Stability of Chymotrypsin and Tobacco Mosaic Virus Decreased by Ultraviolet Radiation

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It is often assumed that when specific activities of various proteins, such as enzymes or viruses, are destroyed by ultraviolet radiation, those irradiated particles or molecules that still remain active are unchanged. The change that causes the loss of a specific activity, therefore, is often referred to as 'primary' or 'initial', as distinct from any further changes such as denaturation, alteration in absorption spectrum, oxidation, etc. (see, for example, Finkelstein & McLaren, 1949; McLaren, 1951).

Results obtained with ultraviolet-irradiated chymotrypsin show that an enzyme molecule can be changed without immediately losing its specific activity. The change shows as a decreased stability of the molecule when exposed to a temperature of 37° at pH 7 and can be measured as an increase in the rate at which proteolytic activity is lost. Thus, two stages in the effect of the radiation can be clearly distinguished, one decreasing stability and the other destroying enzymic activity. A similar phenomenon has been described with a bacteriophage (Kleczkowski & Kleczkowski, 1953).

Decreased stability of irradiated proteins can also find expression in increased sensitivity to the denaturing effect of elevated temperature. This was studied with tobacco mosaic virus, which Oster & McLaren (1950) found to show enhanced sensitivity to heat denaturation after it has been inactivated by ultraviolet radiation. The relationship between the loss of activity and the increase in the heat sensitivity was investigated in the present work.

MATERIALS AND METHODS

Crystallized chymotrypsin (*CTR*). This was prepared from fresh beef pancreas as described by Northrop, Kunitz & Herriot (1948). It was crystallized five times as chymotrypsinogen, converted into chymotrypsin and crystallized twice. The final crystals were dissolved, dialysed for 48 hr. in a cellophan sac against frequent changes of distilled water and dried. Weighed quantities of the dry material were dissolved in 0·067 M phosphate buffer at pH 7 for each experiment.

Tobacco mosaic virus (*TMV*). This was prepared from sap of infected tobacco plants as described by Bawden & Pirie (1943). The final preparation was dialysed against distilled water, placed in a separation funnel, and the liquid crystalline 'bottom' layer was separated and used.

Source of ultraviolet (UV) *radiation.* A Vitreosil low-pressure mercury discharge lamp was used, made by the Thermal Syndicate, Ltd.; this was fitted with a chromium-plated cylindrical reflector. According to the makers' specifications, about 99% of the UV-radiation was of a wavelength 2537 Å. The intensity of radiation at various distances from the lamp was measured with a thermopile.

CTR was irradiated as a 0·2% solution and TMV as a 0·5% or a 0·75% solution, both in 0·067 M phosphate buffer at pH 7. Irradiations were done at room temperature. The solutions were placed in Petri dishes as layers 0·14 cm. deep and were rocked continuously during irradiation. This is assumed to have ensured that all molecules or particles were equally exposed to the radiation. The dishes were 10 or 20 cm. from the lamp, at which distance the intensities of radiation were 3030 and 870 μw/sq.cm., respectively. Evaporation during irradiation was made up by adding water.

Absorption of the radiation of $\lambda = 2537$ Å by the solutions of CTR and TMV was measured with a quartz spectrophometer.

Relative activities of irradiated solutions of CTR. Irradiated solutions of CTR were suitably diluted in 0·067 M phosphate buffer at pH 7. One ml. of each diluted solution was mixed with 1·5 ml. of a 1% solution of casein at pH 7, the mixtures were incubated for 4 hr. at 37° and the amounts of casein rendered unprecipitable by 2% trichloroacetic acid were estimated. The relative activities were obtained graphically by reference to a curve obtained by plotting the amounts of casein rendered unprecipitable by trichloroacetic acid after incubation with non-irradiated control solutions of CTR, against its concentrations, which ranged from 0·0005 to 0·002%.

Relative activities of irradiated solutions of TMV. These were also assayed graphically as described by Bawden & Kleczkowski (1953).

Heat denaturation of TMV. The amounts of TMV denatured by heating in 0·067 M phosphate buffer at pH 7 for various intervals of time in a water bath at 69° were estimated by determining the quantities of precipitates formed during heating and correcting the results by adding 6% to allow for the loss of nucleic acid which remained in solution.

RESULTS

Chymotrypsin (*CTR*)

Ultraviolet-irradiated and control solutions of CTR remained apparently unaltered after a few days' storage at 2° at pH 7. Therefore, the relative activities of the solutions, shown in Table 1, after 48 hr. incubation at 2° can be taken as approximately the same as they were at the beginning of the

incubation. In contrast to the behaviour at 2°, incubation at 37° decreased the activities of all the tested solutions, but it decreased the activity of the irradiated solution relatively more than that of the non-irradiated control. The activity of the irradiated solution fell to about 50 % of its initial value, whereas that of the control fell only to 70 %.

It is obvious, therefore, that some CTR which was still active after exposure to UV-radiation was less stable at 37° and at pH 7 than non-irradiated CTR. The difference was not caused by the presence of any inactivating materials produced by the radiation, because the stability of non-irradiated CTR was not affected when it was mixed with an irradiated solution of CTR. This is seen from the fact that the fall in the activity of such a mixture (in which the contribution of the irradiated component was negligible) at 37° was proportional to that of a non-irradiated control solution of CTR. The relative instability of the residual active enzyme in the irradiated solution seems, therefore, to be due to the fact that the molecules were on the average less stable than those in the non-irradiated solution. There are two possible explanations of this. Irradiated molecules of CTR which still remain active might have been altered by the radiation so that they became less stable, or the original preparation might have contained molecules of different stabilities and the radiation might have acted selectively, inactivating the most stable molecules at a higher rate than the least stable ones. Table 2 shows that the course of UV-inactivation of CTR, until the residual activity fell to 4 % of the original, followed approximately that of a first-order reaction. This excludes the possibility of a selective effect of the radiation, because it means that molecules still active at any given moment did not differ from each other in the probability of being inactivated during the next time unit. It can be concluded, therefore, that at least a proportion of

Table 1. *The effect of incubating* UV-*irradiated chymotrypsin for* 48 *hr. at* pH 7

(Materials: 0·2 % CTR in 0·067 M phosphate buffer at pH 7; (A) non-irradiated, (B) irradiated for 32 min., (C) irradiated for 70 min., (D) a sample of (A) diluted 1/25 in (C). The irradiations were done at a distance of 10 cm. from the lamp.)

	Temperature of incubation (°)	Dilution	Amount of casein digested (mg.)	Relative activity per unit weight*
(A) Non-irradiated CTR	37	1/100	8·1	71
		1/200	6·8	
		1/400	5·1	
	2	1/200	7·5	100
(B) Irradiated CTR	37	1/10	5·0	1·65
	2	1/10	6·6	3·3
(D) Non-irradiated, diluted in irradiated CTR	37	1/10	6·1	2·6
	2	1/10	6·7	3·6

* Obtained graphically using a curve constructed from the data given by non-irradiated CTR that had been incubated at 37°.

Table 2. *The rate of inactivation of chymotrypsin by* UV-*radiation*

(0·2 % CTR in 0·067 M phosphate buffer at pH 7 was irradiated for various intervals of time at a distance of 10 cm. from the lamp.)

Time of irradiation (min.)	Dilution	Amount of casein digested (mg.)	Proportion of remaining activity*	k†	Expected proportion of remaining activity if $k=0{\cdot}104$
0	1/100	8·5	1·00	—	—
	1/200	7·1			
	1/400	5·0			
	1/800	3·2			
5	1/100	7·6	0·62	0·096	0·60
10	1/100	5·9	0·34	0·107	0·365
20	1/20	8·0	0·14	0·098	0·126
30	1/10	6·4	0·04	0·115	0·045
			Mean	0·104	

* Obtained graphically using a curve constructed from the data given by non-irradiated CTR.

† Obtained from the equation $p=e^{-kt}$, where p = the proportion of remaining activity and t = time of exposure to the radiation in minutes.

irradiated molecules that were still active were altered by the radiation.

Finkelstein & McLaren (1948) found that UV-inactivation of CTR followed the course of a first-order reaction, at least until the residual activity fell to about 20% of the original, and showed (Finkelstein & McLaren, 1949) that the quantum yield depends on the pH, being just over 4×10^{-3} at pH 7. The quantum yield (Φ) at this pH can be obtained from the value of the constant k of Table 2, for

$$\Phi = kc/Er, \quad (1)$$

where c is the amount of CTR in moles under each sq.cm. of irradiated area, which was $0{\cdot}685 \times 10^{-8}$ (taking the molecular weight of CTR as 41 000, as it was taken by Finkelstein & McLaren (1949)), E is the amount of energy in einsteins falling during each minute on each sq.cm. of surface area of the irradiated solution, which was $3{\cdot}9 \times 10^{-7}$ (3030 μw/sq.cm., the wavelength being 2537 Å), and r is the proportion of incident radiation absorbed by CTR, which was 0·4.

Substituting all these values, the quantum yield $4{\cdot}5 \times 10^{-3}$ is obtained, agreeing with the value obtained by Finkelstein & McLaren (1949).

Tobacco mosaic virus (TMV)

Table 3 shows that UV-irradiation increased the susceptibility of TMV to denaturation by heat, because greater proportions of irradiated preparations were denatured by heating for 30 min. at 69° than of control solutions, and the amount denatured increased with the increased amount of irradiation. All the irradiated solutions remained clear before they were heated.

The change in heat susceptibility did not coincide with the loss of infectivity, because the amount of denatured material formed in the irradiated solution, the residual infectivity of which was 0·0125 that of the original (i.e. when about 99% of the virus was inactivated), was only slightly greater than that which formed in the non-irradiated solution. Only when irradiation was continued after almost all the virus had been inactivated, was the amount of denatured material increased appreciably, and it then continued to increase as the amount of radiation increased. Evidently a change or changes, which increased the susceptibility of virus particles to heat denaturation, occurred after the particles had been made inactive.

The possibility that the increased heat susceptibility occurred because the surrounding medium contained some materials produced by the radiation, was excluded by the fact that the heat denaturation of non-irradiated TMV was unaffected by mixing the latter with an irradiated solution. The amount of coagulum produced by heating the mixture was exactly equal to the sum of the amounts of coagula obtained when each component was heated separately.

Table 3. *Increasing susceptibility of tobacco mosaic virus to heat denaturation by* UV-*radiation*

(TMV was irradiated and heated as a 0·5% solution in 0·067 M phosphate buffer at pH 7.)

Test no.	Time of irradiation (min.)	Distance from lamp (cm.)	Proportion of remaining activity	Proportion of TMV denatured in 30 min. at 69°
1	Non-irradiated control	—	1·0	0·2
2	24	20	0·0125*	0·22
3	15	10	0·0002†	0·32
4	30	10	undetectable	0·38
5	60	10	undetectable	0·57
6	120	10	undetectable	0·76

* Assayed by infectivity test.

† Computed from the result of test no. 2 by assuming that the fall of activity followed the course of a first-order reaction.

There are two possible ways in which the individual particles could have altered. Each may have undergone a single alteration which increased its heat susceptibility to a maximum, or it may have gone through a series of successive alterations resulting in a gradual increase of heat susceptibility. Results of studying the course of heat denaturation showed that the second possibility is more likely.

Lauffer & Price (1940) found that the heat denaturation of TMV follows the course of a first-order reaction. Table 4 shows that the heat denaturation of both an irradiated and a control solution of TMV approximated to such a course, the rate of denaturation of the former being about 2·7 times greater than that of the latter at 69°. The preparation was not irradiated to such an extent that it attained the maximum increase in heat susceptibility (compare Table 3), so that if single particles had reached their maximum heat susceptibility in a single step, the preparation would have contained a mixture of particles, some with unchanged and some with increased heat susceptibility. That the preparation was not a mixture of components with different rates of heat denaturation is shown by the fact that the course of denaturation approximated to that of a first-order reaction.

The changes in UV-irradiated TMV that increase its susceptibility to heat denaturation are not known, but they seem not to be correlated with any major alteration in antigenic structure nor in the shape nor size of particles. Preparations irradiated as 0·5% solutions for 2 hr. at 10 cm. from the lamp reacted with TMV antiserum in a manner similar to that of control TMV, giving flagellar-type precipitation and the same dilution end-point. Although

electron-micrographs showed no changes in the shape and average length of particles, there were differences in the manner in which the particles were distributed. Whereas in control preparations most particles were separate and distributed at random, electron-micrographs of irradiated virus made at the same concentration showed particles almost all aggregated side-by-side to form two-dimensional sheets of various sizes. This suggests that linkages may have formed between neighbouring particles, as is known to occur with some long-chain polymers when subjected to high-energy radiation (Charlesby, 1953). Electron-micrographs of TMV preparations irradiated as 0·5 % solutions for 24 min. at 20 cm. from the lamp showed that only a very small proportion of particles were aggregated. As about 99 % of the original activity of the preparations was destroyed by the radiation (see Table 3), this shows that the change leading to aggregation was not correlated with the loss of activity.

The quantum yield for inactivation of TMV by UV-radiation cannot be obtained as unequivocally as for CTR, because there is no satisfactory method for dealing with the scatter of radiation by the virus particles. Oster & McLaren (1950) computed the proportion of radiation that should be absorbed by the individual constituents of the virus particles and considered that the excess of optical activity was due to scattering. They obtained $4{\cdot}3 \times 10^{-5}$ as the quantum yield.

Inactivation of TMV by UV-radiation follows approximately the course of a first-order reaction (Oster & McLaren, 1950; Bawden & Kleczkowski, 1953). The quantum yield could, therefore, be computed according to equation (1) if all the values to be substituted into it were known. The value of k can be obtained from the result of test no. 2, Table 3, and equals 0·182 (for $0{\cdot}0125 = e^{-24k}$). E equals 11×10^{-8} einsteins. The amount of TMV, c, under each sq.cm.

Table 4. *The effect of* UV-*irradiation on the rate of heat denaturation of tobacco mosaic virus*

(A 0·75 % solution of TMV in 0·067 M phosphate buffer at pH 7 was irradiated for 1 hr. at a distance of 10 cm. from the lamp and then heated at a temperature of 69°.)

Time of heating (min.)	Proportions remaining in solution: of non-irradiated TMV, Found	of non-irradiated TMV, Computed assuming $k = 0{\cdot}0185$*	of irradiated TMV, Found	of irradiated TMV, Computed assuming $k = 0{\cdot}0495$*
5	—	—	0·76	0·78
10	0·82	0·83	0·61	0·61
20	0·73	0·69	0·39	0·37
40	0·48	0·48	—	—

* From $p = e^{-kt}$, where p is the proportion remaining in solution and t is the time of heating in minutes.

of irradiated surface can be taken as $1{\cdot}75 \times 10^{-11}$ moles (if the average 'molecular weight' of the virus be taken as 4×10^7, as was done by Oster & McLaren). The value of r cannot be settled satisfactorily. Less than 1 % of the incident radiation was transmitted, so that nearly all the radiation was absorbed or scattered. If it is assumed that not less than half of the radiation was absorbed, the value of r will be between 0·5 and 1, and the value of the quantum yield will then be between 3×10^{-5} and 6×10^{-5}.

DISCUSSION

McLaren & Finkelstein (1950) found that the quantum yield for inactivation was higher when a solution of CTR was first irradiated at 3·5°, and then warmed for 2 hr. at 36° and tested at 36°, than when it was irradiated and tested at 3·5°. They concluded that: 'active intermediates exist at low temperature which are inactivated on warming of the solutions irradiated at low temperature'. Decreased stability of some irradiated molecules of the enzyme that still remained active is the cause of this phenomenon, just as it is of increased rate of inactivation of residual activity of CTR solutions irradiated at 20° and then kept at 37° at pH 7, as found in the present work. McLaren & Finkelstein did not consider the possibility of some materials produced by the radiation in the medium inactivating unchanged enzyme molecules, but this was excluded experimentally in this work, and so there is no reason to suspect it to be the cause of the phenomenon described by them.

Bovie (1913) noticed that UV-irradiated egg-white coagulated much more readily on heating than did non-irradiated egg-white, and Stedman & Mendel (1926) observed the same phenomenon with several different proteins of animal and plant origin, although it has also been found that some proteins can, by radiation, be 'desensitized' to heat (Clark, 1925). Clark (1936) distinguishes three stages leading to the heat coagulation of irradiated egg albumin: (1) denaturation by UV-radiation, (2) a reaction of such denatured molecules with water when subsequently heated; this may be similar to usual heat denaturation but occurs at a lower temperature, and (3) flocculation of molecules that passed through the first two stages. However, in the absence of a rigid definition of the word 'denaturation', it seems merely a matter of convenience to which of the two stages it should be applied. The example of UV-irradiated TMV shows the difficulty in deciding when denaturation occurs. The first detectable alteration, or alterations, caused loss of infectivity, which was followed by a series of alterations each contributing to an increase in the rate of another alteration, or alterations, that occurred on subsequent exposure to heat. In the present paper

the alteration occurring during heating is referred to as 'heat denaturation' irrespective of whether the heated preparation was previously irradiated or not.

The ability of UV-radiation to decrease the stability of some proteins, particularly of some enzymes, may be responsible for the sensitization to heat of some living organisms by exposure to sublethal doses of the radiation, such as has been observed with *Paramecium* (Bovie & Klein, 1919; Giese & Crossman, 1946), bacteria (Curran & Evans, 1938) and yeast (Anderson & Duggar, 1939). The same phenomenon was also described with X-ray irradiated chick embryos (Strangeways & Fell, 1928). In contrast to isolated proteins, living organisms can recover from the effect of irradiation if sufficient time is allowed between exposure to the radiation and to heat.

SUMMARY

1. When proteins having specific activities, such as enzymes or viruses, are exposed to ultraviolet radiation, the specific activity need not to be lost as a result of the first of a series of alterations. Chymotrypsin molecules can be altered by the radiation so that their stability at temperatures around 37° at pH 7 is decreased although they are still proteolytically active. The increased rate at which tobacco mosaic virus is denatured on heating after irradiation results from a series of changes following a step which is associated with the loss of specific activity (infectivity).

2. Quantum yields for inactivation of chymotrypsin and tobacco mosaic virus computed from the data obtained in this work agree with those obtained by previous workers.

REFERENCES

Anderson, T. F. & Duggar, B. M. (1939). *Science*, **90**, 358.
Bawden, F. C. & Kleczkowski, A. (1953). *J. gen. Microbiol.* **8**, 145.
Bawden, F. C. & Pirie, N. W. (1943). *Biochem. J.* **37**, 66.
Bovie, W. T. (1913). *Science*, **37**, 373.
Bovie, W. T. & Klein, A. (1919). *J. gen. Physiol.* **1**, 331.
Charlesby, A. (1953). *Nature, Lond.*, **171**, 167.
Clark, J. H. (1925). *Amer. J. Physiol.* **73**, 649.
Clark, J. H. (1936). *J. gen. Physiol.* **19**, 190.
Curran, H. R. & Evans, F. R. (1938). *J. Bact.* **36**, 455.
Finkelstein, P. & McLaren, A. D. (1948). *J. Polym. Sci.* **3**, 223.
Finkelstein, P. & McLaren, A. D. (1949). *J. Polym. Sci.* **4**, 573.
Giese, A. C. & Crossman, E. B. (1946). *J. gen. Physiol.* **29**, 79.
Kleczkowski, J. & Kleczkowski, A. (1953). *J. gen. Microbiol.* **8**, 135.
Lauffer, M. A. & Price, W. C. (1940). *J. biol. Chem.* **133**, 1.
McLaren, A. D. (1951). *Arch. Biochem. Biophys.* **31**, 72.
McLaren, A. D. & Finkelstein, P. (1950). *J. Amer. chem. Soc.* **42**, 5423.
Northrop, J. H., Kunitz, M. & Herriot, R. M. (1948). *Crystalline Enzymes*. New York: Columbia University Press.
Oster, G. & McLaren, A. D. (1950). *J. gen. Physiol.* **33**, 215.
Stedman, H. L. & Mendel, L. B. (1926). *Amer. J. Physiol.* **77**, 199.
Strangeways, T. S. P. & Fell, H. B. (1928). *Proc. Roy. Soc.* B, **102**, 9.

The Purine and Pyrimidine Composition of some Deoxyribonucleic Acids from Tumours

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Since Vischer and Chargaff elaborated procedures for the chromatographic separation and analysis of the purines and pyrimidines, which require only milligram quantities of nucleic acid (e.g. Vischer & Chargaff, 1948; Chargaff, Vischer, Doniger, Green & Misani, 1949), a fairly large number of specimens of deoxyribonucleic acid from a variety of sources have been analysed in different laboratories. Such studies are associated chiefly with the American workers Chargaff and his collaborators (Chargaff, 1950, 1951), with Mirsky and his colleagues (cf. Daly, Allfrey & Mirsky, 1950), and with Marshak (cf. Marshak & Vogel, 1950; Marshak, 1951). In this country Markham & Smith (1949) and Wyatt (1951*b*) have also made valuable contributions to technique and analytical results.

Most of the figures show variations in the purine and pyrimidine contents of the deoxyribonucleic acid specimens from different biological sources, and Chargaff (1950) concluded that his evidence indicates that, while the deoxypentose nucleic acids (DNA) extracted from cells of different organs of one species are very similar in composition, significant differences occur in the DNA from different