iodoacetic acid inhibited the vitamin A esterase and cholesterol esterase, but not the common esterase.

4. The vitamin A esterase of rat liver was more labile towards aging and heat, and the other two esterases were more stable. Acetone-drying at 0–5° preferentially destroyed vitamin A esterase and cholesterol esterase, but not the common esterase.

5. The vitamin A esterase of nuclear and microsomal fractions of chicken liver had similar pH optima at pH 6.6 and 8.6, with maximal activity at pH 8.6, and their K_m values were 0.19 and 0.3 mM respectively. The cholesterol esterase of both sources had the same pH optimum at pH 8.2, and their K_m values were 0.28 and 0.15 mM respectively.

6. The three esterase activities of the nuclear, but not of the microsomal, preparation from chicken liver were solubilized by Lissapol-N; the detergent activated the nuclear cholesterol esterase, but not the vitamin A esterase.

7. Ammonium sulphate fractionation of the Lissapol extract gave 66.6% of the original vitamin A-esterase activity in the fraction between 28.5 and 37.5% saturation, whereas 60% of the common esterase was recovered from the fraction above 50% saturation. No single fraction gave the cholesterol-esterase activity, but on recombination of all fractions 72% of the original activity was recovered.

8. On electrophoresis of a Lissapol extract of chicken-liver nuclei on agar gel vitamin A esterase and cholesterol esterase could be separated.

9. It is suggested that the three activities are due to distinct enzymes.

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REFERENCES

- Aldridge, W. N. (1953). *Biochem. J.* **54**, 442.
 Aldridge, W. N. (1954). *Biochem. J.* **57**, 692.
 Arrhenius, S. (1889). *Z. phys. Chem.* **4**, 226.
 Augustinsson, K. B. (1958). *Nature, Lond.*, **181**, 1786.
 Borgstrom, B. (1954). *Biochim. biophys. Acta*, **13**, 149.
 Byron, J. E., Wood, W. A. & Treadwell, C. R. (1953). *J. biol. Chem.* **205**, 483.
 Cowgill, R. W. (1955). *Biochim. biophys. Acta*, **14**, 583.
 Das, B. R. & Giri, K. V. (1959). *J. Indian Inst. Sci.* **41**, 67.
 Dodgson, K. S., Rose, F. A. & Spencer, B. (1957). *Biochem. J.* **66**, 357.
 Dodgson, K. S., Spencer, B. & Thomas, J. (1955). *Biochem. J.* **59**, 29.
 Fodor, P. J. (1950). *Arch. Biochem.* **26**, 331.
 Ganguly, J., Krinsky, N. I., Mehl, J. W. & Deuel, H. J., jun. (1952). *Arch. Biochem. Biophys.* **38**, 275.
 Krishnamurthy, S., Seshadri Sastry, P. & Ganguly, J. (1958). *Arch. Biochem. Biophys.* **75**, 6.
 Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
 Ludewig, S. & Chanutin, A. (1950). *Arch. Biochem.* **29**, 441.
 McGugan, W. A. & Laughland, D. H. (1952). *Arch. Biochem. Biophys.* **35**, 428.
 Mahadevan, S., Murthy, S. K., Krishnamurthy, S. & Ganguly, J. (1961). *Biochem. J.* **79**, 416.
 Meier, J. R., Siperstein, M. D. & Chaikoff, I. L. (1952). *J. biol. Chem.* **198**, 105.
 Myers, D. K., Schotte, A., Boer, H. & Borsje-Bakker, H. (1955). *Biochem. J.* **61**, 521.
 Niefert, M. L. & Deuel, H. J., jun. (1949). *J. biol. Chem.* **177**, 151.
 Richter, D. & Croft, P. G. (1942). *Biochem. J.* **36**, 746.
 Robinson, R. W. & Hogden, C. G. (1940). *J. biol. Chem.* **135**, 707.
 Schotz, M. C., Rice, L. I. & Alfin-Slater, R. B. (1954). *J. biol. Chem.* **207**, 665.
 Seshadri Sastry, P., Krishnamurthy, S. & Ganguly, J. (1957). *Indian J. med. Res.* **45**, 263.
 Sperry, W. M. & Webb, M. (1950). *J. biol. Chem.* **187**, 97.
 Swell, L., Dailey, R. E., Field, H., jun. & Treadwell, C. R. (1955). *Arch. Biochem. Biophys.* **59**, 393.

Biochem. J. (1961) **80**, 406

The Effects of X-Irradiation in Solution on the Kinetic, Sedimentation and Fluorescent Properties of Glutamic Acid Dehydrogenase

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Investigators of the effects of ionizing radiation on enzyme solutions generally assume that the result of the interaction is an all-or-none loss of

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enzymic activity. This tacit assumption fails to recognize that a change in the measured velocity of an enzymic reaction can also reflect a partial loss in functional activity. Studies of the kinetics of X-irradiated chymotrypsin show changes in the Michaelis constant and the maximum activity,

indicating that both the affinity for the ester substrate and the overall rate of the reaction are modified (Mee, 1958). These results suggest that changes in enzymes produced by irradiation may be more subtle than has previously been appreciated.

Glutamic acid dehydrogenase is a mitochondrial pyridine nucleotide-dependent enzyme known to be sensitive to irradiation in solution (Höhne, Kunkel & Maass, 1955). Its kinetic, sedimentation and fluorescent properties have been characterized (Frieden, 1959*a*, *b*, *c*; Winer & Schwert, 1958). This paper provides an explicit statement of the possible changes produced by irradiation on the experimentally measurable kinetic parameters of glutamic acid dehydrogenase and relates the observed changes in enzyme kinetics to certain structural properties.

THEORETICAL

Assume that an initial total concentration of enzyme, E_T , is characterized by constants K_1 (Michaelis constant) and V_m (maximum velocity) with respect to substrate s . Irradiation may then produce at least three other species of enzyme: $E_2(K_2, V_1)$, $E_3(K_1, V_2)$ and $E_4(K_2, V_2)$ in addition to the original species $E_1(K_1, V_1)$. E_i designates the total concentration of enzyme species i . The overall velocity, v , is given by

$$v = \frac{V_1 E_1}{1 + K_1/s} + \frac{V_1 E_2}{1 + K_2/s} + \frac{V_2 E_3}{1 + K_1/s} + \frac{V_2 E_4}{1 + K_2/s}, \quad (1)$$

where $V_m = E_T V_1$. In terms of reciprocal velocity and reciprocal substrate concentration this reduces to

$$\frac{1}{v} = \frac{1 + (K_1 + K_2)/s + (K_1 K_2)/s^2}{V_1(E_1 + E_2) + V_2(E_3 + E_4) + [(V_1 E_1 + V_2 E_3) K_2 + (V_1 E_2 + V_2 E_4) K_1]/s} \quad (2)$$

Let $\alpha = E_1/E_T$, $\beta = E_2/E_T$, $\gamma = E_3/E_T$, and since $E_T = E_1 + E_2 + E_3 + E_4$ then $(1 - \alpha - \beta - \gamma) = E_4/E_T$. Equation (2) may then be written

$$\frac{1}{v} = \frac{1}{E_T} \frac{1 + (K_1 + K_2)/s + (K_1 K_2)/s^2}{(\alpha + \beta) V_1 + (1 - \alpha - \beta) V_2 + \{[\alpha V_1 + \gamma V_2] K_2 + [\beta V_1 + (1 - \alpha - \beta - \gamma) V_2] K_1\}/s} \quad (3)$$

Inspection of equation (3) indicates that plots of $1/v$ against $1/s$ are hyperbolic, rather than linear, with two branches, one wholly, and the other partly, imaginary. A few representative curves of this type have been given by Dixon & Webb (1958). At $1/v = 0$ the two branches cut the abscissa at $-1/K_2$ and $-1/K_1$ respectively. At $1/s = 0$ the real part of the curve crosses the ordinate at

$$\frac{1}{E_T} \frac{1}{(\alpha + \beta) V_1 + (1 - \alpha - \beta) V_2}.$$

Special case 1: irradiation affects K but not V . $E_3 = E_4 = 0$

$$\frac{1}{v} = \frac{1}{V_1 E_T} \frac{1 + (K_1 + K_2)/s + (K_1 K_2)/s^2}{1 + [\alpha K_2 + (1 - \alpha) K_1]/s} \quad (4)$$

The trace of these hyperbolic functions will depend upon the values of K_1 and K_2 as well as the proportion of original enzyme, α . In this case, curves of differing slope cross the ordinate at a common intercept $1/V_1 E_T = 1/V_m$.

Special case 2: irradiation modifies K or reduces V to zero.

$$\frac{1}{v} = \frac{1}{V_m} \frac{1 + (K_1 + K_2)/s + (K_1 K_2)/s^2}{\alpha + \beta + (\alpha K_2 + \beta K_1)/s} \quad (5)$$

These curves intercept the ordinate at $1/(\alpha + \beta) V_m$: a point which is determined by the relative concentration of unirradiated and K -modified enzyme remaining. With increasing doses of radiation this value of the intercept increases continuously and does not converge on a finite value of $1/V_2$. At low substrate concentrations, the reciprocal velocity will approach the linear asymptote

$$\frac{1}{v} \approx \frac{1}{V_m} \left\{ \frac{\alpha K_2^2 + \beta K_1^2}{[\alpha K_2 + \beta K_1]^2} + \frac{K_1 K_2}{[\alpha K_2 + \beta K_1]} \frac{1}{s} \right\} \quad (6)$$

crossing the abscissa at

$$\frac{1}{s} = -\frac{\alpha/K_1^2 + \beta/K_2^2}{\alpha/K_1 + \beta/K_2} \equiv -\frac{1}{K_m} \quad (7)$$

The observed K_m in this instance will be an average value weighted according to equation (7) and depending upon the relative concentration of E_1 and E_2 as well as the values of K_1 and K_2 .

Special case 3: irradiation reduces V but does not affect K ; $E_2 = E_4 = 0$.

$$\frac{1}{v} = \frac{1}{E_T [\alpha V_1 + (1 - \alpha) V_2]} \{1 + K_1/s\} \quad (8)$$

In this case, straight lines of different slope cross the abscissa at a common intercept $1/s = -1/K_1$. In the instance that $V_2 = 0$

$$\frac{1}{v} = \frac{1}{\alpha V_m} \{1 + K_1/s\} \quad (9)$$

This form of inactivation, depending only upon the relative concentration of undamaged enzyme, α , has implicitly been assumed in most earlier investigations. When s is held constant the velocity becomes proportional to α .

EXPERIMENTAL

Materials. Glutamic acid dehydrogenase was obtained as crystals suspended in sodium sulphate-potassium phosphate from C. F. Boehringer und Söhne GmbH., Mannheim, Germany. Before use the enzyme was dialysed three times against 100 vol.

of 0.05M-potassium phosphate, pH 7.6; these preparations were homogeneous by ultracentrifuge analysis. Diphosphopyridine nucleotide (DPN) and reduced diphosphopyridine nucleotide (DPNH) were also obtained from C. F. Boehringer und Söhne. The DPN was more than 96% pure, as measured by addition of cyanide (Colowick, Kaplan & Ciotti, 1951). The $E_{260\text{ m}\mu}/E_{340\text{ m}\mu}$ ratio of the DPNH was 2.6. Reagent-grade glutamate, α -oxoglutarate, glutarate, succinate and *o*-, *p*- and *m*-phthalate were obtained from the British Drug Houses Ltd.

Enzyme assays. These were carried out at pH 7.6 in 0.08M-potassium phosphate buffer with 3 ml. total volumes. The substrate and coenzyme concentrations were varied as indicated in the text. For the oxidation of DPNH 4 μ g. of enzyme was added to start the reaction; for the reduction of DPN 40 μ g. of enzyme was employed. Velocities were calculated as the change in E at 340 m μ /5 min. at 20°; the velocities were constant over this time interval.

Ultracentrifuge determinations. A Spinco model E analytical ultracentrifuge was employed for the sedimentation analyses. The enzyme was dialysed in

pH 7.3 buffer of the following composition: 0.05M-NaCl, 0.015M- Na_2HPO_4 , 0.005M- KH_2PO_4 . It was centrifuged at 135 000*g*. The rotor temperature of the several runs varied from 15° to 20°. The sedimentation coefficients were corrected to water at 20°.

Fluorescence measurements. The initial fluorescence measurements were made with a mercury-arc source, a quartz-cuvette set in a water bath and a photomultiplier set at 90° to the incident beam and attached directly to a galvanometer. The incident radiation was rendered parallel by a quartz lens and the 365 m μ line was isolated with a Chance filter; the emitted light was passed through a Wratten B₂ filter before striking the photomultiplier in order to minimize the scattered light. Under these circumstances a negligible deflexion was obtained with the protein solutions alone. This apparatus and the ultracentrifuge were made available through the kindness of Dr P. Johnson, Department of Colloid Science. Fluorescent measurements at other wavelengths of incident radiation were also made through the courtesy of Dr G. Weber, University of Sheffield. A low-pressure mercury source was employed and

Fig. 1. Effect of irradiation on glutamic acid-dehydrogenase kinetics: substrates. (A) Reciprocal velocity $(1/v) \times V_m$ against reciprocal glutamate concentration. Standard reaction mixture with 0.63 mM-DPN. Extrapolation of the linear portions gives K_m (glutamate): ●, unirradiated (1.0 mM); ○, 3500 r. (1.9 mM); ▲, 4700 r. (2.5 mM); △, 9500 r. (3.3 mM). Enzyme (380 μ g./ml.) irradiated; V_m 0.083. (B) Reciprocal velocity $(1/v) \times V_m$ against reciprocal α -oxoglutarate concentration. Standard reaction mixture with 0.13 mM-DPNH. K_m (α -oxoglutarate): ●, unirradiated (0.31 mM); ○, 900 r. (0.46 mM); ▲, 1800 r. (0.87 mM). Enzyme (40 μ g./ml.) irradiated; V_m 0.103.

the incident wavelengths were isolated with a grating monochromator. The apparatus has been described (Weber, 1960).

Irradiations. These took place in Pyrex vessels with 210 kv peak unfiltered X-rays. The dose rates were 4000–8000 r./min. The irradiation vessels were calibrated dosimetrically by measuring the oxidation of ferrous ammonium sulphate (Haybittle, Saunders & Swallow, 1956). The enzyme was suspended in 0.05M-phosphate buffer, pH 7.6, and irradiated at 20–30° at a concentration of less than 10 mg./ml. so that the effects were due primarily to the solvent radicals.

RESULTS

Kinetic studies

The effects of X-irradiation on the Michaelis constants and the maximum velocities of glutamic acid dehydrogenase for glutamate, α -oxoglutarate, DPN and DPNH were determined by examining reciprocal velocities as a function of reciprocal substrate and coenzyme concentrations. The plots of these are shown in Figs. 1 and 2 for glutamate, α -oxoglutarate and DPN, DPNH respectively. Irradiation produces an increase in K_m for the substrates and a decrease in V . It produces a decrease in V for the coenzymes without change in

K_m . The hyperbolic shape of the glutamate plot, as evident at high substrate concentration (Fig. 1A), is in accord with theory and indicates that at least one additional, enzymically active species of the enzyme has been created by the irradiation. Inspection of Fig. 1 (A) indicates that changes in K_m for glutamate are apparent before a significant decrease in V is manifest. The hyperbolic shape of the reciprocal velocity and substrate plot is not evident for α -oxoglutarate (Fig. 1B), but this substrate inhibits the enzyme at the high concentrations that would be required to exhibit the deviation from linearity.

The increase in K_m for glutamate observed could be explained by a modification of the binding site for glutamate as a result of irradiation. The properties of this site have been studied with dicarboxylic analogues of glutamic acid as inhibitors of the enzyme (Caughey, Smiley & Hellerman, 1957). Considering the distance between the hydrogen atoms of the two carboxyl groups, the most potent member of each analogous series has been shown to have the mean interproton distance which most closely approximates to that of glutamic acid, which is 7.45 Å. Members with a smaller or larger interproton distance are less effective. The binding site of glutamate was, consequently, said (Caughey *et al.*) to include two cationic centres on the

Fig. 2. Effect of irradiation on glutamic acid-dehydrogenase kinetics: coenzymes. (A) Reciprocal velocity $\times V_m$ against reciprocal DPN concentration. Standard reaction mixture with 10 mM-glutamate. K_m (DPN) 0.31 mM; V_m 0.125. ●, Unirradiated; ○, 9500 r.; ▲, 15 000 r. (B) Reciprocal velocity $\times V_m$ against reciprocal DPNH concentration. ●, Unirradiated; ○, 900 r.; ▲, 1800 r. Standard reaction mixture with 1.2 mM α -oxoglutarate. K_m (DPNH) 0.17 mM; V_m 0.222.

protein, separated by 7.45 Å, which match the anionic carboxylate groups of the substrate. If irradiation modifies the protein structure so that the geometric relationship of the cationic centres is disturbed, this should be reflected in the relative potency of the dicarboxylic acid inhibitors. In Table 1, the relative potency of the members of two series of analogue inhibitors are compared with respect to both irradiated and unirradiated enzyme. The published interproton distances of the inhibitors are also given. It is apparent that the irradiated enzyme does not differ from the unirradiated with respect to inhibitor effectiveness, although K_m for glutamate is increased by three times as a result of irradiation. This is inconsistent with any great modification in the distance of the proposed cationic centres being the cause of the observed increase in K_m .

Ultracentrifuge studies

The kinetic molecular unit of glutamic acid dehydrogenase has a molecular weight of 10^6 . This large unit is dissociated by DPNH, adenosine triphosphate and the metal-chelating agent 1:10-phenanthroline (Frieden, 1959*a, b*). Dissociation of the enzyme is accompanied by loss of activity. Adenosine diphosphate promotes the reconstitution of the units dissociated by DPNH and restores activity.

The effects of irradiation on the sedimentation of glutamic acid dehydrogenase are demonstrated in Fig. 3. Irradiation results in the diminution of the single enzyme protein peak and in the appearance of slower-sedimenting material. The schlieren patterns suggest the formation of at least one major slower component. The sedimentation coefficients of the fast and slow components are given

Table 1. *Comparative effectiveness of substrate analogues as inhibitors of irradiated and unirradiated glutamic acid dehydrogenase*

Enzyme solution (400 µg./ml.) was irradiated with 15 000 r. X-rays. Assay employed a standard reaction mixture with 3.3 mm-glutamate and 0.63 mm-DPN. Results are given as percentage of the initial uninhibited activity of both irradiated and unirradiated enzyme. The maximum velocity of the irradiated enzyme was 0.36 of that of the unirradiated. K_m for glutamate of the unirradiated enzyme was 0.95 mm; for the irradiated enzyme K_m was 2.7 mm. The interproton distances are taken from Caughey, Smiley & Hellerman (1957).

Reagent (mm)	Interproton distance (Å)	Percentage of uninhibited activity	
		Unirradiated	15 000 r.
isoPhthalate (1.8)	7.45	42	45
Phthalate (60.0)	4.25	63	65
Terphthalate (50.0)	8.65	48	50
Glutarate (2.0)	7.65	45	47
Succinate (32.0)	6.55	46	47

Fig. 3. Effect of irradiation on the sedimentation of glutamic acid dehydrogenase. Enzyme (6 mg./ml.) irradiated and placed in the ultracentrifuge cell. (A) Unirradiated enzyme, maximum enzymic velocity 100%. (B) 100 000 r., maximum velocity 55%. (C) 180 000 r., maximum velocity 38%. Activities were measured as described in Table 2. The sedimentation coefficients refer to the main and secondary peaks. Rotor temperature (A) and (B) was 20.6°; for (C) it was 16.0°. Sedimentation was from right to left; photographs were taken at 8 min. intervals.

in the Figure legend. These correspond with those observed when the enzyme is dissociated by high concentrations of DPNH (Frieden, 1959*a*). In addition, at high X-ray doses, very rapidly sedimenting material also appears and indicates that aggregation as well as splitting is promoted by the irradiation. This rapidly sedimenting material is assumed to be protein that has received multiple radical attack.

The values of the corresponding maximum enzyme velocities are also given in the legend of Fig. 3. Coincident with the diminution in the main protein peak there is a significant decrease in enzymic activity. Thus there is a strong correlation of structural modification with functional impairment. Unlike the splitting and inactivation caused by DPNH, the addition of adenosine diphosphate to the irradiated, split enzyme does not restore activity.

Fluorescence studies

The addition of glutamic acid dehydrogenase to DPNH enhances the fluorescence of the latter compound and shifts the peak to shorter wavelengths (Winer & Schwert, 1958). This fluorescence can be obtained with excitation wavelengths in the region of 340 m μ , the broad absorption band of the reduced coenzyme. In addition, the fluorescence of DPNH can be obtained by exciting the enzyme-coenzyme complex at 280 m μ , where the tryptophan and tyrosine groups of the protein absorb strongly. This has been shown for lactic dehydrogenase (Velick, 1958) and we have observed the same for glutamic acid dehydrogenase. These latter observations imply that there is a transfer of electronic energy from the protein to the bound coenzyme. Heating lactic dehydrogenase destroys

the enzyme's ability to enhance the coenzyme fluorescence (Shifrin, Kaplan & Ciotti, 1959).

X-irradiation of glutamic acid dehydrogenase in solution (in the absence of coenzyme), reduces the ability of the enzyme-protein to enhance the fluorescence of DPNH (Table 2). This is observed whether the coenzyme is excited directly at 365 m μ or indirectly at 280 m μ . The decrease in this fluorescence is closely paralleled by the loss in maximum enzyme velocity, V , and suggests a relationship between electronic and catalytic integrity. Irradiation with the same doses did not significantly change E at 279 m μ of the protein or the fluorescence polarization of the enzyme-DPNH mixture.

The addition of glutamate to the enzyme-DPNH complex further increases the coenzyme fluorescence (Winer & Schwert, 1958). Consequently, the effect of enzyme irradiation on this additional fluorescence was explored in doses which produced increases in K_m for glutamate. The results are given in Table 3. Addition of 0.4 mM-glutamate increased the fluorescence by 1.3 times and 4 mM-glutamate increased it by 2.7 times in all instances. Irradiation of the protein has no effect on the relative increase of fluorescence due to the addition of glutamate to the enzyme-coenzyme complex, although K_m is modified.

In addition to augmenting the fluorescence of bound DPNH, native dehydrogenases generally exhibit an intrinsic fluorescence in the region of 340 m μ when excited at 280 m μ (Velick, 1958). The intrinsic fluorescence of glutamic acid dehydrogenase was measured after doses of X-irradiation which diminish the fluorescence of the enzyme-DPNH complex. Data are shown in Table 4.

Table 2. *Effect of X-irradiation on the capacity of glutamic acid dehydrogenase to enhance the fluorescence of reduced diphosphopyridine nucleotide*

Enzyme solution (1.8 mg./ml.) was irradiated. Fluorescence cuvette (A) (exciting wavelength 280 m μ) contained 0.5 ml. of enzyme, 0.1 ml. of 0.1 mM-DPNH and 1.5 ml. of 0.05 M-potassium phosphate buffer, pH 7.6. Fluorescence cuvette (B) (exciting wavelength 365 m μ) contained 2.0 ml. of enzyme, 0.5 ml. of 0.1 mM-DPNH and 8.0 ml. of phosphate buffer. Isolated measurements of enzyme and DPNH were subtracted from the total galvanometer deflexion to obtain the additional fluorescence resulting from their mixture. The adjusted galvanometer deflexion for unirradiated enzyme plus DPNH was designated 100%. E was measured at 279 m μ before and after irradiation. Maximum velocity was measured with 0.63 mM-DPN in a standard reaction mixture containing various concentrations of glutamate.

Dose (r.)	$E_{279\text{ m}\mu}$	Maximum velocity (%)	Fluorescence (%)	Fluorescence polarization
	(A) Exciting wavelength 280 m μ			
0	1.74	100	100	0.20
50 000	1.86	47	45	0.21
160 000	1.95	5	~0	—
	(B) Exciting wavelength 365 m μ			
0	1.80	100	100	0.24
40 000	1.86	73	68	0.23
90 000	—	25	27	—

Table 3. *Effect of enzyme irradiation on the augmentation of reduced diphosphopyridine nucleotide fluorescence when glutamate is added to glutamic acid dehydrogenase plus reduced diphosphopyridine nucleotide; comparison with K_m (glutamate)*

Same conditions as described in Table 2. K_m (glutamate) was determined as described in Fig. 1 (A). F enzyme-DPNH is the fluorescence of enzyme plus DPNH adjusted for the fluorescence of free enzyme and free DPNH. F enzyme-DPNH-glut. is the adjusted fluorescence of enzyme plus DPNH plus glutamate. Two concentrations of glutamate were employed: $G_1 = 0.4$ mM and $G_2 = 4$ mM.

Dose (r.)	K_m (glutamate) (mM)	F enzyme-DPNH	F enzyme-DPNH-glut.	$\frac{F \text{ enzyme-DPNH-glut.}}{F \text{ enzyme-DPNH}}$
(A) Exciting wavelength 280 m μ				
0	1.0	37.5	49.5 (G_1) 98.5 (G_2)	1.3 2.6
50 000	2.3	17.0	24.3 (G_1) 45.7 (G_2)	1.4 2.7
(B) Exciting wavelength 365 m μ				
0	1.0	10.8	14.3 (G_1) 27.8 (G_2)	1.3 2.7
40 000	2.3	7.3	9.6 (G_1) 18.5 (G_2)	1.3 2.5
90 000	2.6	2.9	4.0 (G_1) 8.1 (G_2)	1.4 2.8

Table 4. *Effect of irradiation on the intrinsic protein fluorescence of glutamic acid dehydrogenase*

Same conditions as described in Table 2 except that DPNH was omitted from the fluorescence cuvette. The fluorescence of unirradiated enzyme was designated 100%.

Dose (r.)	Maximum velocity (%)	Fluorescence (%)
0	100	100
50 000	47	91
160 000	5	79

Unlike the enhanced fluorescence, the intrinsic protein fluorescence is not markedly altered by these doses, which also decrease the maximum enzyme velocity.

DISCUSSION

The mathematical statements for the effect of radiation on the experimental kinetic parameters K and V demonstrate that a number of distinct mechanisms can be responsible for any inactivation observed. The elucidation of the mechanisms requires a careful evaluation of the kinetic parameters; the usual determination of irradiated enzyme activity at a single substrate concentration is inadequate. If the concentration of substrate employed is less than optimum and a change in K_m has been effected the sensitivity of the enzyme will appear falsely high. If, on the other hand, optimum substrate concentration is employed, a change in K_m brought about by irradiation will not be recognized. For example, after 9500 r. glutamic acid dehydrogenase that is assayed with 1.1mM-glutamate retains 20% of its activity; the same

enzyme assayed in 11 mM-glutamate has 40% of the activity of the control (Fig. 1A). It is desirable to determine the influence of irradiation on the relative values of the kinetic parameters before investigating the dose-response relationship.

Irradiation increases K_m for the substrates glutamate and α -oxoglutarate. The changes in K_m for glutamate appear before a significant decrease in V (Fig. 1A). This implies that either an ordered sequence of inactivation is obeyed (namely, $E_1 \rightarrow E_2 \rightarrow E_4$) or that the efficiency for modifying K is greater than that for decreasing V . The distribution of the three enzyme species calculated from Fig. 1 and equation 7 is given in Fig. 4. The equation was derived with the assumption that each effective ionization either increases K to form the species E_2 or reduces V to zero to form the species E_4 . The data indicate that the production of E_2 precedes that of E_4 by a considerable margin. On the other hand, the results do not preclude a more complex reaction sequence involving several Michaelis constants and several maximum velocities. A different susceptibility of two separate sites on the enzyme surface to radical attack or an ordered sequence could be responsible for the divergent responses.

The detailed kinetics of glutamic acid dehydrogenase (Frieden, 1959c) involve three substrates and two coenzymes, and are too complex to enable the increases in K_m to be ascribed to any single specific change in the individual rate constants. Nevertheless, it is tempting to assume that the increase in K_m represents decreased substrate affinity. However, irradiation fails to modify the additional fluorescence which glutamate imparts to

Fig. 4. Distribution of enzyme species during irradiation. Calculated from data in Fig. 1 and equation 7, and assuming that irradiation modifies K or decreases V to zero. ●, E_1 , unirradiated enzyme with constants K_1 and V_1 ; ○, E_2 , irradiated enzyme with constants K_2 and V_1 ; ▲, E_3 , irradiated inactive enzyme with V_2 0. K_1 1.0 mM and K_2 3.5 mM.

the enzyme-DPNH mixture, even at limiting glutamate concentrations (Table 3). Assuming that this additional fluorescence is due to the formation of a ternary enzyme-DPNH-glutamate complex, it is evident that observed increases in K_m for glutamate are not accounted for on the basis of reduced substrate affinity alone. This is supported further by the failure of irradiation to change the relative effectiveness of the substrate analogue inhibitors (Table 1).

X-rays appear to promote the splitting of the large glutamic acid dehydrogenase molecule in parallel with a reduction in maximum activity (Fig. 3). Irradiation has also been shown to affect the sedimentation of other proteins. Snail haemocyanin, molecular weight 9×10^4 , is split by the direct action of radiation into units of half that molecular weight (Svedberg & Brohult, 1938; Pickels & Anderson, 1946). The influence of irradiation on the oxygen-binding capacity of haemocyanin in relation to its molecular weight has not been studied. When serum albumin and haemoglobin are irradiated with α -particles in solution, the materials become polydisperse, with the appearance of both faster and slower components in the ultracentrifuge pattern (Brohult, 1940). γ -Irradiation of serum albumin in solution leads only to aggregation (Rosen, Brohult & Alexander, 1957).

The mechanisms by which irradiation effects the splitting of glutamic acid dehydrogenase and its inactivation are not known. Presumably the bonds responsible for the gross structural integrity are sensitive to attack by the radicals formed in solution. Independently, the metal-chelating agent 1:10-phenanthroline is capable also of splitting the enzyme with loss of activity (Frieden, 1959b). As glutamic acid dehydrogenase contains zinc and depends upon the metal for its catalytic, as well as structural, stability (Adelstein & Vallee, 1958), it is tempting to speculate that radicals attack sulphhydryl or other groups which bind zinc to the apoenzyme.

Irradiation not only changes the sedimentation behaviour of glutamic acid dehydrogenase but modifies the electronic properties as well. The ability of the enzyme to augment the fluorescence of DPNH is destroyed coincident with the loss of enzymic activity. It is natural to ascribe the loss of this property to failure in DPNH-binding (Fisher & McGregor, 1960). However, the fluorescence polarization of DPNH is the same on the addition of irradiated or of unirradiated enzyme. If the enzyme had lost its ability to bind DPNH a return of the polarization toward that of free DPNH would be expected. The loss of fluorescence enhancement would also be expected if the optically absorptive groups of the protein were destroyed. The relative constancy of the 279 $m\mu$ absorption before and after irradiation, as well as the preservation of the intrinsic protein fluorescence, eliminates this possibility. It seems most likely that the loss of ability to augment DPNH fluorescence is due to some modification in the protein structure induced by the irradiation.

In contrast with this, the intrinsic fluorescence of glutamic acid dehydrogenase excited at 280 $m\mu$ is not markedly altered by doses of radiation which drastically decrease the enzyme activity. On the other hand, it has been shown for at least one other dehydrogenase that concentrated urea solutions destroy the intrinsic fluorescence of the protein coincident with loss of activity (Shifrin *et al.* 1959); this implies that enzyme activity and a part of the intrinsic fluorescence depends upon the integrity of the secondary structure.

The various fluorescence studies suggest that important alterations in the tertiary structure of glutamic acid dehydrogenase are brought about as the result of irradiation. Disulphide linkages, metal-ion bridges and the general stabilization of structure due to electrostatic forces would all seem to be susceptible (Linderstrøm-Lang & Schellman, 1959), but identification of the specific structural elements which are attacked by the aqueous radicals will require further experimentation.

SUMMARY

1. An explicit mathematical statement for the effects of irradiation on the kinetic parameters K (Michaelis constant) and V (maximum velocity) of enzymes is provided.

2. Irradiation of glutamic acid dehydrogenase, in solution, increases K and decreases V for the substrates glutamate and α -oxoglutarate. V is decreased and K is unchanged for the coenzymes diphosphopyridine nucleotide and reduced diphosphopyridine nucleotide (DPNH).

3. The increase in K for glutamate is not associated with a change in the relative inhibitory power of dicarboxylic analogues of glutamate.

4. Irradiation reduces the main protein peak seen in the ultracentrifuge and produces slower sedimenting material. This is consistent with a splitting of the large kinetic molecular unit into sub-units. At very high doses rapidly sedimenting material, presumed to be aggregates, is produced. The decrease in the main component is coincident with a decrease in enzymic activity.

5. Irradiation of the enzyme reduces its ability to enhance the fluorescence of DPNH. This is observed whether wavelength of the exciting light is 365 m μ or 280 m μ . The change observed parallels a decrease in the maximum velocity, V .

6. The increase in K for glutamate is not accompanied by a decrease in the augmented DPNH fluorescence observed on the addition of glutamate to enzyme-DPNH mixtures. The decrease in maximum enzyme velocity is not paralleled by a marked fall in the intrinsic protein fluorescence.

7. It is suggested that the primary action of the aqueous radicals formed on irradiation leads to modification in the tertiary structure of the enzyme with resulting loss in maximum velocity.

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REFERENCES

- Adelstein, S. J. & Vallee, B. L. (1958). *J. biol. Chem.* **233**, 589.
 Brohult, S. (1940). *Nova Acta Soc. Sci. upsal.* **12**, no. 4.
 Caughey, W. S., Smiley, J. D. & Hellerman, L. (1957). *J. biol. Chem.* **224**, 591.
 Colowick, S. P., Kaplan, N. O. & Ciotti, M. M. (1951). *J. biol. Chem.* **191**, 447.
 Dixon, M. & Webb, E. C. (1958). *Enzymes*, p. 95. London: Longmans, Green and Co. Ltd.
 Fisher, H. F. & McGregor, L. L. (1960). *Biochim. biophys. Acta*, **38**, 562.
 Frieden, C. (1959a). *J. biol. Chem.* **234**, 809.
 Frieden, C. (1959b). *J. biol. Chem.* **234**, 815.
 Frieden, C. (1959c). *J. biol. Chem.* **234**, 2891.
 Haybittle, J. L., Saunders, R. D. & Swallow, A. J. (1956). *J. chem. Phys.* **25**, 1213.
 Hühne, G. J., Künkel, H. A. & Maass, H. (1955). *Naturwissenschaften*, **42**, 488.
 Linderström-Lang, K. U. & Schellman, J. A. (1959). In *The Enzymes*, vol. 1, p. 443. Ed. by Boyer, P. D., Lardy, H. & Myrback, K. New York: Academic Press Inc.
 Mee, L. K. (1958). *Radiation Res.* **9**, 151.
 Pickels, E. G. & Anderson, R. S. (1946). *J. gen. Physiol.* **30**, 83.
 Rosen, D., Brohult, S. & Alexander, P. (1957). *Arch. Biochem. Biophys.* **70**, 266.
 Shifrin, S., Kaplan, N. O. & Ciotti, M. M. (1959). *J. biol. Chem.* **234**, 1555.
 Svedberg, T. & Brohult, S. (1938). *Nature, Lond.*, **142**, 830.
 Velick, S. F. (1958). *J. biol. Chem.* **233**, 1455.
 Weber, G. (1960). *Biochem. J.* **75**, 335.
 Winer, A. D. & Schwert, G. W. (1958). *Biochim. biophys. Acta*, **29**, 424.

Biochem. J. (1961) **80**, 414

The Metabolism of Specifically Labelled Glucose in Bovine Ketosis

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Bovine ketosis in dairy cows is accompanied by hypoglycaemia in degrees varying from slight to severe. Shaw (1943, 1956) demonstrated that glucose tolerance and uptake were normal in the mammary gland of ketotic cows. He concluded

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that the overall rate of glucose utilization, especially by the mammary gland, is not impaired in ketosis. Studies with labelled glucose have also suggested that the overall rate of glucose metabolism is not decreased during ketosis (Kronfeld, Tombropoulos & Kleiber, 1959b). Nevertheless, the pathway of glucose catabolism may be abnormal.