ULTRA-VIOLET IRRADIATION OF TRIOSEPHOSPHATE DEHYDROGENASE

by

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A study, of a qualitative nature, of the kinetics of inactivation of crystalline muscle triosephosphate dehydrogenase (TPD) by polychromatic ultraviolet light, has previously been reported upon[1].

The following work was carried out principally with monochromatic radiation, of wave-length 2537 Å, and includes measurements of the quantum yield for inactivation based on a provisional determination of the molecular weight of the enzyme. The interpretation of the mechanism of inactivation by ultraviolet light still leaves much to be desired; however, the emphasis placed in recent years on quantitative measurements both on enzymes and dipeptides by McLAREN and his collaborators[2] is providing data which should undoubtedly prove useful in the elucidation of the structure and behaviour of proteins and enzymes.

With a view to extending existing information on the nature of the active groups of TDP and of the inactivation process itself, a series of experiments was performed with samples of enzyme which were completely oxidized and therefore exhibited no enzymatic activity in the absence of a reducing agent; and with samples of enzyme in the presence of an excess of cysteine so that all reducible –SS groups were transformed to –SH.

The effect of the reduction of the enzyme-bound diphosphopyridine nucleotide (DPN), (see below), has also been investigated. During this latter phase of the investigation, it was found that irradiation in the near ultraviolet (3100–3800 Å), following reduction of the enzyme-bound DPN, resulted in a partial reactivation of the enzyme. Preliminary results of this photoreactivation process have been reported elsewhere[3].

MATERIALS

Triosephosphate dehydrogenase was prepared by the method of CORI *et al.*[4] and recrystallized three times in 66% saturated ammonium sulphate at p_H 8.6. For use, the crystal suspension was centrifuged, the supernatant decanted and the crystals dissolved to required concentration in the appropriate buffer. Concentrations were determined from the height of the absorption maximum at 2780 Å.

Diphosphopyridine nucleotide (DPN) was prepared by the method of WILLIAMSON AND GREEN[5] as modified by OCHOA[6] and was about 38% pure. A sample of about 44% purity from the Schwartz laboratories was used as well.

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dl-Glyceraldehyde-3-phosphate (GAP) was prepared as the dioxane brominated compound according to the method of FISCHER AND BAER[7] with the kind advice and assistance of Mlle. B. TCHOUBAR. A generous sample was also made available by Dr BAER. Purities of the DPN and the *d*-component of GAP (about 40%) were measured enzymatically in the presence of arsenate. *The d-glyceraldehyde* used in one part of the work was prepared by Mr B. C. LOUGHMAN of Cambridge and made available through the courtesy of Dr D. M. NEEDHAM, F.R.S.

Buffers were made up according to CLARK AND LUBBS, using all-glass double distilled water; p_H measurements were with the hydrogen electrode.

LIGHT SOURCE AND METHOD OF IRRADIATION

The source of radiation was a Gallois low-pressure mercury lamp, with peak emission at 2537 Å, consisting of a 2-mm tube wound in the form of a flat spiral 2 cm in diameter. The irradiation cell was simply a 5-mm quartz Beckman cell, supported in a metal jig 6 cm from the lamp with the face of the cell perpendicular to the axis of the spiral. A short-focus quartz lens interposed between the source and the cell rendered the incident beam sufficiently parallel so that the variation of intensity from the position of the entrance to the exit window was only about 3%. A linear thermopile with a 1-mm slit was used to "scan" the area occupied by the utilized portion of the face of the cell and showed that the intensity was quite uniform over this area.

The characteristics of the lamp, as given by the manufacturer, indicate an output of 65% of the emitted energy at 2537 Å, 5% between this wavelength and 3130 Å, and the remainder at 3650 Å and in the visible. A series of tests, using a glass and a CCl_4 filter, coupled with an examination of the spectrum, showed that actually only 50% of the emitted energy was located at 2537 Å and that this constituted 90% of the energy emitted below 3130 Å. Intensity of the 2483 line was less than 1% of that at 2537 and the 1849 line was so feeble that a considerable exposure was required to reveal its presence. The enzyme itself does not absorb above 3100 Å and tests showed that no measurable inactivation was perceptible when light below this wavelength was eliminated.

Energy incident on the face of the cell was measured by means of a Zeiss thermopile, the effective area of which was limited by means of a slit, and which was calibrated against a Hefner lamp. The energy incident on the face of the cell was calculated to be $4.1 \cdot 10^{17}$ quanta/min/cm^2 at 2537 Å for a lamp current of 26 milliamperes and was maintained constant by regulation of the current.

GENERAL PROCEDURE

The irradiation cell contained about 1.2 ml of enzyme solution. Prior to exposure 0.1 or 0.2 ml was drawn off to serve as a control; similar volumes were drawn off following 2, 4 and 6 minutes exposure times. Enzyme activities of the four samples were then measured simultaneously in the Beckman spectrophotometer by following the rate of reduction of DPN. The components of the reaction mixture were generally about $3 \cdot 10^{-5}$ *M* pyrophosphate buffer p_H 8.5; $6 \cdot 10^{-6}$ *M* sodium arsenate and $2 \cdot 10^{-7}$ *M* each of DPN and *d*-GAP, in a total volume of 3 ml. The reaction was usually started by the addition of GAP.

From the optical density readings at 1, 2 and 3 minutes the initial velocity of the reaction was calculated from the derivative at the origin of a cubic equation fitted to the curve of optical density *vs.* time. Initial velocities were then transformed to equivalent enzyme concentrations by means of calibration curves made up in the same way.

The cell compartment of the spectrophotometer was maintained as close to room temperature as possible by means of a circulating coil and thermostatic bath. The temperature in each cell was checked immediately following the reaction and temperature corrections made to initial velocities where necessary[8].

ABSORPTION SPECTRUM OF TPD

Curve a of Fig. 1 shows the absorption spectrum of the enzyme for a concentration of 0.8 mg/ml in 0.025 *M* phosphate buffer p_H 5.8. The spectrum remains unaltered in the p_H range 4.5 to 8.6.

Part of the absorption is due to DPN with which the enzyme is stoichiometrically combined in the ratio of 1 mole of DPN to 50,000 g TPD[9]. At 2537 Å it can be calculated that about 35% of the total absorption is due to enzyme-bound DPN.

Ultraviolet irradiation results in a general rise in the height of the curve as is

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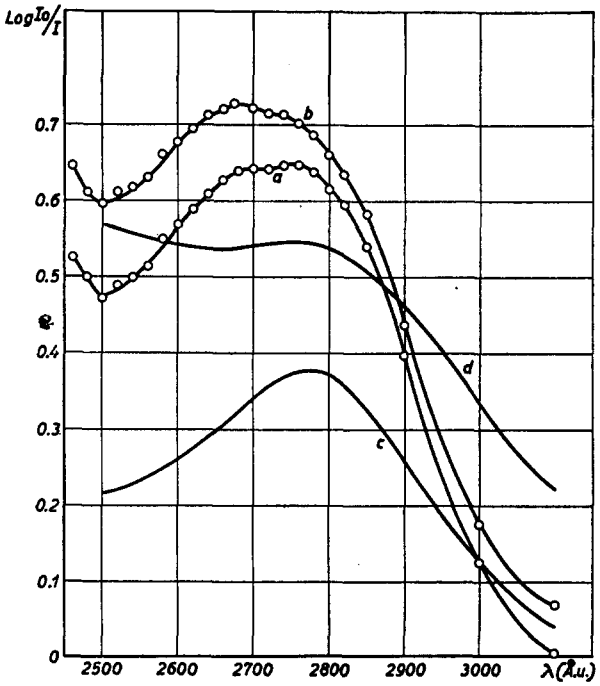

Fig. 1. Absorption spectrum of triosephosphate dehydrogenase (a) 0.8 mg/ml in 0.025 *M* phosphate buffer p_H 5.8; (b) same, following 6 minutes irradiation with 90% loss in activity; (c) spectrum of a sample of enzyme, p_H 6, with most of bound DPN removed and (d) same, following 52 minutes irradiation

the spectrum following 52 minutes irradiation at p_H 7.

ORDER OF REACTION FOR INACTIVATION

Using polychromatic light TPD has been shown to follow a first-order inactivation course[1]. In most of the present experiments inactivation was usually followed to only 50% completion which is often insufficient to distinguish between a 0, 1st or 2nd order reaction. Since calculations of the quantum yield are dependant on the order of the reaction, a few measurements were made to 90% inactivation by irradiating at a distance of about 3 cm from the lamp and thoroughly mixing the enzyme solutions prior to extraction of samples for activity measurements. Fig. 2 shows that the first order rate is closely followed.

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illustrated by the spectrum of the sample, Curve b of Fig. 1, following 6 minutes irradiation at an intensity sufficiently high to reduce the activity about 90%.

Irradiation was not accompanied by any coagulation or discoloration. No apparent relationship was observed between rate of inactivation and rate of increase of absorption. The increase in absorption continues long after inactivation is complete and even following removal of the source of radiation, indicating the presence of secondary reactions. The process is most likely a degradation of the protein molecule due to photo-oxidation by hydroxyl radicals in the presence of oxygen[10].

Curve c of Fig. 1 shows the spectrum of a sample of TPD from which most of the DPN has been removed by dialysis against distilled water following reduction of the bound DPN[11]. As a result the minimum at 2500 Å is considerably reduced. Curve d shows the form of

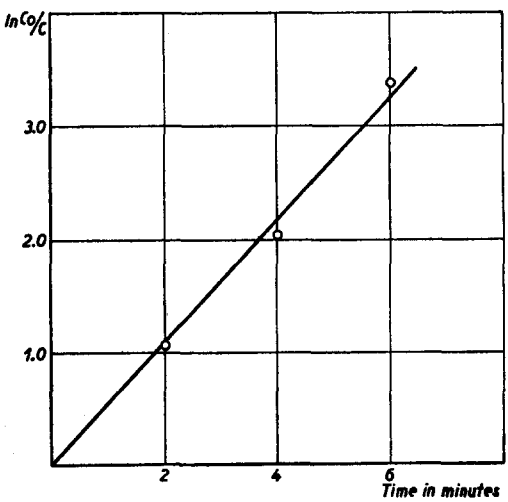

Fig. 2. Plot of $\ln C_0/C$ *vs.* time of irradiation for TPD, showing that inactivation follows a first-order course (Enzyme concentration 0.68 mg/ml in phosphate buffer p_H 6)

QUANTUM YIELD AND p_H VARIATION

The quantum yield is defined as

$$\varnothing = \frac{\text{number of molecules inactivated}}{\text{number of quanta absorbed}}$$

If account is taken of the decrease in enzyme concentration with time of irradiation, then, following a procedure used by FINKELSTEIN AND MCLAREN[12]

$$\varnothing = \frac{C_o \ln C_o/C}{I_a t}$$

where C_o is the initial enzyme concentration, C the concentration at time t and I_a the number of quanta absorbed per unit of time. Now $1/t \times \ln C_o/C = k_1$, the inactivation rate constant, which is merely the slope of the line in Fig. 2. Hence

$$\varnothing = C_o k_1 / I_a$$

It is assumed that reactants and products absorb alike which is equivalent to the assumption that a molecule that suffers an increase in absorption has already been inactivated; and that such molecules, or the products of their decomposition (if any) exert no influence on the inactivation process.

The molecular weight of the enzyme is an uncertain factor. VELICK AND RONZONI[13] calculate a minimum molecular weight of 100,000 based on amino acid analysis. However TRISTRAM[14] has cast doubt on the validity of this method for molecular weights in excess of 40,000. TAYLOR[15] has calculated a value of 118,000, but the method is as yet unpublished.

Attempts to measure the molecular weight by sedimentation and diffusion measurements were not successful; in particular the enzyme does not appear to be homogeneous in the ultracentrifuge[3]. CORI *et al.*[4] had previously attempted, without success, to use sedimentation and diffusion measurements for a calculation of the molecular weight.

A provisional measurement, using the light scattering method*, yielded a value of about 47,000. In view of the finding of TAYLOR *et al.*[9] that 50,000 g of enzyme are combined with 1 mole of DPN, and the arguments of various authors[16] to the effect that DPN is bound with dehydrogenases in the ratio of 1:1, the value of 50,000 has been adopted for calculations of the quantum yield.

Table I shows the values of the inactivation rate constants k_1 and the corresponding quantum yields at several p_H values grouped about the isoelectric point

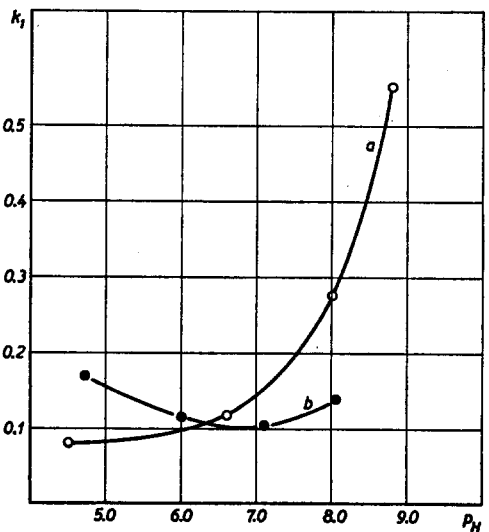

Fig. 3. Inactivation rate-constant, k_1, as a function of p_H for the ultraviolet inactivation of TPD (a) using polychromatic light and (b) using monochromatic radiation, of wavelength 2537 Å

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of the enzyme, p_H 6.6[4] at which point the rate is a minimum. The variation of k_1 with p_H is quite different from that found with polychromatic light[1]. Fig. 3 shows the two curves drawn to the same scale. A somewhat similar finding has been reported for pepsin[17]. It is evident that the p_H dependance of quantum yield varies with the wavelength.

TABLE I

INACTIVATION RATE CONSTANT, k_1 AND QUANTUM YIELD, ø FOR TPD AT 2537 Å

Enzyme concentration 0.68 mg/ml in 0.025 *M* buffer, corresponding to $4.1 \cdot 10^{15}$ molecules/cm² of light path. Incident energy $4.1 \cdot 10^{17}$ quanta/min/cm². Quanta absorbed = $I_a = 1.35 \cdot 10^{17}$ quanta/min.

pH	C_o (molecules/cm²)	k_1 (min^{-1})	$\varnothing = \frac{C_o k_1}{I_a}$
Acetate 4.7	$4.1 \cdot 10^{15}$	0.173	$5.2 \cdot 10^{-3}$
Phosphate 6.0	$4.1 \cdot 10^{15}$	0.116	$3.5 \cdot 10^{-3}$
Phosphate 7.1	$4.1 \cdot 10^{15}$	0.103	$3.1 \cdot 10^{-3}$
Phosphate 8.05	$4.1 \cdot 10^{15}$	0.138	$4.15 \cdot 10^{-3}$

RELATION OF CONCENTRATION TO ø

One determination of quantum yield was made using an initial concentration C_o of 1.85 mg/ml at p_H 6.0. The value of k_1 was 0.052 and the absorption at 2537 Å was 70.5% corresponding to an I_a of $2.9 \cdot 10^{17}$ quanta/min. The value of ø works out to be $1.9 \cdot 10^{-3}$ (see discussion).

INFLUENCE OF STATE OF –SH GROUPS

Unlike the yeast enzyme crystallized by WARBURG AND CHRISTIAN[18], muscle TPD, even when utilized immediately following final recrystallization, is appreciably inactivated due to oxidation of many of its –SH groups. Maximum activity is attainable only in the presence of a suitable reducing agent. A number of measurements were therefore made with the object of studying the effect of reduction of reducible –SS groups on the inactivation of the enzyme. Cysteine was selected as reducing agent because it absorbs little in this region; several tests showed that its reducing ability on the enzyme was unaffected during the periods of irradiation used.

Fig. 4 shows the results for an old enzyme preparation which had inactivated spontaneously over a period of many months so that its activity in the absence of cysteine was practically imperceptible. A sample containing 0.8 mg/ml in 0.025 *M* phosphate buffer at p_H 6.0 was irradiated for 2, 4 and 6 minutes; the three samples withdrawn following each irradiation period, along with a control, were then added

* I should like to express my thanks to Dr J. TONNELAT and Mlle. S. GUINAND for carrying out these measurements.

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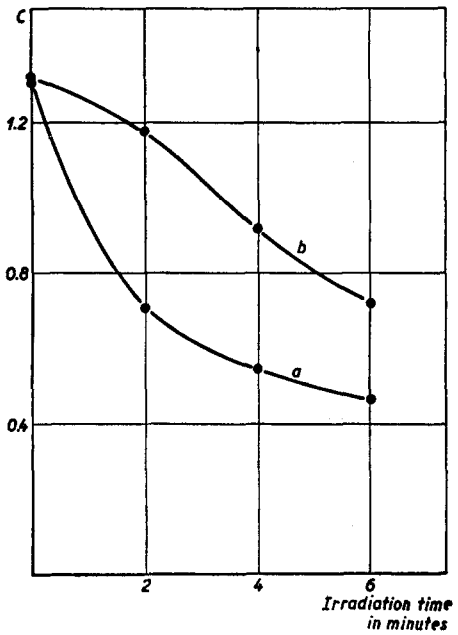

Fig. 4. Inactivation of oxidized TPD as a function of time of irradiation (a) enzyme incubated in cysteine following irradiation and (b) enzyme incubated in cysteine prior to and during irradiation. Activities measured in presence of cysteine in both cases

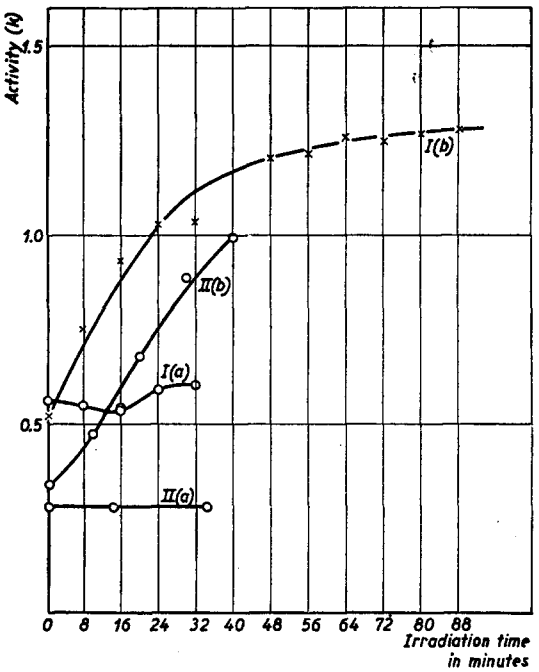

Fig. 5. Influence of enzyme-bound DPN(H) on the activity of TPD following irradiation in the near ultraviolet. (For details see text)

to the reaction mixtures in the Beckman cells which now contained $4 \cdot 10^{-6}$ *M* cysteine hydrochloride previously adjusted to p_H 8.5 with NaOH. Following an incubation period of 7–10 minutes, DPN was added and the reaction was then started with the addition of GAP. A calibration curve of enzyme activity *vs.* concentration was made up in the same way. Curve a of Fig. 5 shows the decrease of enzyme concentration as a function of time of irradiation. Curve b of the same figure shows the behaviour of the same sample incubated in the presence of $2 \cdot 10^{-5}$ *M* cysteine prior to irradiation. It is obvious that the presence of cysteine prior to and during irradiation exerts an appreciable protective effect on the enzyme. However the rate of inactivation is in neither case a first order one. Results for irradiation prior to incubation in cysteine are closest to a 2nd order reaction, while those for irradiation following cysteine incubation are not far from a 0 order reaction.

When the above procedure was carried out with a sample of enzyme that was only partially oxidized, the protecting effect of the cysteine was even more striking. Unfortunately the concentrations of enzyme used (0.6–0.9 mg/ml) were such that the activities measured were far beyond the extrapolation range of any of the calibration curves available. The results for one such experiment are therefore given in Table II, where the activities are given as the 1-minute optical density readings in the Beckman.

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TABLE II

EFFECT OF CYSTEINE ON ULTRAVIOLET INACTIVATION OF TPD

0.9 mg/ml in 0.025 *M* phosphate buffer p_H 6 irradiated in (a) presence and (b) absence of cysteine. Activities, following irradiation, measured in pyrophosphate-cysteine buffer and expressed in terms of the optical density of reduced DPN 1 minute after start of reaction.

Time of irradiation (minutes)	Activity	
	(a)	(b)
0	0.674	0.679
2	0.663	0.594
4	0.653	0.442
6	0.641	0.294

It is of interest to note that, although sample (a) of Table II was incubated with cysteine at p_H 6 the control activities of (a) and (b) are the same; the activities of both were, of course measured at p_H 8.5.

INFLUENCE OF ENZYME-BOUND DPN

In view of the fact that approximately 35% of the absorption of the enzyme is due to its DPN content, it becomes of interest to inquire whether the DPN influences the inactivation process. At p_H 8.5 it has been shown that the reduced DPN (DPNH) remains bound to the enzyme[11,8] with a dissociation constant which, though small, is finite and approximately equal to the dissociation constant for the oxidized DPN[19]. However, it can then be dialyzed away against distilled water, final p_H about 6[11].

A number of measurements of inactivation rate were therefore made with the bound DPN previously reduced*. GAP was used initially for enzymatic reduction of the bound DPN. However, with the exception of one experiment, the results were complicated by the appearance of appreciable absorption in the ultraviolet due in some unknown manner to the GAP**.

d-Glyceraldehyde (GA) which showed only very slight absorption in this region was then used to replace GAP. Because of the much slower rate of reduction by GA as compared to GAP[20] the enzyme concentration was first made up to about 5–6 mg/ml in *M*/200 pyrophosphate buffer containing about *M*/100 sodium arsenate. This was divided into two portions, GA added to one to a final concentration of about *M*/50 and the reduction of DPN followed in the Beckman. About 13 minutes sufficed to reduce half the theoretical amount of bound DPN, corresponding to an optical density at 3400 Å of 0.062/mg enzyme for a light path of 1 cm. The solutions were then diluted

* It has been found that the quantity of bound DPN which can be reduced decreases with increasing oxidation (inactivation) of the enzyme[11]. For the sample of enzyme used here about 70% of the bound DPN could be reduced in the presence of GAP.

** In the final experiments on photoreactivation this difficulty disappeared; its origin remains unclear.

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to concentrations of 0.6–0.9 mg/ml in 0.025 *M* phosphate buffers at p_H 6 and p_H 8. Irradiations were then carried out as above for 2, 4 and 6 minutes, followed by measurements of residual activity. At p_H 6 the difference between the rates of inactivation of enzyme containing oxidized DPN (TPD–DPN) and enzyme containing reduced DPN (TPD–DPNH) was only 5%, while at p_H 8 the difference was 7%. Taking into account inherent instability of the enzyme, these differences are certainly not superior to the accuracy of the measurements. The best that can be said, therefore, is that the reduction of the bound DPN does not sensibly affect the inactivation rate of the enzyme.

Note: In these experiments the TPD–DPNH controls showed markedly lower activities than those of TPD–DPN from which they were prepared, due to inhibition of GAP oxidation by GA. With a final GA concentration of $3 \cdot 10^{-3}$ *M* and a GAP concentration of $4 \cdot 10^{-4}$ *M* the reduction of initial velocity varied from 26 to 37% which is reasonably well in agreement with the recently reported findings of NEEDHAM *et al.*[21] of 30% inhibition for a GA: GAP ratio of 10:1.

EFFECT OF IRRADIATION IN NEAR ULTRAVIOLET

The reduction of the enzyme bound DPN results in the appearance, at 3400 Å of the characteristic absorption maximum of DPNH. In this spectral region, as is evident from curve a in Fig. 1, the enzyme itself exhibits no absorption. The effect of irradiation in this region was therefore investigated, using as source an ordinary mercury arc with appreciable emission at 3130, 3340 and 3660 Å. Radiation below 3100 was eliminated by means of a glass filter. Enzyme concentrations used were of the order of 1 mg/ml in pyrophosphate-arsenate buffer as above and reduction of the bound DPN was effected with GAP. No difficulties were encountered with GAP absorption this region. About 10–12 minutes was required for reduction of about 70% of the bound DPN, following which the absorption of the TPD–DPNH in a 5 mm irradiation cell was 10, 18 and 10% respectively at 3130, 3400 and 3650 Å, as measured against a control to which no GAP was added.

Following definite intervals of irradiation, aliquots of 0.1 ml were drawn off and activities measured using the enzyme to start the reaction instead of the GAP as heretofore. Curves Ia and Ib of Fig. 5 show the variation of enzyme activity (as measured by the initial velocity *k*) with time of irradiation.

While the activity of the TPD–DPN remains approximately constant, that of the TPD–DPNH increases gradually, attaining a limiting value in about 40 minutes. The time required to reach maximum activity was occasionally less than 40 minutes; the extent of increase in activity varied from 100–200%.

Curves IIa and IIb illustrate an analagous experiment in which, however, the irradiating beam was limited to the region around 3660 by a Wood's filter. At this wavelength the filter transmission is 38% while the height of the absorption band of the reduced DPN is only 50% of that at the maximum at 3400 Å. Notwithstanding the consequent decrease in irradiation intensity and the amount of light absorbed, the rate of photoreactivation of the TPD–DPNH is about the same.

The above results were selected for illustration of the phenomenon of photoactivation from a number of series in which the activities of the controls were about the same for the TPD–DPN and TPD–DPNH. On several occasions, however, it was noted that the TPD–DPN control exhibited a net lower activity than the TPD–DPNH, in

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some instances 40% less. This is in sharp contrast to the situation where GA was used for reduction of the DPN and the TPD–DPNH controls demonstrated a lower activity due to inhibition of GAP oxidation by GA. It may be that in the present instance the higher initial activities of TPD–DPNH are due to protection of the active groups, by GAP, similar to the protection by DPN[22]*.

Irradiation in a refrigerated room at 4° C did not show any significant difference in rate of increase of activity. The maximum activity reached appeared to be lower generally than at 22° C, but it is not possible to say that this is more than fortuitous.

The bound DPNH is unaffected by the irradiation and, following photoreactivation of the enzyme, its absorption band remains unaltered and it can all be reoxidized with sodium pyruvate and lactic dehydrogenase. Between 2500 and 2800 Å, however a small but definite increase in absorption was noted; in view of the difficulty mentioned above regarding GAP absorption in this region, it is not certain whether this increase is associated with the enzyme.

It has been shown that, when TPD has undergone considerable spontaneous inactivation, it can then be partially reactivated by heating at temperatures as high as 60° C or even higher[23]. The enzyme used in the present work was not sufficiently inactivated to show this phenomenon.

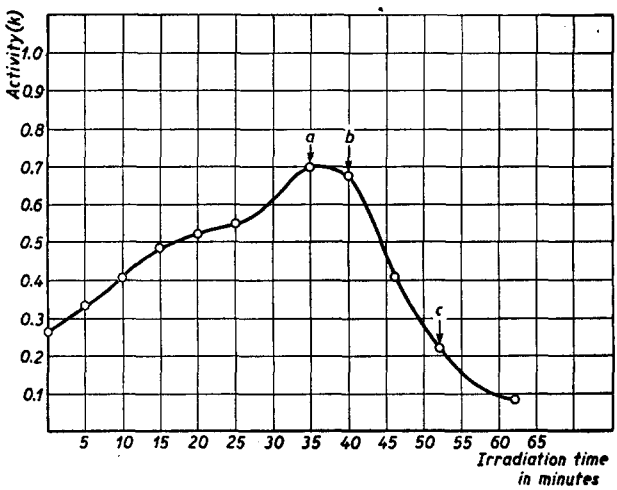

Fig. 6. Variation of activity of TPD–DPNH with time and type of irradiation: Up to arrow a, irradiation source was ordinary mercury arc with glass filter; from a to b without glass filter; from b to c, 2537 Å resonance lamp; at c, ordinary arc with glass filter; see text for further details

In view of the known dependance of TPD activity on intact –SH groups, it would appear most likely that some reduction of inactive –SS groups to the –SH form is involved in the process of photoactivation. And, in fact, when the enzyme was fully reduced in the presence of an excess of cysteine, no reactivation was obtained upon irradiation.

Fig. 6 illustrates another experiment with enzyme activity increasing with time of irradiation up to the time indicated by the arrow a, 35 minutes. At this point the glass filter was removed and the enzyme slowly inactivates. At the time indicated by the arrow b, the solution was placed in front of the mercury resonance lamp with high ultraviolet emission at 2537 Å; the rate of inactivation is speeded up to such an extent that in 12 minutes the activity had fallen below what it was originally. Following this latter irradiation the enzyme itself now showed absorption above 3100 Å (see Fig. 1), comparable in magnitude to that of the bound DPNH. At the time indicated by the arrow c, therefore, the enzyme was again placed in front of the ordinary mercury arc

* During the course of a study of the inhibition of several enzyme systems by narcotics, carried out in collaboration with A. J. ROSENBERG and to be published elsewhere, it was noted that GAP does protect against inhibition, although not as well as DPN, in the case of TPD.

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with glass filter; no further reactivation is noted; instead the enzyme continues to inactivate, although more slowly, reaches a limiting value and then remains unaltered.

Several tests were also carried out with TPD–DPN partially inactivated by irradiation at 2537 Å with the consequent appearance of enzyme absorption above 3100 Å. Subsequent irradiation with the ordinary mercury arc and glass filter resulted in further inactivation to a lower limit as above.

Assuming that the GAP present in the TPD–DPNH samples plays no secondary role, the phenomenon of photoreactivation is probably due to a reduction of –SS groups by the bound DPNH provoked by the absorption of energy by the latter, which has been shown[22] to be bound in the neighbourhood of the active groups of the enzyme. The fact that the reactivation attains only a limiting value, which is still far below that of the fully reduced enzyme, may be explained on the basis of the finding of CORI *et al.*[19] according to which there are two active types of sites, one of which binds the DPN much more tightly than the other. Only those –SS groups would be reduced, as a result of irradiation, which bind the DPN tightly. The existence of two types of active sites has been independently postulated from studies of the heat activation of this enzyme[23].

The absence of photoactivation at the time indicated by the arrow c in Fig. 6 shows that inactivation by ultraviolet light is not due to reversible oxidation of –SH groups by free radicals formed during irradiation of the solution. This does not, of course, exclude the possibility of some kind of irreversible oxidation (see further discussion below).

The fact that additional inactivation takes place at the point c in the figure, as well as for ultraviolet inactivated TPD–DPN subsequently irradiated in the near ultraviolet, is of some interest. It is a common observation that proteins or enzymes subjected to the action of ultraviolet light until they are partially denatured or inactivated, are subsequently unstable and, even following the removal of the irradiation source, continue to inactivate at a rate considerably faster than that of a non-irradiated control of the same activity. This behaviour has been verified in the case of TPD[1]. This secondary effect is also considerably enhanced by heating. The fact that it is, as well, by near ultraviolet radiation fits in with the observation that the enzyme solution itself now shows absorption in this region and indicates that it is most likely due to reactions between the absorbing fragments resulting from the prior ultraviolet inactivation, and those molecules which are still active.

DISCUSSION

From Table I it is seen that, at the isoelectric point of the enzyme, p_H 6.6, the quantum yield for inactivation is $3.2 \cdot 10^{-3}$, which is about what one would expect on the basis of values reported for other enzymes of comparable molecular weights. Such a low value of $\varnothing$ is not all unusual in the light of the recent measurements of MANDL *et al.*[24] of the quantum yields for splitting of peptide bonds, which come out to be of the same order of magnitude. The conclusion of these authors is that the absorption of light by any chromophore in the protein molecule may lead to inactivation.

MCLAREN[25] has recently proposed the following relationship between the molecular weight M and the quantum yield $\varnothing$

$$\varnothing = Q\,M^{-\frac{2}{3}}$$

where Q is a constant. No suitable derivation of the equation was found; it is based

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rather on the observed fact that the plot of log ($\varnothing \cdot 10^7$) *vs.* log M gives an approximate straight line with slope $-^2/_3$. For TPD the results fit McLAREN's graph better for an assumed molecular weight of 50,000 than 100,000 and might therefore be regarded as further evidence in support of a molecular weight of 50,000. However the relationship is based on quantum yield measurements for a bacteriophage, tobacco mosaic virus, *B. coli* and urease. The value of $\varnothing$ for urease can certainly not be considered as established (see below); if the others are omitted and only the results for enzymes considered, the relationship falls down, a fact which is not surprising in view of the complexity of the various factors involved in the determination of $\varnothing$.

The diminution of $\varnothing$ with increased concentration for TPD is not unexpected in view of the previously reported variation[1] of inactivation rate constant k_1 with change in concentration. Assuming a constant quantum yield and using the formula given above for the calculation of $\varnothing$ it is a simple matter to calculate the variation of k_1 *vs.* concentration The variation found previously was greater than that expected, although it must be admitted that polychromatic light of unknown intensity was used. The present quantitative result tends, however, to substantiate the previous.

A variation of $\varnothing$ with concentration is by no means unusual in photochemical reactions. It is one of the factors which must be studied thoroughly for the interpretation of any such reaction[26] and may be due to various factors such as secondary reactions, transferance of energy between molecules, Franck-Rabinowitch effects of intermolecular origin, etc. The importance of eliminating these effects has been emphasized by WARBURG[27] who advises the use of "optically thin" solutions such as was in fact used by KUBOWITZ AND HAAS[28] in their study on urease. With the exception of one or two experiments where the concentration has been varied 2 to 3 fold no effort has been made to investigate this effect in the case of enzymes.

TPD is not a very good enzyme on which to conduct such an investigation because of the difficulty of maintaining suitable controls, especially in dilute solutions. Aldolase, on the contrary, the enzyme which catalyzes the splitting of hexose diphosphate to triose-phosphates, is better suited for this purpose. The quantum yield for this enzyme has been measured[29] over a 24-fold range in concentration, from 0.075 to 2 mg/ml. No really marked variation in $\varnothing$ was found.

It is, of course, possible that in the case of TPD the concentration effect is related in some way to the reactivity of its –SH groups. The existence of an intermolecular effect of such origin has been demonstrated during a study of the activation by heat of this enzyme[23].

The results of KUBOWITZ AND HAAS[28] and LANDEN[30] on urease, another –SH enzyme, are of interest in this connection. LANDEN's values for $\varnothing$ vary from one-fourth to four times those of KUBOWITZ AND HAAS. An inspection of their techniques shows that the most significant difference in their methods was the use by LANDEN of concentrations of the irradiated solutions about 80 times as high as those of KUBOWITZ AND HAAS*.

For ficin, another –SH enzyme, the quantum yield drops 8% for an increase in concentration from 0.5 mg/ml to 1.0 mg/ml[31]. Since the original authors make no claim

* The argument has been advanced that LANDEN's results may be the more accurate because his solutions were stirred during irradiation. But, where the optical absorption of the solution is well below 100%, the need for stirring is not at all obvious. The results for aldolase illustrate this point well.

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of a change of ø with concentration, it is not certain that this difference is more significant than the experimental errors.

Further evidence of the role of concentration is provided by studies of the heat inactivation of TPD[23] where it has been shown that the rate of inactivation is markedly diminished with an increase in initial concentration. CASEY AND LAIDLER have more recently studied this problem in greater detail for pepsin[32] and show that the effect of concentration on the velocity of inactivation is due to modifications of the potential energy of the molecules as a function of the concentration.

The protective effect of cysteine against inactivation may be due to competition with the active protein groups for free radicals produced by the rupture of peptide linkages and the action of light of wave-length 2537 Å on dissolved oxygen[33]. Such a mechanism appears to account for the behaviour of ultraviolet irradiated serum albumin[10]. The fact, however, that a completely oxidized sample of enzyme (the activity of which is placed in evidence only in the presence of a reducing agent such as cysteine) inactivates even more rapidly than one which is only partially oxidized, is not entirely in accord with such a mechanism.

An alternative hypothesis is that the –SH groups, when oxidized to the –SS form, alter the local configuration of the molecule by the formation of new linkages in the protein chain. The probability of the transmission of absorbed energy from the chromophores to other parts of the molecule may then be enhanced with a consequent increase in the rate of inactivation. The work of SANGER[34] on insulin and of MARTIN[35] AND GORDON[36] on wool provide evidence for the existence of interchain –SS bonds. In the presence of cysteine the number of such bonds is diminished, and their formation impeded, with the consequent slowing down of the rate of inactivation. The results represented in Fig. 4 are in agreement with such an explanation. The enzyme used here was practically completely oxidized; its control activity, in the presence of cysteine, was about equal to that of the enzyme used to obtain the results of Table I, for an equal protein concentration. In the absence of cysteine during irradiation it inactivates more rapidly than the sample of Table I, whereas when irradiated in the presence of cysteine, it inactivates less rapidly. Although calibration curves were not available for the results of Table II, they can be seen to follow the same behaviour.

It is somewhat puzzling that the rate of inactivation is no longer first order; this could equally as well be in accord with either of the two mechanism outlined above. In contrast the –SH enzyme ficin has been found to follow a first-order inactivation course when activities were measured in the presence of H_2S following irradiation[31].

With regard to the phenomenon of photoactivation it was pointed out previously[3] that this may be of some significance biologically. Some results reported since then in a preliminary note by CALCUTT are of interest in this regard[37]. The rate of killing of cultures of *Paramecium bursaria* by sulphydryl inhibitors was found to be considerably enhanced upon subsequent exposure to light of wavelength above 3300 Å and is ascribed by the author as being due to a greater availability of –SH groups under the conditions of illumination.

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SUMMARY

1. The inactivation of triosephosphate dehydrogenase by ultra-violet light of wave-length 2537 Å is first-order. The quantum yield at this wave-length and at the isoelectric point is $3.2 \cdot 10^{-3}$.

2. The quantum yield appears to depend upon the concentration of the irradiated solution.

3. In the presence of the reducing agent, cysteine, partial protection against inactivation is obtained; but the inactivation rate is no longer 1st order.

4. The rate of inactivation is not affected by prior reduction of the enzyme-bound coenzyme DPN.

5. Following reduction of the bound DPN, irradiation in the near ultraviolet results in a partial increase in enzyme activity.

6. The nature of this photoactivation process, as well as the other observations, is discussed.

RÉSUMÉ

1. L'inactivation de la triosephosphate déhydrogenase par la lumière ultraviolette d'une lonqeur d'onde de 2537 Å est une réaction du premier ordre; le rendement quantique à cette lonqeur d'onde et au point isoélectrique est de $3.2 \cdot 10^{-3}$.

2. Le rendement quantique paraït dépendre de la concentration de la solution irradiée.

3. En présence d'un agent réducteur, comme la cystéine, se manifeste une effet protecteur contre l'inactivation; en outre la réaction n'est plus du premier ordre.

4. Le taux d'inactivation n'est pas modifié par la réduction préalable du coenzyme lié à l'enzyme.

5. Après réduction du coenzyme lié à l'enzyme, l'activité enzymatique est augmentée par irradiation dans le proche ultraviolet.

6. La nature du processus de photoactivation, aussi bien que les autres observations, sont discutées.

ZUSAMMENFASSUNG

1. Die Inaktivierung der Triosephosphatdehydrogenase durch ultra-violettes Licht von 2537 Å ist eine Reaktion erster Ordnung. Die Quantenausbeute bei dieser Wellenlange und am isoelektrischen Punkt betrifft $3.2 \cdot 10^{-3}$.

2. Die Quantenausbeute scheint von der Konzentration der irradierten Lösung abhängig zu sein.

3. In Gegenwart eines reduzierenden Stoffes, wie Cystein, tritt ein gegen die Inaktivierung schützendes Effekt auf; dabei ist der Vorgang nicht mehr erster Ordnung.

4. Die Wirkung der Inaktivierung wird von der vorhergehenden Reduktion des am Ferment gebundenen Coferments nicht geändert.

5. Nach der Reduktion des Coferments wird die Fermentäktivitat durch Irradiation im nahen Ultraviolett gesteigert.

6. Die Natur des Photoaktivierungsvorganges, wie auch die anderen Beobachtungen, werden besprochen.

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