The Reduction of Diphosphopyridine Nucleotide by Sodium Dithionite

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It is well known that diphosphopyridine nucleotide (DPN) can be reduced by sodium dithionite to give a product, DPNH, identical with that obtained by enzymic reduction. DPN can also be reduced by ionizing radiations under special conditions (Swallow, 1953), but the product was found to possess no coenzyme activity when tested with lactic dehydrogenase (Stein & Swallow, 1954). Barron, Johnson & Cobure (1954), however, using alcohol dehydrogenase, showed that the product contained some of the active form. The reason for the apparent discrepancy is not yet clear. Sodium borohydride produces a mixture of reduced forms (Mathews & Conn, 1953).

The mechanism of the various chemical methods of reduction is of interest in connexion with the mechanism of dehydrogenase action, and the observation that dithionite reduction in alkaline solution gives a bright yellow substance instead of DPNH is therefore of particular importance (Adler, Hellström & Euler, 1936). Recently, Yarmolinsky & Colowick (1954; see also Colowick, 1954) have produced evidence that the yellow substance is not a stable free radical, as was previously thought, but an addition product of a derivative of dithionite with the pyridine ring of DPN.

In this paper are presented some magnetic data which confirm that the yellow substance cannot be a free radical. Some other properties of the yellow substance are also reported.

METHODS

Magnetic measurements were made by the micromethod of Theorell (1942). The apparatus consists of a narrow thin-walled glass tube divided by a central transverse septum so that different solutions may be placed at either end, air bubbles being rigorously avoided. The tube is suspended horizontally between the pole pieces of an electromagnet (field $\approx 19\,000$ gauss) and carries an extension by means of which the position of the tube may be observed with the aid of an optical system. With water in one side of the tube, it is necessary to correct for slight variations in the diameter by placing a suitable solution in the other side, such that a minimal deflexion is observed when the current is switched on. This solution is left in place throughout the experiments. The apparatus is calibrated by replacing the water with a known solution of a paramagnetic substance, and observing

the deflexion when the current is switched on. The unknown solutions are measured in the same way. The author is indebted to Dr P. George for permission to use the instrument, and for advice during the experiments.

Irradiations were with 190 kvp X-rays from a Victor Maximar therapy set. There was no added filtration. The dose rate was measured by irradiating dilute solutions of ferrous ammonium sulphate in 0.1N-H₂SO₄ in the same place. From the amount of Fe²⁺ oxidized, and the known yield of the reaction (15.5 ions oxidized/100 ev absorbed, according to Hochanadel, 1952) the dose rate was calculated to be 1400 R/min.

DPN for most of the work was 'cozymase 90', stated to be 90% pure, from the Sigma Chemical Company, U.S.A. Some of the experiments with lactic dehydrogenase were with DPN stated to be 100% pure, from C. F. Boehringer & Söhne, Germany.

Partly purified pig-heart lactic dehydrogenase was kindly given by members of the Biochemistry Department, University of Cambridge.

All chemicals were of A.R. purity.

RESULTS

Paramagnetism of the yellow substance

A solution of yellow substance was prepared by mixing equal volumes of 0.016M DPN in 0.3N-NaOH and 0.2M-Na₂S₂O₄ in 0.3N-NaOH. After standing for 2 min., the solution was aerated for 8 min. to destroy excess Na₂S₂O₄. Control solutions were prepared containing DPN without Na₂S₂O₄ and Na₂S₂O₄ without DPN. Results are given in Table 1.

Table 1. *Demonstration that the yellow substance is not paramagnetic*

Solution	Deflexion on instrument (scale divisions)
(NH ₄) ₂ Fe(SO ₄) ₂ (0.0022M) in 0.05N-H ₂ SO ₄	+ 38
Water	+ 5
DPN in 0.3N-NaOH	- 7
Na ₂ S ₂ O ₄ in 0.3N-NaOH (aerated)	- 8
Yellow substance	- 8*

* If the yellow substance had been a free radical, the deflexion would have been +5 scale divisions.

The mass susceptibility (χ) of ferrous ammonium sulphate is 32×10^{-6} and of water -0.720×10^{-6} . Thus, by the additivity law, the mass susceptibility of the calibration solution is calculated to be

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-0.693×10^{-6} . Hence, 33 scale divisions correspond to a difference in mass susceptibility of 0.027×10^{-6} . From this, the mass susceptibility of the aerated $\text{Na}_2\text{S}_2\text{O}_4$ -NaOH control solution must be -0.736×10^{-6} .

The molar susceptibility (M_X) of a free radical with one unpaired electron is 1270×10^{-6} . From the additivity law, allowing for the small diamagnetic contribution of the DPN molecule by means of Pascal's constants (Selwood, 1943), it is calculated that if the yellow substance had been a free radical its solution would have a mass susceptibility of -0.720×10^{-6} and therefore have given a deflexion of +5 scale divisions. As the deflexion was -8 scale divisions, unchanged from the control, it is apparent that the yellow substance cannot be a free radical.

Attempts at the further reduction of the yellow substance

The yellow substance is stable only in alkaline solution. On neutralization it reverts to the oxidized form of DPN (DPN^+) and $\text{Na}_2\text{S}_2\text{O}_4$ (Hellström, 1937). On the other hand, Yarmolinsky & Colowick (private communication) have discovered that when the yellow substance is anaerobically neutralized and then incubated for 60 min. at 37° , the reduced form of DPN (DPNH) appears in good yield. We have repeated this work under the appropriate conditions, and have confirmed Yarmolinsky & Colowick's result.

Irradiation with X-rays

Solutions of the yellow substance were prepared in alkaline solution, and the pH was adjusted to 10.2 with NaHCO_3 . After oxidizing excess $\text{Na}_2\text{S}_2\text{O}_4$ by aeration, the solutions had the correct absorption maximum at $355 \text{ m}\mu$. and retained about 80% of their optical density after standing for 1 hr. at room temperature. On adjusting the pH to 8.0 with boric acid, the absorption band at $355 \text{ m}\mu$. disappeared, and no band at $340 \text{ m}\mu$. was seen, indicating that the solutions contained no DPNH . There was enough dissolved air present to oxidize the $\text{Na}_2\text{S}_2\text{O}_4$ formed. On the basis of these preliminary observations it was decided to irradiate solutions of the yellow substance at pH 10.2, and examine the irradiated solutions for the presence of DPNH by adjusting the pH to 8.0. Solutions were given an X-ray dose of 80000R and were cooled in ice-water during irradiation.

Irradiation of $3 \times 10^{-4} \text{ M}$ solutions of the yellow substance in the presence of dissolved oxygen or hydrogen or in the absence of air resulted in a decrease in the optical density at $355 \text{ m}\mu$. accompanied by an increase at $320 \text{ m}\mu$. On adjusting the pH to 8.0, it was found that the curves differed from those of unirradiated controls similarly adjusted

only by a small increase in absorption in the region of $300 \text{ m}\mu$. There was no absorption band at $340 \text{ m}\mu$. The absorption curves have been published elsewhere (Swallow, 1954b).

Irradiation in the presence of 0.5 M ethanol but in the absence of air gave qualitatively different results (Fig. 1). The peak at $355 \text{ m}\mu$. appears to be

Fig. 1. Effect of X-irradiation on an air-free solution of the yellow substance in the presence of 0.5 M ethanol. Absorption curves of solutions of yellow substance. O, control solution, pH 10.2; ●, control solution, adjusted to pH 8; x, irradiated solution, pH 10.2; +, irradiated solution, adjusted to pH 8.

almost unaffected, but a large increase occurs near $290\text{--}300 \text{ m}\mu$. Air was bubbled through the solution and the curve again measured, but it was found to be unchanged, showing that $\text{Na}_2\text{S}_2\text{O}_4$ (which has an absorption maximum at $313 \text{ m}\mu$.) was not present. On adding boric acid to give a final pH of 8.0, the optical density diminished considerably both at $360 \text{ m}\mu$. and at $290 \text{ m}\mu$., but the final absorption curve was markedly different from that of the control, and different from the corresponding curves obtained after irradiations under the other conditions (Swallow, 1954b). Interpretation of the results in detail is not easy, but it is clear that DPNH is not present in any of the solutions prepared by irradiating the yellow substance.

Chemical properties of the yellow substance

The yellow substance being of interest in connexion with enzyme action, it seemed desirable to test its reactivity with substances of biochemical interest, including especially some of the substrates which are capable of being oxidized or reduced by dehydrogenases.

Two 3 ml. portions of a $3 \times 10^{-4}M$ solution of yellow substance were prepared in carbonate-bicarbonate buffer at pH 10.3, and, after aeration to destroy excess $Na_2S_2O_4$, nitrogen (O_2 -free) was bubbled through to remove oxygen. To one portion was added 0.05 ml. of acetaldehyde that had been neutralized with NaOH. The rates of decrease in optical density at 360 $m\mu$. are shown in Fig. 2. The ultraviolet absorption curve was measured after the reaction was completed, and it was found that the solution did not have an absorption band at 340 $m\mu$. The reaction of the yellow substance with acetaldehyde formed within the solution by the dissociation of the acetaldehyde-sodium bisulphite compound

Fig. 2. Rate of reaction of yellow substance with acetaldehyde and glycine. $\times$, control solution; O , with acetaldehyde present; $\bullet$, with glycine present.

was also investigated, and a somewhat similar result obtained. An attempt was made to detect butane-2:3-diol amongst the reaction products, but none was found. It can be stated that there was less than 10% of the diol which might have been expected if it had been a reaction product. The full details of these experiments are given elsewhere (Swallow, 1954b).

The reaction of the yellow substance with a variety of compounds was investigated. For each experiment, three samples of $3 \times 10^{-4}M$ yellow substance were prepared, air-free, in carbonate-bicarbonate buffer at pH 10.2. Two of the solutions contained the added substance under test at 0.05M concentration, while the third solution was a control to allow for the normal slow decrease in concentration of the yellow substance. The rates of decrease of optical density at 360 $m\mu$. were followed for about 90 min. at 19–23°. None of the following compounds reacted significantly with the yellow substance: ethanol, acetone, formic acid, acetic acid, pyruvic acid, lactic acid, β -hydroxybutyric acid, succinic acid, maleic acid and cysteine. Glycine was the only substance which definitely reacted (Fig. 2). The absorption curve after reaction with glycine showed no maximum at 340 $m\mu$., proving that DPNH is not a reaction product.

The action of lactic dehydrogenase on the yellow substance

The yellow substance was originally prepared in strongly alkaline solution, and it was therefore impossible to determine whether it possessed coenzyme activity (Adler *et al.* 1936). Pig-heart lactic dehydrogenase, however, is active at pH 10.2, and has now been used to test the yellow substance prepared at that pH.

Fig. 3. Test of yellow substance for activity as a coenzyme. $\bullet$, rate of reaction of yellow substance with pyruvate in the presence of lactic dehydrogenase; +, reaction of yellow substance with pyruvate alone; O , reaction with enzyme alone; $\times$, control curve, yellow substance with no enzyme or pyruvate. At 61 min., 0.4 mg. DPNH added to complete system.

Fig. 3 shows the result of a typical experiment in which 0.05 ml. lactic dehydrogenase solution was added to a $3 \times 10^{-4}M$ solution of yellow substance in the presence of $3.3 \times 10^{-3}M$ sodium pyruvate (total volume 3 ml.). There is a slow decrease in optical density at 360 $m\mu$. but this may not be significantly greater than the decrease observed when no enzyme was present. Fig. 3 also gives the control curves showing the decrease in optical density at 360 $m\mu$. for solutions of yellow substance alone, and for yellow substance with enzyme but no pyruvate.

After 61 min., 0.4 mg. DPNH was added to the solution containing enzyme and pyruvate, and it can be seen that the DPNH is rapidly oxidized, proving that the enzyme was still active in the solution.

A crude estimate based on the spectrophotometrically determined activity of the enzyme against DPNH gave the result that if the enzyme does oxidize the yellow substance (which cannot, of course, be regarded as proved) then it does so at less than 5% of the rate at which it oxidizes DPNH.

In an attempt to determine whether lactic dehydrogenase will reduce the yellow substance to DPNH in the presence of excess lactate, it was

found that for a $3 \times 10^{-4} \text{M}$ solution of yellow substance which was 1.1M in lactate, the optical density at $340 \text{ m}\mu$. increased slowly when lactic dehydrogenase was present. Interpretation of the result is difficult, as the effect could be due to reduction by the enzyme of DPN^+ formed by decay of the yellow substance. The interpretation is further complicated by the fact that a $2.3 \times 10^{-4} \text{M}$ DPN^+ solution in the presence of 1.1M lactate at $\text{pH } 10.2$ was found to have an absorption curve different from the curve of DPN^+ added to that of lactate (Fig. 4). It must be concluded from Fig. 4 that DPN^+ reacts with lactate at $\text{pH } 10.2$. It was found spectrophotometrically that it does not react at $\text{pH } 7.8$.

Fig. 4. Chemical reaction of sodium lactate with DPN. +, Absorption curve of DPN; O, absorption curve of lactate; x, absorption curve of DPN + lactate, $\text{pH } 10.2$.

DISCUSSION

Adler *et al.* (1936) suggested that the yellow substance obtained by reducing DPN with sodium dithionite in alkaline solution was a stable free radical. This hypothesis was rendered untenable by Yarmolinsky & Colowick's (1954) demonstration that sulphite-free solutions of the yellow substance give one mole of sulphite on neutralization under special conditions. Yarmolinsky & Colowick (1954; see also Colowick, 1954) advanced other evidence which pointed to the conclusion that the yellow substance is an addition compound between DPN and a derivative of dithionite. The demonstration reported here that the yellow substance is not paramagnetic confirms that it cannot be a free radical.

It has been shown previously (Swallow, 1954*a*) that X- or γ -rays in the presence of ethanol or certain other substances will, in the absence of air, act as a powerful reducing agent. In particular they will reduce DPN to a form which absorbs at $340 \text{ m}\mu$., but which does not possess coenzyme activity. The failure to reduce the yellow substance to DPNH or other species absorbing at $340 \text{ m}\mu$. is therefore compatible with the idea that it is not a half-reduced form of DPN. It should also be mentioned that it has been found impossible to reduce the yellow substance by the palladium-foil technique of Ubbelohde (1949) and Parravano (1951).

If the yellow substance is not a free radical it is perhaps not surprising that it is unreactive towards several chemical compounds. The lack of coenzyme activity is also to be expected. The incidental demonstration that under appropriate conditions DPN appears to react chemically with lactic acid may be of fundamental interest in connexion with the mechanism of lactic dehydrogenase action.

The demonstration that the yellow substance is not a free radical destroys one of the pieces of evidence that dehydrogenases work by a free-radical mechanism.

SUMMARY

1. The yellow substance obtained by reducing diphosphopyridine nucleotide (DPN) with sodium dithionite in alkaline solution is not paramagnetic, and therefore not a free radical.
2. Although on neutralization the yellow substance normally reverts to DPN^+ and sodium dithionite, it is confirmed that under special conditions DPNH can be formed.
3. Air-free solutions of the yellow substance in the presence of ethanol cannot be reduced by X-rays.
4. The yellow substance reacts with acetaldehyde and glycine but is otherwise unreactive. It possesses little or no coenzyme activity.

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The Retention of Metabolic Radioactive Carbonate

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There is urgent need for a procedure for calculating the combustion rate of a ^{14}C metabolite from the amount of ^{14}C carbon dioxide expired. In order to carry out such a calculation one must know the amount of ^{14}C retained in the bicarbonate, carbonate and carbamino compound pools of the body as a function of the amount of ^{14}C carbon dioxide which appears in the respiratory carbon dioxide.

When ^{14}C carbon dioxide is produced from a metabolite only in the circulating blood, or (to a good approximation) when ^{14}C carbon dioxide is produced in a few localities and is presented to most of the body as blood ^{14}C bicarbonate, it is possible to calculate the combustion rate directly from a preliminary experiment in which the retention of an instantaneous dose of ^{14}C bicarbonate, administered intravenously, is established as a function of elapsed time. This is the method used by Kornberg (1953) and Kornberg, Davies & Wood (1954) to calculate the rate of conversion of ^{14}C -urea into ^{14}C carbon dioxide by the gastrointestinal bacteria of the intact cat.

This paper considers the possibility of deriving, from the equation describing the retention of an intravenous dose of ^{14}C bicarbonate, an equation approximately describing the retention of a hypothetical dose of ^{14}C bicarbonate introduced in the 'soft tissue compartment' of the real animal, the latter equation being the one which is suitable for calculating the combustion rate of a metabolite which is burned generally throughout the soft tissues of the body. The procedure followed is to establish the desired equation for an 'ideal' cat, and then to use the observations of Kornberg (1953) to find out to what extent the ideal situation is present in the real cat.

THEORY

The properties of the ideal cat. Fig. 1 represents the situation as we might wish it to be in the cat. Since there are three terms in the exponential

equation which represents the retention of an intravenous dose of ^{14}C bicarbonate (Kornberg, 1953) and since we know that there is a central compartment which represents the circulating blood, we would like the other two terms of the three-term equation to reflect the presence of two discrete peripheral compartments. One of the two peripheral compartments we expect to represent the solid carbonate of bone with its slow rate of turnover and the other we expect to represent the

Fig. 1. Kinetic model for retention of ^{14}C introduced into the total acid-volatile CO_2 of the cat. A_B (roughly the blood compartment) contains A_B mg. of carbonate carbon and the fraction (y) at any time (t) of the original ^{14}C activity which was introduced at $t=0$. It loses $(k_3 + k_4)$ mg. C/min. as expired CO_2 and its rate of ^{14}C loss to the expired air is $\frac{k_3 + k_4}{A_B}(y)$ /min. Its rates of ^{14}C loss to

A_1 and A_2 are $\frac{k_1}{A_B}(y)$ and $\frac{k_2}{A_B}(y)$ respectively. A_1 (roughly the bone compartment) contains A_1 mg. carbonate carbon and the fraction (z) of the original ^{14}C dose. It gains k_4 mg. $^{14}\text{CO}_2$ C/min. by metabolic CO_2 production within A_1 , and loses ^{14}C to A_B at the rate of $\frac{k_1 + k_4}{A_1}(z)$ /min. A_2 (roughly the soft tissue compartment) is analogous to A_1 .