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PHYSICO-CHEMICAL INVESTIGATION OF THE MECHANISM OF
ENZYME INACTIVATION.
PART III. THERMODYNAMIC ANALYSIS OF STRUCTURAL AND
CHEMICAL CHANGES IN PROTEIN DURING RADIATION INACTIVATION.

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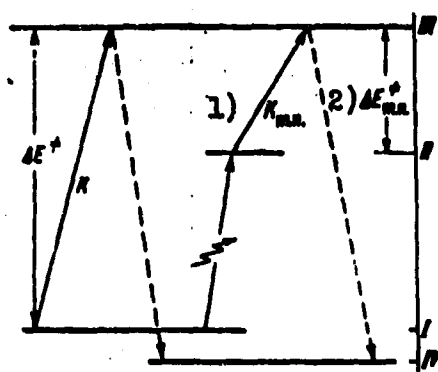
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At the present time it has been established that the inactivation of a number of enzymes in the presence of radiation effects takes place in two stages, in the first of which long-lived latent damage develops which does not affect the enzymatic function of the protein and is realized in visible damage (inactivation) only in the second stage — in the presence of supplementary non-radiation effects (1-4). One of the types of latent damage is realized upon the action of oxygen on the anaerobically irradiated enzyme (1-4); the other type, upon heating the irradiated protein in an aqueous medium ("thermal" after-effect)(2-5).

As regards what structural and chemical changes take place in the protein molecule during the individual stages of inactivation, at present practically nothing is yet known. However, the revealed discreteness of the various stages of radiation inactivation permits charting the course of the study of these changes during each of the stages individually. In the present report is proposed such a method of analysis, investigated with the example of the radiation inactivation of myosin and pepsin — enzymes which are quantitatively the most studied in the present connection (6). This method consists of applying the method of thermodynamic analysis of protein denaturing proposed by Eyring and Stearn (7, 8) to the thermal radiation after-effect reaction. The information obtained as a result of such an analysis characterizes the structural and chemical changes in the irradiated protein which take place in the second stage of inactivation — upon realization of the latent "thermal" damage. From the comparison of these changes with those that take place upon thermal inactivation of intact protein containing no latent damage, conclusions can also be drawn, in accordance with the differential effect, concerning disturbances of the structure which are

brought about in the first stage of the radiation effect upon the formation of latent damage.

This is depicted schematically in the illustration. Under number III in the illustration is designated the state of the "activated complex", from which the protein has already spontaneously passed over into the inactivated state. A whole set of various conformations of the protein structure fits the enzymatically active state; the transition from these to the activated complex requires the surmounting of energy barriers varying in magnitude and entropy changes. It is usually assumed that the activated complex in all these cases is the same; the difference consists only in the greater or lesser difficulty of its formation from one or another initial state of the protein.



Legend: 1) $K_{t.a.}$
2) $\Delta E^*_{t.a.}$

Energy diagram of the two-stage inactivation of the molecules of enzymes in which latent damage of the "thermal" type (right section of the illustration) is created in the presence of radiation effects.

I — initial enzyme state; II — enzymatically active state with latent radiation damage; III — "activated complex"; IV — inactivated state. $K_{t.a.}$ and $\Delta E^*_{t.a.}$ — rate constant and energy of activation of the thermal after-effect reaction; sigsag arrow — first stage of inactivation (creation of latent damage). On the left a diagram of the thermal inactivation of intact enzymes is given for comparison; K and ΔE^* — rate constant and energy of activation of this process.

We also will assume (see drawing) that molecules containing latent radiation damage pass over, when heated, into an activated complex state identical with the one that arises upon thermal inactivation of these proteins from any other enzymatically active conformations. Of course, it is

not possible to substantiate this hypothesis exactly. However, in the case of myosin the results of our preceding work (9), in which it was shown that for this object the activated complex is one and the same for inactivation proceeding from different initial states of the intact protein, serves as a serious argument in its favor.

A. Second Stage of Radiation Inactivation

The kinetic and thermodynamic characteristics of the inactivation of intact enzymes and of the thermal after-effect reaction in the case of γ -irradiation, which correspond to the experimental data given in (6), are given in the table. Within the scope of the concepts of Eyring and Stearn the conclusion should be drawn from analysis of this table that activation in the thermal after-effect reaction consists of the rupture of one disulfide bond and approximately four hydrogen bonds. Of course it is not ruled out that, along with the hydrogen bonds, not the disulfide bond but some other covalent bond is ruptured here, and moreover within the limits of accuracy of the experiments the whole series of $\Delta H_{t,a.}^{\ddagger}$ and $\Delta S_{t,a.}^{\ddagger}$ values obtained can be correlated. The assumption concerning the rupture of only some hydrogen bonds is, however, clearly contradicted by these data; in this case myosin, for example, would have $n_H/n_S = 2.25$. The arguments in favor of the rupture of specifically the disulfide bond can be obtained from examination of the results of radiation inactivation and in other aspects.

B. First Stage of Radiation Inactivation

From comparison of the structural changes taking place upon inactivation of intact proteins and proteins containing latent radiation damage it should be concluded that the formation of latent damage (first stage of inactivation) is accompanied by the rupture of $\sim 10-15$ hydrogen bonds without the rupture of covalent bridges. This conclusion correlates well with the retention, at this stage of protein damage, of its enzyme activity, for the maintenance of which the completeness of the molecule's tertiary structure is apparently mainly responsible.

The physical process which can lead to simultaneous rupture of a number of hydrogen bonds in the irradiated protein molecule was pointed out in their time by Flatsman and Franck (10). It consists of the polarisation of the intramolecular medium as a result of the sudden formation of an electric charge upon ionization of the protein molecule. The quantitative evaluation of this effect, which was derived by the authors, agrees well with the values we obtained of the number of ruptured hydrogen bonds in the first stage of inactivation. It should, however, be pointed out that, in the opinion of the authors, one polarisation effect only is insufficient for the inactivation of practically all the protein molecules except perhaps the ones smallest in size (10, 11). This conclusion is connected with their denial of the role of migration processes in the radiation damage of protein. Naturally it is difficult to explain the great effectiveness of radiation inactivation.

**Kinetic and Thermodynamic Characteristics of the Thermal Inactivation
of Intact Enzymes and Thermal After-effect Reactions in
-Irradiated Myosin and Pepsin Solutions**

Enzyme	°C	lg K	ΔF^\ddagger kcal/mol	ΔH^\ddagger kcal/mol	ΔS^\ddagger kcal/de- gree·mol	$n_S = \frac{S^\ddagger}{12}$	$n_H = \frac{H^\ddagger - 24}{4}$	$\frac{n_H}{n_S}$
Thermal Inactivation of Intact Enzymes								
Myosin	35	-4.02	25.9	97	237	19.8	18.3	0.92
	25	-6.35	26.1	97	238			
Pepsin	60	-4.5	26.5	90	190	15.8	16.5	1.04
Thermal After-effect in Irradiated Solutions								
Myosin	30	-3.5	22.7	38.4	52	4.3	3.6	0.84
Pepsin	58	-3.05	24.1	36.0	36.5	3.0	3.0	1.0

tion with an ion yield close to unity, as is observed in many enzymes, by the local "fusion" of 10-15 hydrogen bonds in any arbitrary section of the protein molecule. However, the situation can change in the presence of migration of the charge along the protein with its localization at the junction sites of the structure that are responsible for the configuration in the native state; for example, at the S-S bridges. In this case polarisation would lead to deformation of this section, which could contribute to its subsequent rupture, in particular the heat-activated rupture of the disulfide bond. Note: The possibility of localization of the charge at the disulfide bond in irradiated protein molecules now has already been proved experimentally (12) during the second stage of inactivation.

CONCLUSIONS

1. A method for studying the structural and chemical changes during the individual stages of the radiation inactivation of enzymes is proposed, which consists of applying the Eyring-Stearn method of thermodynamic analysis of protein denaturing to the thermal radiation after-effect reaction.

2. It has been shown by this method that in the process of formation of latent damage in the myosin and pepsin molecules 10-15 hydrogen bonds are ruptured, but the covalent bonds are not ruptured; in the second stage of inactivation — upon realization of the latent damage — the rupture of one covalent bond (apparently the disulfide bond) and 3-4 hydrogen bonds takes place.

3. The hypothesis was expressed that the rupture of the hydrogen bonds in the first stage of inactivation takes place due to the Platsman-

Franck polarization effect, but only after preliminary migration and localization of the charge at "weak" sites of the structure, particularly at the S-S bridges. "Fusion" of sections of the protein molecule in this region during the first stage of inactivation guarantees the possibility of carrying out the second stage.

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