phenyl phosphate and $1.3 \cdot 10^{-3}$ M for di-*p*-nitrophenyl phosphate under the usual assay conditions. However the monoesterase activity was the more strongly inhibited by inorganic phosphate, and was the only one of the two enzymes to be inhibited by monomethyl phosphate. K_i for inorganic phosphate was approx. $3 \cdot 10^{-4}$ M for the monoesterase and $5 \cdot 10^{-3}$ M for the diesterase. The initial rate of breakdown of *p*-nitrophenyl phosphate was strongly reduced by the presence of high concentrations of monomethyl phosphate as expected for an alternate substrate. The rate of breakdown of di-*p*-nitrophenyl phosphate was unaffected. The kinetic parameters for the two enzymes are such that initial rate measurements for diesterase were not appreciably affected by monoesterase action on the product.

Since the discovery of repression of alkaline phosphatase in *E. coli*[6,7], repression of enzymes by inorganic metabolites has been detected in several microorganisms; sulfate and phosphate have been found to repress hydrolases for their respective monoesters in *A. aerogenes*[8,9]. Coordinate repression by complex organic end-products has been recognized in several metabolic pathways[10]. In the present system an inorganic metabolite appears to produce coordinate repression of enzymes involved in its own production.

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Frick Chemical Laboratory,
Princeton University,
Princeton, N.J. (U.S.A.)

RICHARD WOLFENDEN
GEORGE SPENCE

1 W. A. DRUSHEL AND A. R. FELTY, *Am. J. Sci.*, 43 (1917) 57.
2 O. BAILLY, *Bull. Soc. Chim.*, 25 (1919) 240.
3 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
4 A. GAREN AND C. LEVINTHAL, *Biochim. Biophys. Acta*, 38 (1960) 470.
5 M. MALAMY AND B. L. HORECKER, *Biochem. Biophys. Res. Commun.*, 5 (1961) 104.
6 A. TORRIANI, *Biochim. Biophys. Acta*, 38 (1960) 460.
7 T. HORIUCHI, S. HORIUCHI AND D. MIZUNO, *Nature*, 183 (1959) 1529.
8 D. H. RAMMLER, C. GRADO AND L. R. FOWLER, *Biochemistry*, 3 (1964) 324.
9 F. M. HAROLD, *J. Gen. Microbiol.*, 35 (1964) 81.
10 B. N. AMES AND R. G. MARTIN, *Ann. Rev. Biochem.*, 33 (1964) 235.

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Identification of the water radicals involved in X-ray inactivation of enzymes in solution and determination of their rate of interaction with the enzyme

The inactivation of enzymes irradiated in dilute aqueous solution is due to the action of water radicals ($OH^{\cdot}$, e^-_{aq}, O_2^- *etc.*). It is the purpose of the present paper to demonstrate that in cases where the enzyme inactivation is primarily due to one type of water radical, it is possible from competition experiments to identify this

radical. It will also be shown that with the aid of absolute rate constants now available[1] for the interaction of water radicals with organic compounds it is possible to determine the absolute rate constant for the interaction of the responsible water radical with the enzyme.

In the kinetic treatment it is assumed that the enzyme inactivation is caused primarily by one species of water radicals denoted X. X will disappear in reactions with the enzyme, E, with added protective compounds, P, and with buffer components and impurities, B. The rate constants for these reactions are denoted k_E, k_P and k_B, respectively. The enzyme inactivation by X(Eqn. 1) occurs with a rate constant k_I.

$$E + X \xrightarrow{k_I} E_{\text{inact.}} \tag{1}$$

It is assumed that X will react with active and with inactivated enzyme molecules at the same rate. This will generally be the case when the yield of inactivation is low[2]. Under such conditions the enzyme inactivation will be an exponential function of the dose, and it can be shown[2-5] that the dose reducing the enzyme activity to 37% of the initial activity, D_{37}, is given by the expression:

$$D_{37} = \frac{k_E}{k_I} E_0 + \frac{k_P}{k_I} P_0 + \frac{k_B}{k_I} B_0 \tag{2}$$

where E_0, P_0 and B_0 denote the initial concentrations. From this it follows that

$$D_{37P} - D_{37C} = \frac{1}{k_I} \cdot k_P P_0 \tag{3}$$

where D_{37P} and D_{37C} are the D_{37} doses observed in the presence and absence of protector, respectively. If experiments are carried out in which a constant concentration of enzyme is irradiated in the presence of different protective substances, a straight line will be obtained when $D_{37P} - D_{37C}$ is plotted *versus* the product $k_P P_0$ (see Eqn. 3). In contrast if similar plots are made for water radicals which play little or no role in the enzyme inactivation, or if two or more radicals are involved in the enzyme inactivation, a straight line will not be obtained, and the points will be widely scattered. This follows from the fact that when different protective compounds are used, the relative rates with which they react with the various types of water radicals usually differ greatly.

When the active radical has been identified, its rate of interaction with the the enzyme can be determined. It follows from Eqn. 2 that if the D_{37} dose is plotted *versus* E in experiments where P and B are kept constant, a straight line will be obtained with a slope of k_E/k_I. Consequently, if k_I has been determined with the aid of Eqn. 3, k_E can readily be obtained. It should be pointed out that the procedure is valid only if the protective substances act exclusively by a radical scavenger mechanism.

The procedure outlined will be demonstrated with trypsin as an example. Under the conditions used trypsin was inactivated as an exponential function of the dose. When the D_{37} doses were plotted against enzyme concentration, a straight line was found (Fig. 1A), as predicted by Eqn. 2. Evidence was obtained that the protective substances acted predominantly by a radical scavenger mechanism. This follows from the fact that a straight line was obtained when the D_{37} doses were

plotted against increasing protector concentrations at constant enzyme concentration (see Eqn. 2). A typical example is demonstrated in Fig. 1B.

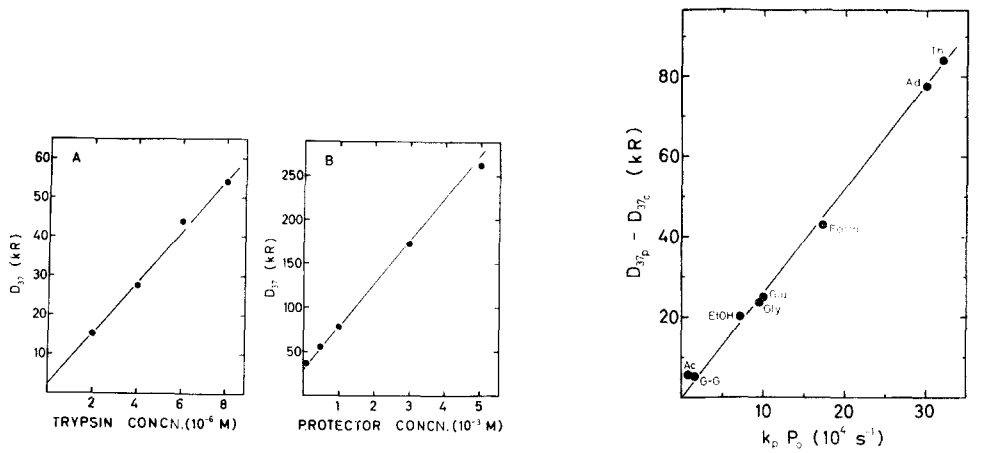

Fig. 1. X-ray inactivation of trypsin in solution. Crystalline trypsin (Sigma, Cleveland, Ohio) was irradiated in 0.01 M phosphate buffer, pH 6.0, in erlenmeyer flasks at 4°, in the presence of air. The solution was irradiated with an X-ray therapy set with factors 220 kV, 20 mA, using 0.5 mm of Cu filtration. The dosimetry was carried out with the Fricke dosimeter. The enzyme activity was assayed by measuring the splitting of benzoyl arginine ethyl ester at 253 mμ, according to SCHWERT AND TAKENAKA[8]. A molecular weight of 23 800 was used for trypsin[9]. The D_{37} doses were determined from dose–inactivation curves. A. D_{37} as a function of enzyme concentration. B. D_{37} as function of increasing glycylglycine concentration.

Fig. 2. Protection of trypsin by various substances. The D_{37} doses in the presence of $1.0 \cdot 10^{-4}$ M of the various protective substances were determined from plots as in Fig. 1B. The enzyme concentration was $0.4 \cdot 10^{-5}$ M. Otherwise conditions as in Fig. 1. Ac, acetone[10]; G-G, glycylglycine[11]; Gly, glycerol[11]; Glu, glucose[12]; EtOH, ethanol[10]; Form, sodium formate[10,13]; Ad, adenine[14]; Th, thymine[14] (the references give the sources for the rate constant k_P, for the interaction of OH· radicals with the respective substances).

In Fig. 2 the results obtained with different protective substances are plotted according to Eqn. 3. It is seen that a straight line was obtained when the relevant rate constants for the OH· radical were used. No similar plot could be made for the O_2^- radical, which was formed in similar quantities as the OH· radical, as rate constants for the interaction of O_2^- with the protective substances used are not available. The results in Fig. 2 strongly indicate that the OH· radical is the one mainly responsible for the inactivation of trypsin under the present conditions. If O_2^- radicals were active the points in Fig. 2 would inevitably have been far more scattered. Furthermore under the present conditions the yield of inactivation is approximately the same in oxygen and in nitrogen[6].

From the slope of the line in Fig. 2 the rate constant for the inactivation of the enzyme by OH· radicals, k_I, was found to be $1.8 \cdot 10^9$ $M^{-1} \cdot sec^{-1}$. This value was obtained by using the value $G_{OH} = 2.25$[7]. From the slope of the line in Fig. 1A, k_E/k_1 was found to be 14, from which k_E was calculated to be $2.5 \cdot 10^{10}$ $M^{-1} \cdot sec^{-1}$.

The procedure here outlined is applicable to the study of enzymes in general and also to other systems where it is desired to identify the water radicals primarily responsible for a certain type of radiation damage.

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Norsk Hydro's Institute for Cancer Research,
The Norwegian Radium Hospital,
Montebello, Oslo (Norway)

TORE SANNER
ALEXANDER PIHL

1 M. EBERT, J. P. KEENE, A. J. SWALLOW AND J. H. BAXENDALE, *Pulse Radiolysis*, Academic Press, New York, 1965.
2 T. SANNER AND A. PIHL, *Radiation Res.*, 19 (1963) 12.
3 F. HUTCHINSON AND D. A. ROSS, *Radiation Res.*, 10 (1959) 477.
4 J. A. V. BUTLER, A. B. ROBINS AND J. ROTBLAT, *Proc. Roy. Soc. London, Ser. A.*, 256 (1960) 1.
5 T. SANNER AND A. PIHL, *European J. of Biochem.*, 1 (1967) 33.
6 T. SANNER, *Radiation Res.*, 26 (1965) 95.
7 A. O. ALLEN, in M. HAISSINSKY, *Actions Chimiques et Biologiques des Radiations*, Vol. 5, Academic Press, New York, 1961, p. 11.
8 G. W. SCHWERT AND Y. TAKENAKA, *Biochim. Biophys. Acta*, 16 (1955) 570.
9 P. DESNUELLE, in P. D. BOYER, H. LARDY AND K. MYRBÄCK, *The Enzymes*, Vol. 4, Academic Press, New York, 1960, p. 119.
10 J. K. THOMAS, *Trans. Faraday Soc.*, 61 (1965) 702.
11 G. E. ADAMS, J. W. BOAG, J. CURRANT AND B. D. MICHAEL, p. 131 in ref. 1.
12 J. V. DAVIS, W. GRIFFITHS AND G. O. PHILLIPS, p. 181 in ref. 1.
13 E. J. HART, J. K. THOMAS AND S. GORDEN, *Radiation Res., Suppl. 4*, (1964) 74.
14 G. SCHOLES, P. SHAW, R. L. WILLSON AND M. EBERT, p. 151 in ref. 1.

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Fructose 1,6-diphosphate and 3',5'-cyclic AMP as positive effectors of pyruvate kinase in developing embryos

In the case of feed-back control, the end product inhibits the activity of the first enzyme of a biosynthetic chain and consequently blocks the enzymatic processes leading to its own synthesis. The investigations of YATES AND PARDEE[1] have established the inhibition of aspartate carbamoyltransferase (EC 2.1.3.2) by CTP in the chain of CTP biosynthesis. The inhibition of L-threonine dehydratase (EC 4.2.1.16) by L-isoleucine has been established by UMBARGER AND CHANGEUX[2–4] in the chain of isoleucine biosynthesis. The inhibitory action of ATP and citrate on the activity of phosphofructokinase (EC 2.7.1.11) has been suggested for the mechanism of Pasteur effect[5–8].

In contrast to feed-back control, the "feed-forward" control suggests the activation of the end reaction in a metabolic chain by the product of the first reaction in that metabolic chain. HESS, HAECKEL AND BRAND[9] were the first to report the possibility of this type of regulation in the control of glycolysis in yeast.

In this report, results are presented on the activation of pyruvate kinase (EC 2.7.1.40) by Fru-1,6-P_2 ("feed-forward" control) in developing loach *(Mis-*