Aldolase, 0.7×10^{-5}M.

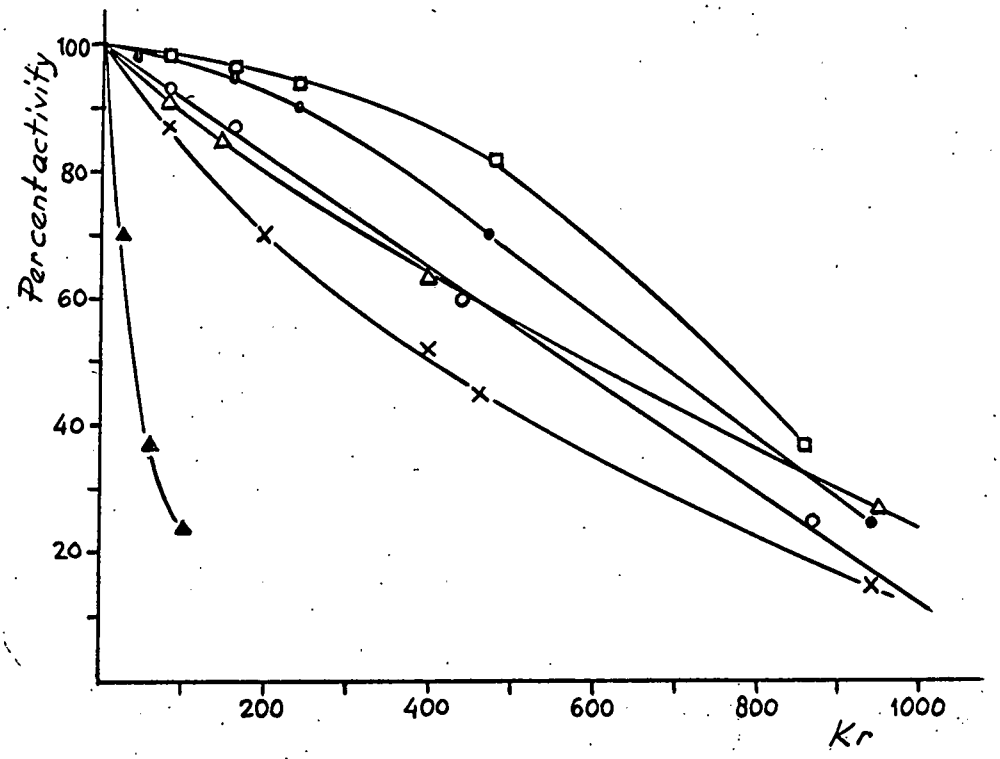

Fig 3

Aldolase inactivation by x-rays in presence of various protective compounds. Aldolase. 0.7×10^{-5}M.

△—△	Control	
□—□	AET	1.75×10^{-3}M
•—•	Cysteamine	"
○—○	Glutathione	"
△—△	Cystamine	"
×—×	Cystamine	0.87×10^{-3}M

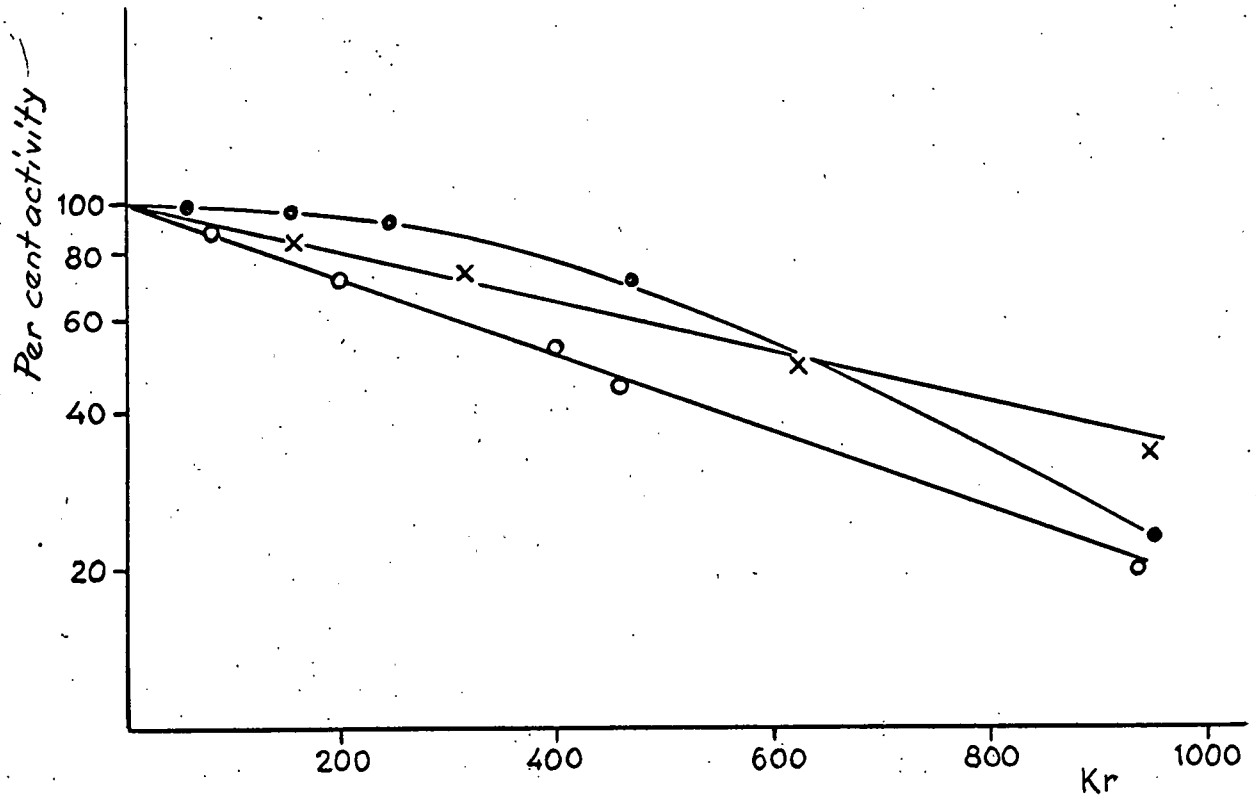

Fig. 4

Aldolase inactivation by x-rays in presence of cysteamine and cystamine. Aldolase, 0.7×10^{-5}M.

·——·	Cysteamine	1.75×10^{-3}M
×——×	Cystamine	"
O——O	Cystamine	0.87×10^{-3}M

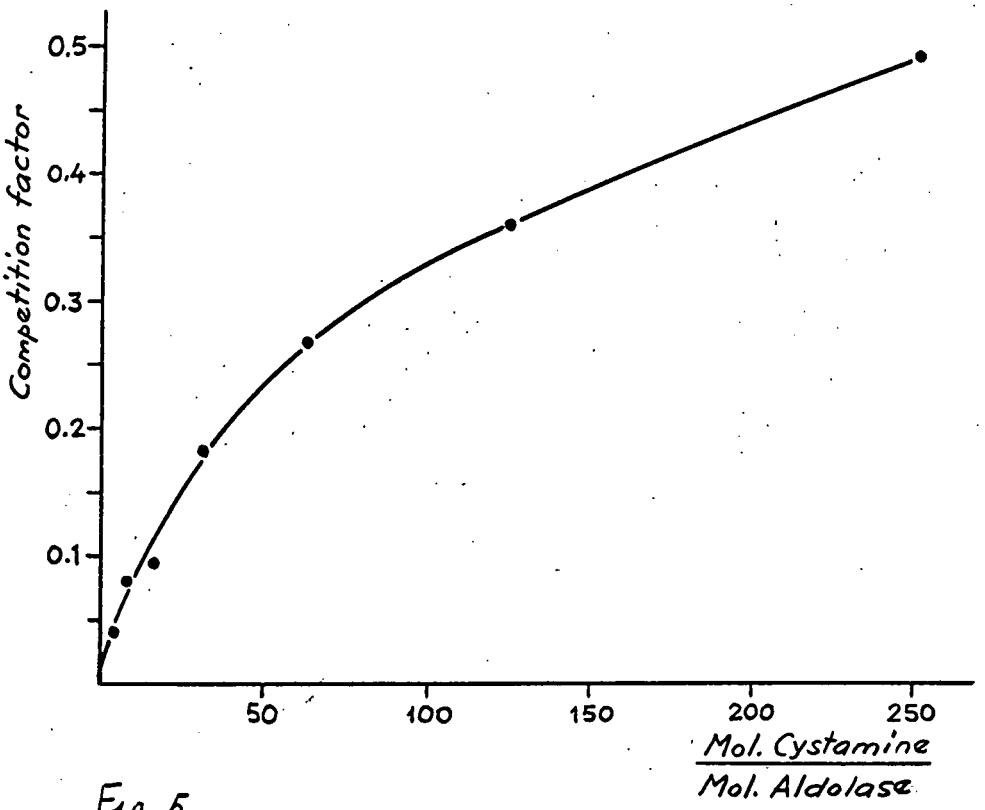

Fig. 5

Protective effect of cystamine at various concentrations. Aldolase, 0.7×10^{-5}M. Competition factor = $\frac{\log. I_c - \log. I_p}{\log. I_c}$

(I_c and I_p as in fig. 1)

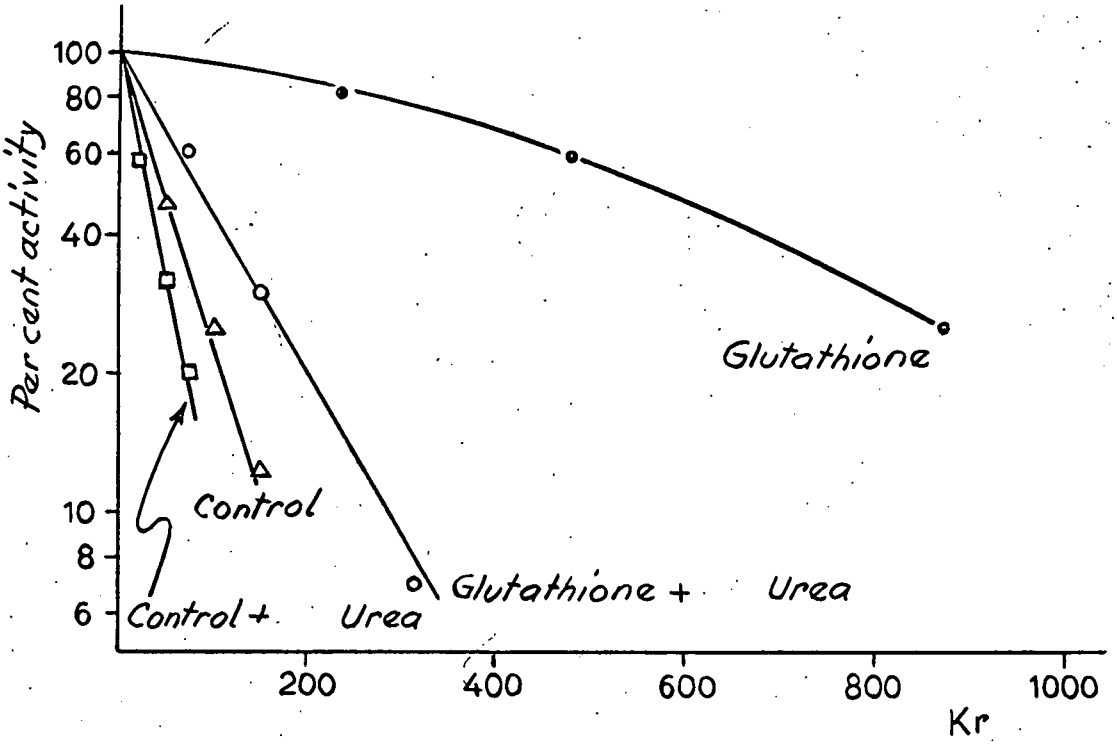

Fig. 6

Protective power of glutathione in the presence of urea.

△ — △ Aldolase 0.7×10^{-5}M

□ — □ " " + urea 3.75 M

• — • " " + glutathione 1.75×10^{-3}M

○ — ○ " " " " + urea 3.75M