THE INACTIVATION OF TRYPSIN BY X-RAYS.

By HARRY CLARK and JOHN H. NORTHROP.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

The inactivation of trypsin by the rays from the active deposit of radium emanation has been studied by Hussey and Thompson.[1] They find that in dilute solution trypsin is inactivated at a rate which is proportional to the intensity of the radiation and to the concentration of the solution; that for constant intensity the reaction would follow the simple exponential law which indicates a monomolecular reaction. In concentrated solution, it is not inactivated. They state also that a 2 hour exposure to hard, filtered x-rays produces no effect although soft, unfiltered rays produce considerable inactivation.

Although physical theory indicates that radiation produces changes in matter through the medium of ionization, it has not been shown conclusively that various apparently characteristic effects, produced by various kinds of rays, result from corresponding characteristic intensities and distributions of ionization. For this reason it has seemed desirable to study the inactivation of trypsin under soft x-ray.

The experiments to be described were made on solutions of Fairchild's trypsin powder. These solutions, of various degrees of trypsin concentration, were made up in .05 M, pH 5.4 acetate buffer. The results are in good accord with those of Hussey and Thompson.

Method and Apparatus.

The x-ray outfit has been described.[2] A broad-focus Coolidge tube is operated on 60 cycle current rectified by kenetrons; it is enclosed in a lead-lined cabinet and kept cool by a fan. This outfit is capable of continuous operation without a variation of voltage or current exceed-

[1] Hussey, R. G., and Thompson, W. R., *J. Gen. Physiol.*, 1922–23, v, 647.

[2] Clark, H., *Am. J. Roentgenol. and Radium Therap.*, 1924, xi, 445.

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ing 2 per cent. In this work it was run at 30 kv. (peak) and 22 milliamperes, the mean target distance being 14.7 cm.

It proved rather difficult to provide conditions suitable for exposing the trypsin. Even with the solution close to the tube, exposures of several hours duration were required to produce any considerable effect; consequently the spontaneous inactivation was also considerable, even at a temperature of about 0°C. To keep the solution at this temperature necessitated efficient cooling apparatus and careful shielding from the heat of the tube.

A strip of sheet lead was set into a shallow round tray of enamelled iron, 10 cm. in diameter, so as to divide it into two equal compartments, after which the tray was dipped in hot paraffine to coat the surface and to make the compartments water-tight. The control solution was put in one side and the solution to be radiated in the other. A sheet of lead was placed over the control to shield it from the x-ray, and to ensure perfect shielding it was allowed to overlap the radiated solution slightly, thus casting on it a shadow about 5 sq. cm. in area. The cross-section of one-half of the tray being 39.2 sq. cm., only 87 per cent of the radiated solution was actually exposed. This factor will have to be considered later.

The tray was supported in a small water bath through which a strong current of ice water was maintained by means of a motor-driven gear pump. To shield the solutions from the heat of the tube and to prevent evaporation, a metal frame, carrying four sheets of thin paper parallel and about .5 cm. apart, was so placed over the tray as to close it to air currents. The fan which served to cool the tube maintained a current of air between the sheets of paper. With this arrangement the solutions were kept continuously at a temperature of about .5°C. 15 cc. of solution were used in each run, the depth being .382 cm.[3]

[3] In the first experiments, two of which are here used, the whole tray was used for the solution to be radiated, the control being kept on ice elsewhere. The conditions of radiation were, therefore, different from those described; the depth was slightly less, the distribution of radiation slightly different, and the difference in temperature between the two solutions may have been as great as .5°C. These runs are certainly comparable with the others to a good degree of approximation. No calculations are based on them, however; they are used in this paper only to show the general magnitude of the effect.

At proper times during each run, the solutions were stirred and 1.5 cc. samples were taken. Whenever a sample of radiated solution was taken, an equal volume of control solution was added to keep the depth constant. This process was compensated by calculation; since few samples were taken, the correction was negligible in some cases and small in the others. The trypsin content of the samples was measured by Northrop and Hussey's viscosity method.[4] This method involves sufficient dilution to insure that all of the enzyme exists in the active state. The determinations are independent, therefore, of the state in which it existed in the original sample.

RESULTS.

The results of five runs are shown in Fig. 1 in which logarithms of trypsin content per cc., in arbitrary units, are plotted against the length of exposure in hours. The circles refer to the control solution (spontaneous decay) and the dots to the exposed solution (x-ray plus spontaneous decay). Each point is the mean of two determinations. Nos. 1, 2, and 4 were ordinary solutions. No. 3 was almost wholly inactivated by heat before use, and No. 5 was dialyzed before use. Nos. 2 and 3 are the ones referred to in Foot-note 3.

The percentage concentrations shown in figures on the chart for the various solutions refer to the dry weight of trypsin powder used in making them. The dry material contains an unknown and somewhat variable amount of foreign material, and the enzyme itself suffers spontaneous inactivation both in the dry state and in solution; in consequence, the figures given do not correspond accurately to the initial values plotted.

Quantitative work with enzymes entails usually an uncertainty of several per cent. With the exception of a few points, the various straight lines represent the courses of the various runs within the expected limits of error. Although straight lines may not represent the law of inactivation accurately, they furnish the simplest basis for discussion.

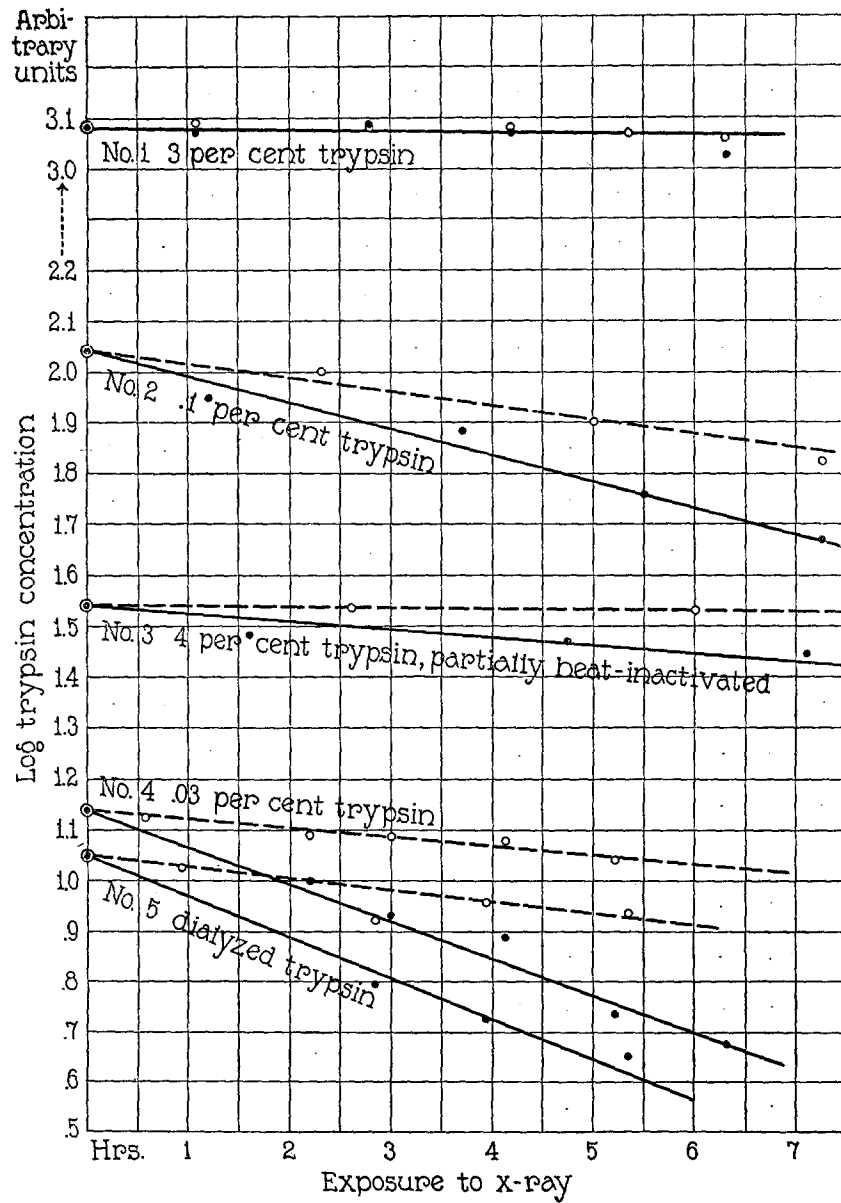[4] Northrop, J. H., and Hussey, R. G., *J. Gen. Physiol.*, 1922-23, v, 353.

FIG. 1. Inactivation of trypsin solutions by x-rays.

DISCUSSION.

Theory of Enzyme Solutions.—Northrop has shown[5] that, in general, in solutions of trypsin containing foreign material of certain sorts the trypsin exists partly in the combined or inactive state, and partly in the free or active state. The reaction is reversible and obeys the law of mass action. Therefore, the point of equilibrium depends both on the concentration of trypsin and on that of the foreign matter. In concentrated solution most of the enzyme is combined; in dilute solution it is mostly active. The rate of spontaneous inactivation of combined trypsin is negligible compared to that of free trypsin.

X-Ray Inactivation and the State of the Enzyme.—The chart shows that the inactivation by x-ray and the spontaneous inactivation run parallel within reasonable limits; both are slight in Nos. 1 and 3, and great in Nos. 4 and 5. No. 2 lies between the extremes. If No. 3 be left out of account, the effects are seen to increase with the dilution over the range studied. All of the solutions were made from the same stock of trypsin powder. With the exception of Nos. 3 and 5, they represent, therefore, about the same ratio of enzyme to foreign matter. The ratio of free to combined trypsin increases consequently with the dilution. Before use, No. 3 was almost entirely inactivated by heat, which presumably did not alter the amount or the condition of the impurities to any great extent. Although trypsin is present in this solution in very small amount, it exists almost entirely in the combined state; which accounts for the stability under both x-ray and heat. Solution 5 was dialyzed several times before use to remove as much of the impurity as possible. The chart discloses nothing peculiar about the behavior of this solution; apparently the amount of impurity in the stock was not sufficient to hold any appreciable part of the trypsin in combination at very low concentration, so dialysis was unnecessary. On the basis of all of these experiments, and of Northrop's theory,[5] we conclude that x-ray affects predominantly free trypsin.

On the more concentrated solutions, the effects are too small to throw any light on the law of inactivation. Straight lines fit Experiments 4 and 5 fairly well, though the latter is represented by too few

[5] Northrop, J. H., *J. Gen. Physiol.*, 1921–22, iv, 261.

points to be of much value.   The exponential law and a monomolecular law are indicated.   Both of these conclusions are in accord with those of Hussey and Thompson.[1]

Ionization Theory.—It is quite generally accepted on the basis of physical theory that x-rays effect primary changes in matter only through the ionization produced by the emission of electrons with high speed; *i.e.*, beta rays.   Gamma rays are, of course, merely hard x-rays. On this view, x-rays of all wave-lengths and the beta and gamma rays of radioactive substances should produce the same changes if their intensities were so adjusted as to produce equal rates of ionization per unit volume.

The ionization of liquids and solids by radiation cannot be measured directly, but it has been shown[6] that substances composed only of light elements, water, air, organic substances, in whatever state, are ionized in proportion to their density.   From measurements made in air we can, therefore, estimate the ionization of the enzyme solution.

Hussey and Thompson used radium emanation enclosed in glass tubes thick enough to stop all of the alpha particles.   That they were not so thick as to interfere appreciably with the passage of beta particles is indicated by the fact that several different tubes gave the same results.   The slight interference may be considered as cancelling the almost negligible effect of the gamma rays.   The trypsin solution surrounded the tube in a layer sufficiently thick to stop practically all of the beta rays.   As a first approximation then it may be assumed that the ionization of the solution resulted from total absorption of the beta rays from Radium B and C.

Moseley and Robinson[7] state that the beta rays from B and C in equilibrium with 1 gm. of radium produce 9×10^{14} pairs of ions per second in air, if completely absorbed.   To reduce the time unit from seconds to hours, and the curies to millicuries, we multiply this number by 3,600 and divide by 1,000.   Thus, one millicurie-hour represents 3.24×10^{15} pairs of ions.   2.95 cc. of solution were used; the number of pairs of ions per cc. per millicurie-hour is, therefore, 1.10 $\times 10^{15}$.   From the curves given by Hussey and Thompson, it may be seen that 240 millicurie-hours reduced the activity of the trypsin

[6] Crowther, J. A., *Proc. Roy. Soc. London, Series A*, 1907–08, lxxx, 186.

[7] Rutherford, E., Radioactive substances and their radiations, 1913, 230.

to half value ($\log_{10} 2 = .301$). Multiplying $1.10 \times 10^{15} \times 240$, we find the corresponding number of pairs of ions per cc. to be 2.63×10^{17}.

Leaving this result for the moment, let us estimate the total ionization per cc. required to reduce the trypsin to half value by the x-rays in our experiments. The rate of ionization in air was measured by apparatus previously described.[2] At a target distance of 27 cm. but with all other conditions as in the work with the enzyme, 5.42×10^{12} pairs of ions were produced per second per gm. of air. The possible influence of the metal tray on the enzyme was investigated by taking readings with and without the empty tray directly under the ionization chamber and no measurable effect was found. The effect of scattering, with these very soft rays and under the conditions of the work, is negligible. The proof of this is given in the report of certain other experiments[8] where the conditions of measurement were the same as in the present case except that a different tube was used and also a filter of thin bristol board practically identical in stopping power with the four sheets of paper used in the present work. In fact the actual ionization value used in that work differs from the one here used by only about 10 per cent, that being the difference in efficiency between the old tube and the new one.

The ionization per cc. of enzyme solution per hour may be taken as equal to that in a gm. of air in the same time and under the same circumstances. To estimate it, we must interpolate the value at 27 cm. to the actual target distance, 14.7 cm., by the inverse square law and multiply by two correction factors. The first of these factors, .87, has already been computed; it accounts for the shadow cast on the radiated solution by the lead screen. The second factor, .89, takes account of the falling off of the ionizing power of the rays in traversing the solution. This was studied with the ionization chamber and a sheet of wax of the same weight per cm.[2] as the solution. The absorption by the wax amounted to 22 per cent, and the factor, .89, represents, therefore, the average relative intensity throughout the solution. Since the chamber was stationary, the use of this factor is consistent with the use also of the mean target distance. Multiplying 5.42×10^{12} by the square of ($27 \div 14.7$), by .87, by .89, and by 3,600 (seconds to hours), we get 5.09×10^{16} for the number of pairs of ions produced

[8] Clark, H., and Sturm, E., *J. Exp. Med.*, 1924, xl, 517.

per hour in 1 cc. of solution. Experiment 4 (see Fig. 1) represents the concentration nearest that used by Hussey and Thompson (.0375 per cent). The difference between the ordinates of the two curves of Experiment 4 is .301 or $\log_{10} 2$ at $5\frac{1}{2}$ hours, which is, therefore, the time required for half-inactivation by x-ray alone. This corresponds to $5\frac{1}{2} \times 5.09 \times 10^{16}$ or 2.80×10^{17} pairs of ions per cc.

The ratio of this value to the one estimated above for the work with radium emanation is 1.065. The agreement is better than could have been expected. If not fortuitous, it substantiates the hypothesis already stated, that radiations of various sorts do not produce characteristic effects—only ionization. It indicates also that the trypsin in the commercial preparation is a single substance rather than a mixture of various substances; otherwise the two different stocks considered in the estimate could scarcely have given comparable results.

Nature of the Process of Inactivation.—Under ionization of random distribution, the probability, P, that at least one free electron will appear in (or disappear from) a certain small portion of the solution, within the time interval dt, is given by

$$P = n\,v\,dt$$

where n is the number of electrons (*i.e.* the number of pairs of ions) set free in 1 cc. of solution in 1 second; and v is the volume of the portion considered. If n is large, and if we consider a large number, N, of portions, all of the same volume, v, then P becomes the actual fraction of them in which at least one free electron appears in time dt; that is,

$$P = -\frac{dN}{N} = n\,v\,dt,$$

$$-\log_e N = n\,v\,dt + k,$$

and, since $N = N_0$, when $t = 0$,

$$-\log_e N_0 = k,$$

and

$$\log_e \frac{N_0}{N} = n\,v\,t.$$

This is the simple exponential law previously referred to. No theory of multiple or successive ionizations would yield this simple relation. For "inactivation" to half-value $\dfrac{N_0}{N} = 2$ and $v = \dfrac{.692}{nt}$.

Setting nt equal to 2.80 $\times$ 10^{17}, the number of pairs of ions per cc. corresponding to half-inactivation of free trypsin as found in our experiments, we find that v = 2.47 $\times$ 10^{-18} cc. This is 5,000 times the volume of the molecule of oleic acid[9] or 60 times that of the molecule of egg albumin.[10] If combined trypsin is inactivated at all, the rate, compared to that of free trypsin, is very small. According to the theory given above, v must be correspondingly small. It need not be absurdly small, however; if it were equal to the volume of the molecule of oleic acid, nearly 3 years would be required for half-inactivation.

Two hypotheses may now be considered—that inactivation results (1) from the loss of an electron and (2) from the gain of an electron. The theory given above holds good in either case. That free trypsin is believed to exist as a positive monovalent ion[11] may help us to make a choice. There is no reason for supposing that the actual volume of the trypsin radical is different in the free and combined states. The probability that a beta particle will knock an electron out of this radical should then be independent of state, and consequently, on the first hypothesis, free and combined trypsin should be inactivated at the same rate. On the second hypothesis (inactivation by gaining an electron), the probability may not be independent of state. By virtue of its positive charge, the trypsin radical in the free state is doubtless able to capture a free electron from a considerable distance, whereas positive ions appearing in the neighborhood would be repelled. v would then be the volume of the sphere of influence of the ion. The negligible inactivation of combined trypsin may mean that in this condition the radical is neutralized electrically; that it makes, therefore, no attempt to appropriate free electrons which appear in or near it.

This second hypothesis, together with the assumption that trypsin in combination is electrically neutral, explains the facts qualitatively. Inactivation of free trypsin appears, therefore, to result from electrical neutralization of the ion by the addition of one electron.

[9] Langmuir, I., *J. Am. Chem. Soc.*, 1917, xxxix, 1848.

[10] du Noüy, P. L., *J. Biol. Chem.*, 1925, lxiv, 595.

[11] Northrop, J. H., *J. Gen. Physiol.*, 1923–24, vi, 337.

SUMMARY.

1. The inactivating effect of soft x-rays on trypsin in solutions of various degrees of concentration has been studied.

2. It has been found to run parallel with spontaneous heat inactivation. It is almost, if not entirely, confined to the free or active trypsin.

3. Under radiation of constant intensity, the inactivation follows the simple exponential law which indicates a monomolecular reaction.

4. Estimates have been made of the amount of ionization required to inactivate trypsin to half value in these experiments and in those of Hussey and Thompson, who employed the beta rays from Radium B and C. The close agreement corroborates the idea that the effect is a function of ionization only.

5. The nature of the process of inactivation is discussed; inactivation seems to result from electrical neutralization of the trypsin ion.