The Radiation Chemistry of Ethanol and Diphosphopyridine Nucleotide and its Bearing on Dehydrogenase Action

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One of the most striking features of radiation chemistry is that the products are often those which would be produced by biological systems acting on the same substrate (Keller & Weiss, 1951; Weiss & Scholes, 1951; Rigg, Scholes & Weiss, 1952). An example is the oxidation of ethanol to acetaldehyde. This reaction has been induced by 27 MeV. α -particles (McDonell, 1951) and can also be carried out by liver and yeast alcohol dehydrogenases in the presence of diphosphopyridine nucleotide (DPN). It is tempting to suppose that the mechanism of such reactions may bear some resemblance, especially as changes induced by ionizing radiations can often be explained by free-radical mechanisms, and such mechanisms have also been suggested for the action of certain enzymes (Waters, 1943, 1948).

It was thought that a study of the action of γ -radiation on ethanol and DPN might give results relevant to the action of the alcohol dehydrogenases and, especially, might show whether chain reactions of the type suggested by Waters (1943, 1948) can take place.

EXPERIMENTAL

Irradiation arrangements. These were somewhat similar to those described in a previous paper (Swallow, 1952). Two sources of ^{60}Co γ -radiation of strength 100 mc. and 430 mc. were used, and also a 250 mg. source of radium. In order to obtain reproducible results, all quantitative irradiations were performed with solutions (1 ml.) irradiated in flat-bottomed tubes 1 cm. in diameter and 9.25 cm. high, fitted with greased ground-glass stoppers and immersed in a thermostat at $20 \pm 0.1^\circ$. When the irradiation tubes were at a certain distance from the 100 mc. ^{60}Co source, the dose rate was calculated from the previous measurement (Swallow, 1952) to be 400 r./hr., allowance having been made for decay of the source (half-life, 5.3 yr.). This was the dose rate for the results shown in Fig. 1. With other arrangements of sources and irradiation vessels the dose was estimated by measuring the amount of acetaldehyde produced from ethanol by irradiation, and comparing this with the amount produced by irradiation at the measured dose rate. The results shown in Figs. 2-4 and the results showing the effect of DPN on acetaldehyde yield and the effect of de-aeration on yield were at a dose rate of 725 r./hr.

Chemicals. Ethanol was obtained from the Distillers Company Ltd. and was stated to be 96.1% (v/v) ethanol and to contain only 0.0005% (w/w) aldehydes. As sodium pyrophosphate of A.R. purity was not obtainable, a commercial grade was recrystallized before use. HCl was redistilled. Water for solutions to be irradiated was distilled from alkaline permanganate. Preliminary experiments were performed with commercial DPN (L. Light and Co.) and later experiments with 55% pure DPN, kindly supplied by the Biochemistry Department of the University of Cambridge. The DPN used for the actual experiments described in this paper was from the Sigma Chemical Company, U.S.A., and was 90% pure. Other chemicals were of A.R. purity.

Deoxygenation of solutions. The method for deoxygenation and saturation with O_2 -free N_2 or H_2 has been described previously (Swallow, 1952). It was necessary to make allowance for the decrease in ethanol concentration during deoxygenation. It was calculated that after the deoxygenation of 2 ml. of solution containing 3.0 m-moles ethanol, the remaining 1 ml. of solution would contain approx. 0.5 m-mole.

Measurement of acetaldehyde. The method is fundamentally that of Barker & Summerson (1941), originally intended for the measurement of lactic acid. It consists of the colorimetric measurement of the purple colour produced when *p*-hydroxydiphenyl reacts with acetaldehyde in conc. H_2SO_4 . It was found that H_2O_2 (which is a possible radiation product) interferes when ethanol is present, and DPN also interferes in the presence of ethanol. The modification adopted eliminates both sources of interference. Other aldehydes interfere, but are not present in irradiated solutions.

A stream of N_2 is bubbled through the aqueous acetaldehyde solution and acetaldehyde is condensed in a test tube containing a mixture of 6 ml. conc. H_2SO_4 , 0.9 ml. water and 0.05 ml. copper sulphate solution (4% $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$). The aqueous solution is maintained at 24° and the condensing solution at about -20° . Bubbling is continued for 20 min., by which time all acetaldehyde has been removed from the aqueous solution and most of it collected in the H_2SO_4 . This is allowed to warm to room temperature and 0.1 ml. of a *p*-hydroxydiphenyl solution (1.5 g./100 ml. 0.125N-NaOH) is added as close to the surface of the liquid as possible. The test tube is well shaken to disperse the precipitated reagent through the solution, and after standing for at least 1 hr. it is heated in boiling water for 90 sec. After addition of 3 ml. conc. H_2SO_4 the intensity of the purple colour is measured with a colorimeter at about 560 μ . A Hilger Biochem Absorptiometer was used for this purpose. The intensity is proportional to the amount of acetaldehyde within the

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range 0–24 μg . In order to obtain an absolute value for the amount of acetaldehyde in an irradiated solution, it is necessary to calibrate with known quantities of acetaldehyde on the same day as the unknown is measured. This was the procedure adopted for the points on Figs. 1 and 2, which are considered to be accurate to within $\pm 10\%$. When it is desired only to compare the yields from several irradiations it is not necessary to calibrate, provided all measurements are made on the same day.

Ultraviolet absorption curves. The curves shown on Figs. 3 and 4 were obtained by diluting the irradiated solution (1 ml.) with 3 ml. water and measuring the optical density with a Unicam photoelectric quartz spectrophotometer SP500. Distilled water was used as a blank. The author is indebted to Dr A. S. Jones for permission to use the instrument.

RESULTS

Production of acetaldehyde

A 1% aqueous solution of ethanol (6 ml.) was irradiated with a dose of approximately 2×10^6 r., and the aldehyde formed characterized by preparation of the 2:4-dinitrophenylhydrazone. The melting point was 164–165°. The melting point of authentic acetaldehyde 2:4-dinitrophenylhydrazone prepared by the same method was 165° and the melting point of a mixture of the two products also 165°, thus proving that the only aldehyde formed by irradiation is acetaldehyde.

An attempt was made to test for 2:3-butylene glycol in irradiated solutions. If the presence of this substance could be demonstrated it would be evidence of the existence of $\text{C}_2\text{H}_4\text{OH}$ radicals in the system. The periodic acid method as given by Shriner & Fuson (1948) is interfered with by hydrogen peroxide and gave no positive results. There does not appear to be any method which would unambiguously detect quantities as small as those expected in the system and so, rather than develop a special method, the approach was abandoned.

The irradiations described above were of unbuffered aqueous ethanol, but in order to obtain results relevant to the alcohol dehydrogenases it was decided to irradiate under the same conditions of pH, type of buffer and temperature as are required for the action of these enzymes. All quantitative results on both ethanol and DPN were obtained with solutions made up in 0.01M-sodium pyrophosphate adjusted to pH 7.8 with HCl, and were at 20°.

It was shown that the production of acetaldehyde from 0.5M-ethanol in the presence of dissolved air is proportional to dose up to 66 000 r. (Fig. 1). From these results it was calculated that the yield of acetaldehyde under these conditions is 5.4 molecules/100 eV. absorbed.

The effect of deoxygenation is to decrease the yield of acetaldehyde to about 1.8 molecules/100 eV. It was thought possible that this might

have been due to the concentration of ethanol in the de-aerated solution not being exactly 0.5M (the calculation of this concentration being only approximate); the effect of ethanol concentration on yield was therefore investigated for air-saturated solutions. The result is shown on Fig. 2. It is apparent that the yield is not greatly dependent on concentration, and the decrease in yield on de-aeration cannot be explained by a change in ethanol concentration, and must therefore be due to the removal of oxygen.

Fig. 1. The effect of γ -ray dose on the yield of acetaldehyde from 0.5M-ethanol.

Fig. 2. Oxidation of ethanol to acetaldehyde by γ -radiation. Effect of ethanol concentration on yield (dose 52 000 r.).

The addition of DPN to 0.5M-ethanol had little effect on the yield of acetaldehyde in the presence of air. DPN was neutralized with sodium hydroxide before making up solutions which were given a dose of approximately 30 000 r. The yield of acetaldehyde from 0.5M-ethanol alone was 6.6 μg . and when the DPN concentration was 2.7×10^{-3} M the yield was 7.0 μg . When the DPN concentrations were 2.7×10^{-4} M and 2.7×10^{-5} M the yields were 7.2 μg . and 6.7 μg ., respectively.

DPN had a much greater effect on yield of acetaldehyde in the absence of air. With a dose of about 30 000 r. the yield for ethanol alone was $2.2 \mu\text{g.}$ and with $2.7 \times 10^{-3} \text{M-DPN}$ the yield increased to $6.6 \mu\text{g.}$ With $2.7 \times 10^{-4} \text{M-}$ and $2.7 \times 10^{-5} \text{M-DPN}$ the yields were 7.2 and $4.0 \mu\text{g.}$, respectively.

It was thought possible that the increased yield in the presence of DPN might be due to the production of acetaldehyde from DPN itself. That this is not the case was shown by irradiating $5.3 \times 10^{-4} \text{M-DPN}$ solutions with a dose of about 30 000 r. Neither in the presence nor in the absence of oxygen was more than a trace of acetaldehyde produced, and this may have been due to ethanol impurity in the DPN.

Fig. 3. Absorption curves of DPN after irradiation with a dose of 120 000 r. $\times-\times$, Air-saturated solution; $\circ-\circ$, oxygen-free solution; $\square-\square$, solution saturated with hydrogen; $+--+$, air-saturated solution containing 0.5M-ethanol and (identical curves) DPN with no irradiation.

Production of reduced DPN

Oxidized DPN (DPN^+) has an absorption band at about $260 \text{ m}\mu$, and the reduced form (DPNH) has in addition an absorption band at $340 \text{ m}\mu$. Racker (1950) used the absorption at $340 \text{ m}\mu$ as a measure of the amount of DPNH present in mixtures of the oxidized and reduced form. It was decided to measure the ultraviolet absorption curve of irradiated $2.7 \times 10^{-4} \text{M-DPN}^+$ solutions in the region $270-420 \text{ m}\mu$, in order to see whether DPNH is formed. Results are shown in Figs. 3 and 4.

It can be seen (Fig. 3) that the absorption curve of DPN^+ irradiated with a dose of 120 000 r. in the presence of dissolved air is very little different from the curve of non-irradiated DPN^+ , and the presence of 0.5M-ethanol completely protects it even from this small effect of irradiation. In the absence of air

and ethanol no band due to DPNH appears when $2.7 \times 10^{-4} \text{M-DPN}^+$ is irradiated with a dose of 120 000 r. Neither is DPNH formed when $2.7 \times 10^{-4} \text{M-DPN}^+$ is irradiated in the presence of hydrogen with a dose of 120 000 r. All four irradiations were repeated with a dose of 30 000 r.: even smaller effects were observed.

When a DPN^+ solution containing 0.5M-ethanol is irradiated in the absence of dissolved oxygen with a dose of about 30 000 r., the characteristic absorption band at $340 \text{ m}\mu$ appears (Fig. 4). Longer irradiation (60 000 r.) produces some increase in absorption at $340 \text{ m}\mu$ but absorption increases at about $290-300 \text{ m}\mu$. With still further

Fig. 4. Absorption curves of oxygen-free DPN solutions irradiated in the presence of 0.5M-ethanol. $+--+$, No irradiation; $\times-\times$, dose 30 000 r.; $\circ-\circ$, dose 60 000 r.; $\square-\square$, dose 120 000 r.

irradiation (120 000 r.) the characteristic absorption at $340 \text{ m}\mu$ disappears and the absorption at $290-300 \text{ m}\mu$ increases still further. These irradiations show that DPNH is the first product formed, and this itself appears to be further affected by radiation with the production of a substance absorbing at $290-300 \text{ m}\mu$.

DISCUSSION

Waters (1943, 1948) has suggested free-radical equations to explain the action of certain enzymes. In the particular case of the alcohol dehydrogenases, the following reactions (p. 256) are an important part of one mechanism put forward.

These equations have so far little experimental support, but it can be seen that if the free $\text{C}_2\text{H}_4\text{OH}$ radical could be produced under the same conditions of pH, temperature and concentration of reactants as are required for enzyme action, then it might be

and it is likely that the agent responsible for producing it is the C_2H_4OH radical. There is insufficient evidence available to be able to write equations explaining the production of DPNH by this radical, but in any case the alcohol dehydrogenases cannot work by this particular reaction because they are not inhibited by dissolved oxygen.

Theorell & Bonnichsen (1951) have shown that liver alcohol dehydrogenase (ADH) forms a complex with DPN, both in the oxidized and in the reduced form. This complex reacts with ethanol as follows (Theorell & Chance, 1951):

There is much recent evidence that enzymic oxidations and reductions are free-radical reactions (George, 1952; Parravano, 1951), and as there are many points of resemblance between the enzymic and the γ -ray reductions of DPN, it seems likely that reaction (5) might be found to take place by a free-radical mechanism. Work is in progress to obtain further information on the radiation chemistry of this system.

SUMMARY

1. Gamma radiation acts on aqueous ethanol to produce acetaldehyde. The effect of dose and ethanol concentration on the yield is described for air-saturated solutions.

2. The yield of acetaldehyde in oxygen-free solutions is smaller than in the presence of air, and when DPN^+ is added the yield increases and DPNH appears. DPN^+ solutions do not produce DPNH when irradiated alone in air, or in the absence of oxygen, or in the presence of hydrogen.

3. When DPN^+ is added to air-saturated ethanol solutions the acetaldehyde yield is almost the same as in the absence of DPN^+ and DPNH does not appear.

4. It is considered that the free C_2H_4OH radical is responsible for the production of DPNH. This reaction is inhibited by dissolved oxygen and does not occur as a step in the action of the alcohol dehydrogenases.

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Pancreatic Trypsin Inhibitor

1. PREPARATION AND PROPERTIES

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Two specific trypsin inhibitors are known to be present in pancreas; one, soluble in 2.5% trichloroacetic acid, is a dialysable polypeptide which was crystallized by Kunitz & Northrop (1936). The other inhibitor is non-dialysable, and precipitated by trichloroacetic acid; it was obtained as a hetero-

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geneous product in only small amounts (Kazal, Spicer & Brahinsky, 1948). The polypeptide inhibitor of Kunitz & Northrop formed a crystalline compound with trypsin, devoid of either inhibitor or trypsin activity. Its molecular weight was reported to be about 6000, but no evidence for purity of the final product was presented, and the analytical figures were admitted to be of uncertain significance.