ON THE MECHANISM OF PYRIDINE NUCLEOTIDE REDUCTION BY DITHIONITE*, **

by

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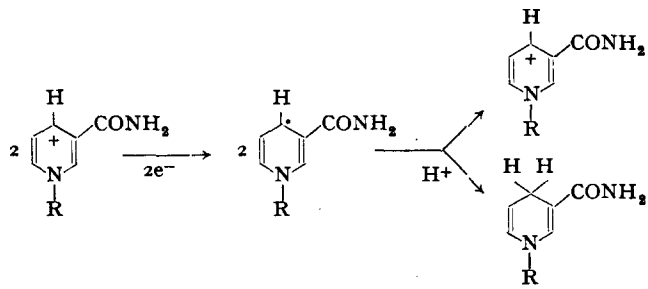
The reduction of a pyridine nucleotide by sodium dithionite (hydrosulfite) in mildly alkaline medium takes place with the formation of a transient yellow intermediate which may be stabilized by strongly alkaline conditons[1]. It has been suggested that the yellow intermediate, Y§, formed in the course of dithionite reduction of DPN represents a "half-reduced", free-radical form of DPN[2-5] and that this form of the coenzyme may be of significance in respiration[3], possibly being stabilized on certain apodehydrogenases and responsible for their enzymic activity[6,7]. The formation of DPNH from Y has been assumed to occur by dismutation of two free radicals (Equation A) to yield one molecule each of DPN and DPNH[4,5]. The DPN could be recycled according to this view.

(A)



The possibility has been considered by earlier investigators[2] that Y might be a sulfur-containing compound rather than a free radical. The present paper offers

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§ Abbreviations: DPN or DPN_{ox} for oxidized diphosphopyridine nucleotide; DPNH or DPN_{red} for reduced diphosphopyridine nucleotide; R for ribose-pyrophosphate-adenosine; NMN for nicotinamide mononucleotide; Y and Y′ for the yellow intermediate(s) formed in the course of reduction of DPN by dithionite and by hydroxymethyl sulfinate, respectively; tris for tris-(hydroxymethyl)aminomethane; ADH for yeast alcohol dehydrogenase; μM for micromoles.

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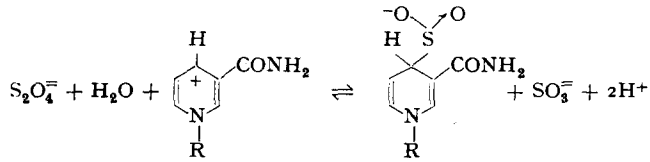
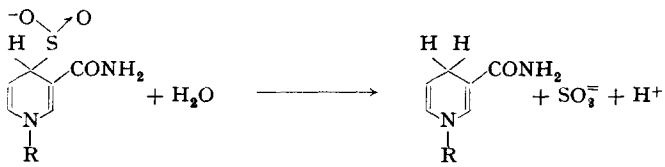
evidence that Y is the sulfinate derivative of DPNH*, resulting from the transfer of a portion of the dithionite ion to carbon 4 of the nicotinamide ring of DPN. The formation of DPNH from Y is believed to occur by hydrolysis rather than by dismutation (Equations B and C).

(B) 

(C) 

Preliminary accounts of this work have appeared[8,9].

RESULTS

Formation and characteristics of Y

The formation of Y from DPN and dithionite can be conveniently followed spectrophotometrically by measuring optical densities at 400 mμ. At this wave length the absorption of Y is not appreciably interfered with by absorption due to DPN, DPNH (see Fig. 1) or dithionite. In a medium containing sufficient dithionite and alkali, DPN is completely converted to Y and the reaction is prevented from going further. Under these conditions the formation of Y exhibits a logarithmic time-course over an interval during which the fraction of dithionite disappearing is small (Curve "Y", Fig. 2).

The absorption spectrum of Y in 0.05 *N* NaOH (Fig. 1) shows a broad low band extending out into the visible region, in agreement with the observations of earlier workers[1]. This has an extinction maximum of $3.2 \cdot 10^6$ cm^2 mole^{-1} at about 357 mμ. In addition, the extinction of Y in the neighborhood of 260 mμ is strikingly increased over the values characteristic of DPNH or DPN. Since similar rises in extinction appear upon forming the NMN analog of Y, the spectral changes in the formation of Y may be entirely attributed to effects upon the nicotinamide ring. The large contribution of the nicotinamide moiety to the 260 mμ absorption of Y is retained at pH 11. At this same pH the nicotinamide moiety of a number of tertiary N^1-substituted nicotinamide compounds makes no contribution whatsoever to their 260 mμ absorption. This applies to DPNH and to DPN adducts with cyanide[10], with carbonyl compounds[11], with hydroxylamine**, or with bisulfite*** (measuring the light absorption of the last mentioned at neutrality, where the compound is stable[10,12]). Y also differs from the DPN compounds just listed in that it fails to show detectable

* Adducts between DPN and various carbonyl reagents are named as derivatives of DPNH. Thus Y (or Y') is referred to as sulfinyl DPNH.

** R. M. BURTON AND N. O. KAPLAN, unpublished data.

*** Unpublished observations of the authors.

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fluorescence with exciting light of wave length 2537 A ("Mineralight") or 3650 A (Coleman photofluorometer). However, the above-mentioned observations appear insufficient to rule out a tertiary N^1-substituted nicotinamide compound. Thus Y behaves more like DPNH than like DPN in that it is stable in alkali, forms no cyanide addition complex and resists cleavage by *Neurospora* DPNase[13].

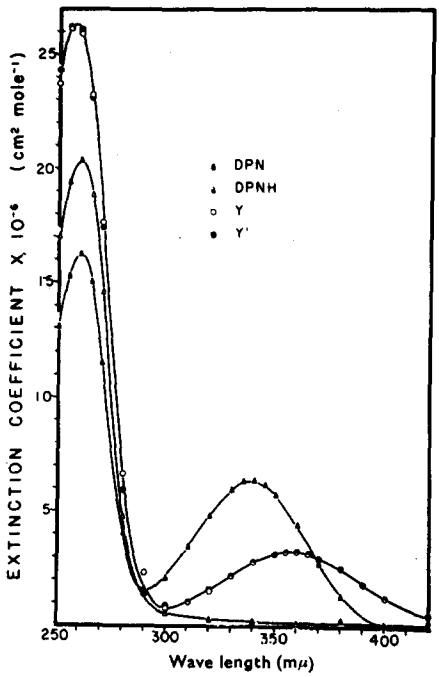

Fig. 1. Absorption spectra of DPN, DPNH, Y, and Y'. DPN (Pabst) was a sample of 86% purity by weight and served as a source of DPNH, Y, and Y'. The spectrum of DPN was measured in unbuffered solution, of DPNH in 0.1 *M* tris pH 10, and of Y and Y' in 0.05 *N* NaOH. Appropriate corrections were made for the absorption of the reducing agent or its oxidation products and for incompleteness of conversion of DPN to Y' at low hydroxymethyl sulfinate concentrations. Because of the instability of Y and Y' the points for these curves represent extrapolated values obtained from several readings at each wave length. The extinctions at and below 290 mμ in the case of Y' were determined with the aid of a photomultiplier, necessitated by the high absorption of the reductant. See MATERIALS AND METHODS for further details.

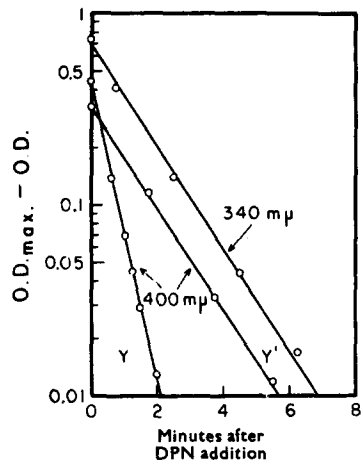

Fig. 2. Logarithmic time course of Y and Y' formation. In each case the reaction was initiated in a cuvette of 1 cm light path by the addition of an aqueous solution of DPN to an alkaline solution of the reductant, final volume 3 ml. Curve Y: 1.1 μ*M* of DPN, 11 μ*M* of sodium dithionite in 0.05 *N* NaOH. Curves Y': 0.9 μ*M* of DPN, 80 μ*M* of sodium hydroxymethyl sulfinate in 0.17 *M* glycine buffer, pH 10.5.

Aerobic decomposition of Y

Solutions of Y freed of dithionite by oxygenation[2] readily decompose in air to yield DPN. The reaction is first order with respect to Y and is strongly dependent on pH and the nature of the buffer (Fig. 3). From Fig. 3 it appears that the lability of Y is influenced to very different degrees by the different ionic species of the buffer L-arginine.

The reaction yields almost entirely DPN regardless of which of the indicated buffers is present. A variable but small amount of DPNH may appear on the aerobic neutralization of solutions of dithionite-free Y. The DPNH appears independently of whether or not the solution is freed of sulfite. Under a variety of aerobic conditions the yields of DPNH have never exceeded 50% of the total nucleotide present, and

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usually the yield has been no more than 10–15%. This observation is in agreement with the earlier results of SCHLENK *et al.*[5]. In general, after the complete decomposition of Y all the nucleotide may be accounted for as the sum of DPN and DPNH. The observed partial decomposition of Y to DPNH in the absence of any reducing agent requires that Y be at a reduction level either half-way between DPN and DPNH or at the reduction level of DPNH itself. The very low yields of DPNH obtained upon neutralization of Y in air suggest that under the usual aerobic conditions the principal reaction which Y undergoes involves autoxidation.

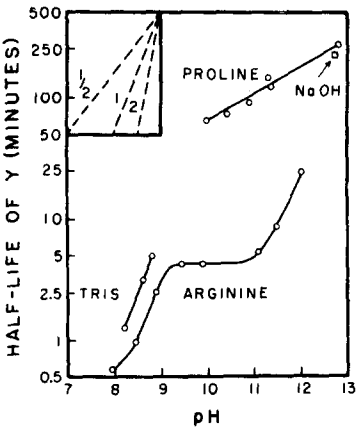

Fig. 3. Buffer and pH effects on the stability of Y under aerobic conditions. The approximate half-life of Y in solutions of the indicated buffers at room temperature (*ca.* 25°) was computed (by extrapolation when necessary) from the rates of decrease of 400 mμ absorption of dithionite-free solutions of Y. The buffers tris (pK = 8.1) L-arginine ($pK_1 = 9.0$, $pK_2 = 12.5$), and L-proline (pK = 10.6) were 0.1–0.2 M; NaOH was a 0.05 N solution for which the pH was estimated by calculation. The insert gives the slopes expected for decomposition reactions in which the rate-limiting step involves the participation of the indicated numbers of hydrogen ions per molecule of Y.

Anaerobic decomposition of Y

Solutions of Y were found to exhibit greater stability anaerobically than when open to the air. However, even under anaerobic conditions, Y becomes very unstable at neutral or slightly alkaline reactions. For example, at 26° the half-life of Y in tris buffer is less than half a minute at pH 7.4 in the presence of air, while Y has a half-life of 4 minutes at this same pH and temperature in the absence of air.

As may be seen from Table I, the major nucleotide product of the *anaerobic* decompositon of Y is not DPN but DPNH. This finding has been confirmed by SWALLOW[14]. Similar results may be obtained with preparations which have been freed of sulfite. In the experiment of Fig. 4 neutralization of sulfite-free Y is carried out anaerobically and the nucleotide reactant and product followed by measuring optical densities at 400 and 340 mμ, preliminary to enzymic assay of the yield of DPNH and DPN.

TABLE I

YIELD OF DPNH FROM DITHIONITE-FREE Y UPON ANAEROBIC NEUTRALIZATION

Experiment number	*1*	*2*	*3*	*4*	*5*	*6*	*Average*
Yield of DPNH expressed as per cent of initial DPN	73	81	85	86	91	93	85

The procedure in a typical experiment (No. 3) was as follows: Equal volumes of 0.03 M DPN and 0.04 M sodium dithionite in 0.3 N NaOH were mixed. After 2 minutes the solution was oxygenated for 8 minutes and 1.0 ml of the yellow solution was diluted in 8.1 ml 0.1 N NaOH in a Thunberg tube. This was evacuated, sealed, and 0.9 ml 1 M KH_2PO_4 was tipped in from the Thunberg top. After incubation for one hour at 37° the tube was chilled, opened, and the contents assayed for DPNH. The final pH was 7.8.

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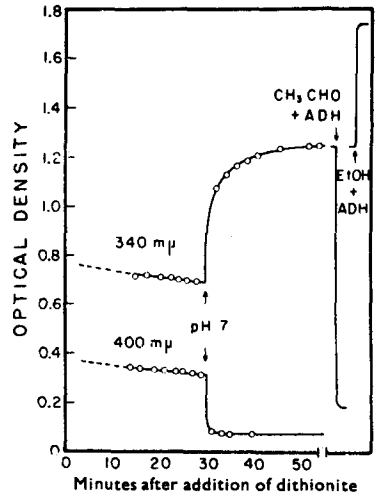

Fig. 4. Anaerobic neutralization of dithionite-free, sulfite-free Y. To 2.5 μM of DPN (Pabst) in 5.4 ml of 0.5 M tris-glycine buffer, pH 9.9, 20 μM of sodium dithionite were added making a final volume of 6 ml. One minute was allowed for color development and 1 ml of 1 M $BaCl_2$ was added. The mixture was oxygenated for two minutes and filtered with suction through fine sintered glass. Turbidity in the filtrate due to $BaCO_3$ formation was prevented by adding 3 ml 1 M Na_2SO_4 and refiltering as before. Of the clear yellow filtrate 2.5 ml were removed to an anaerobic cuvette of 1 cm light path with a Thunberg top containing 0.3 ml 2 N H_2SO_4. The cuvette was evacuated and sealed. Y and DPNH were followed spectrophotometrically with a Model B Beckman spectrophotometer by measuring optical densities at 400 and 340 mμ against a water blank. Neutralization was effected by tipping in the acid and an hour later air was admitted and samples taken for enzymic assay of DPN and DPNH with alcohol dehydrogenase and ethanol or acetaldehyde, respectively. The readings have been corrected to values appropriate for a final volume of 3 ml. It may be calculated from this graph that for every 0.25 μM of DPN initially present, at neutralization time 0.21 μM may be accounted for as Y, and after neutralization 0.17 μM appear as DPNH and 0.08 μM as DPN.

The observation that Y may be converted almost stoichiometrically to DPNH in the absence of any reducing agent may be taken as evidence that Y is at the reduction level of DPNH. The data do not conform to the view that Y represents a half-reduced intermediate, but suggest instead that Y is the product of an addition reaction between DPN and dithionite or a derivative of dithionite.

Stoichiometric release of sulfite from Y

When freed of contaminating sulfite by barium treatment, Y yields sulfur dioxide upon acidification. Preliminary experiments, in which barium-treated Y was acidified directly, indicated the expected dependence of the yield of sulfur dioxide upon the amount of Y present. The molar ratios of sulfur dioxide released to Y acidified averaged 0.65 in four experiments with varying initial DPN concentrations. To reduce the possibility that this result is due to a solubilizing effect of nucleotide on barium sulfite, experiments were performed which demonstrate that decomposing Y simply by neutralization suffices to release barium-precipitable sulfite in nearly stoichiometric amounts. In each experiment of Table II the sulfite formed upon neutralization of a solution of Y was precipitated with barium and the precipitate analyzed for sulfite. The control and experimental solutions are identical except with respect to the order of addition of acid and alkali. A mole for mole release of sulfite from Y is approximated in these experiments, for which the average of six determinations of sulfite found after neutralization is 91% of theoretical; the small amounts of sulfite found without neutralization are probably accounted for by slight hydrolysis of Y occurring even at the alkaline pH.

This result suggests that Y possesses a sulfur residue, containing probably one atom of sulfur which neutralization liberates as sulfite. It provides no information as to whether the linkage of sulfur to carbon is direct or through oxygen.

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TABLE II

STOICHIOMETRY OF RELEASE OF SULFITE FROM Y UPON NEUTRALIZATION

For each experiment Y was prepared by mixing equal volumes of 0.1 M $Na_2S_2O_4$ (in 0.5 N NaOH) and 0.05 M DPN. After 2 minutes the solution was freed of dithionite by oxygenating for 8 minutes and the resulting sulfite precipitated by the addition of 0.3 ml 1.5 M $BaCl_2$ to 1.2 ml of solution. The precipitate was centrifuged down for 5 minutes at 25,000 g in the cold and 0.2 ml of the clear yellow supernatant liquid introduced into each of four small centrifuge tubes at room temperature. One aliquot was brought to pH 8 with neutral tris-HCl buffer and, after decolorization was complete, brought back to pH 13 with NaOH. To the remaining aliquots a solution of tris-NaCl was added to bring them to the same salt concentration and pH as in the neutralized realkalized fraction. One of the unneutralized aliquots served as a blank. The remaining two aliquots provided internal standards. To each was added freshly prepared solutions of $NaHSO_3$ at levels of 2 and 4 μM/ml, respectively. All solutions, at a final volume of 1 ml, were centrifuged as before and the supernatant liquids discarded. In the first three experiments the precipitates were suspended in water and the color reagent was added. The assay for sulfite was as described under MATERIALS AND METHODS. In the second set of three experiments the sulfite was released into solution from the precipitates by adding a solution of Na_2SO_4 to each centrifuge tube, stirring, and recentrifuging. The supernatant liquids were then assayed for sulfite. The concentrations of Y are based on initial DPN throughout. Values for sulfite are corrected for the per cent recovery of added sulfite.

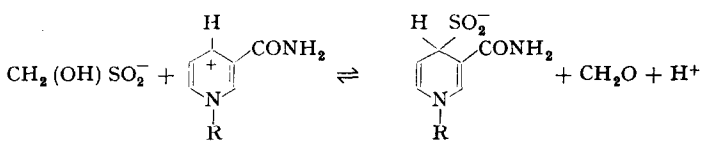
Expt. No.	*Per cent recovery of added sulfite*	*Moles SO_3 found per mole of Y*	
		without neutralization	*after neutralization*
1	76	0.10	0.79
2	89	0.09	0.83
3	67	0.10	0.93
4	95	0.05	0.78
5	65	0.04	1.06
6	72	0.00	1.06

Reduction of DPN with sulfinates

The exact nature of the sulfur-containing addition compound, Y, is suggested by a consideration of the "aldehydic character" (*i.e.*, potentiality for carbonium ion formation) of the 4-position of the nicotinamide ring of DPN (see DISCUSSION). Dithionite is known to react with certain aldehydes in alkaline solutions to form what are believed to be sulfinate compounds, $-\overset{|}{\underset{|}{C}}-SO_2^-$ [15-18] which, like Y, are stable to oxygenation. The best known example of such reactions,

(D) $$^-O_2S\text{–}SO_2^- + CH_2O + H_2O \rightleftharpoons CH_2(OH)SO_2^- + SO_3^= + H^+$$

is considered to yield hydroxymethyl sulfinate. The analogous reaction between dithionite and DPN (Equation B) would yield sulfinyl DPNH. If Y and sulfinyl DPNH are identical, alkaline conditions might be expected to favor a transfer of the sulfinate moiety of various organic sulfinates to DPN, *e.g.*,

(E) 

The expectation that sulfinates may react with DPN as described by Equation E to yield the same yellow compound produced by the action of dithionite is confirmed by the finding that hydroxymethyl sulfinate and amino imino methane sulfinate react with DPN at pH 10 or above to form yellow compounds which appear to be identical with Y. The yellow compound resulting from the reaction of DPN with hydroxymethyl sulfinate, we designate Y'. Conditions for its formation are described under MATERIALS AND METHODS. The absorption spectra of the yellow derivatives, insofar as it was possible to compare them, appear to be identical (*cf.* Fig. 1).

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Following formation of the yellow DPNH derivative by either of the above-mentioned sulfinates, enzymically active DPNH may be formed, as anticipated, simply by neutralization. These reductants, unlike dithionite, are stable to oxygenation and were not removed. The yields of DPNH were therefore appreciable even upon aerobic neutralization since regeneration of the yellow intermediate was constantly taking place. The possibility that dithionite generated from the reductant is responsible for Y′ formation is inconsistent with the extreme stability of solutions of hydroxymethyl sulfinate to oxygenation and with the absence of dithionite's characteristic absorption peak at 313 mμ in unoxygenated solutions.

Sulfinate transfer from Y to formaldehyde

Another point of similarity between Y and Y′ is their reactivity with formaldehyde. The reaction with formaldehyde provides the most satisfactory evidence for the hypothesis that Y is a sulfinate of DPNH. According to this view the addition of formaldehyde to Y should reverse Reaction E and yield as products DPN and hydroxymethyl sulfinate. In a typical experiment a 0.1 mM solution of Y in 0.02 N NaOH was half decomposed in about two minutes by the addition of formaldehyde at 2.0 mM final concentration. That DPN is formed has been confirmed by the cyanide reaction[10]. Advantage was taken of the reducing action of the sulfinates upon silver ion to identify the other product, by its electrophoretic mobility on paper, as

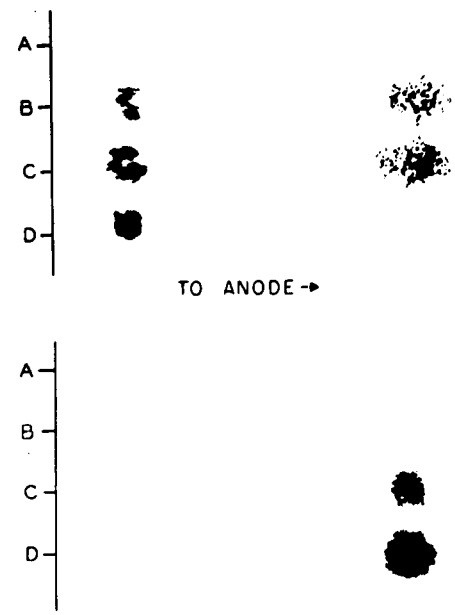

Fig. 5. Identification of hydroxymethyl sulfinate as a product of the reaction of formaldehyde with Y (above) and with amino imino methane sulfinate (below) by paper electrophoresis. In each case the paper was a strip of Whatman No. 3, 7 cm wide; the solvent was 0.05 N NaOH. The paper was spotted with solutions of approximately the same alkalinity as the solvent. The electrophoresis apparatus and technique was as described by MARKHAM AND SMITH[19]. A potential of 500 volts was maintained for 1.5 hours following which the paper was air-dried to dampness and sprayed with 5% ammoniacal $AgNO_3$. Development of the spots was not immediate. When it was complete, the paper was washed in "rapid liquid fixer" (Eastman Kodak) for 5 minutes, in running tap water for 20 minutes, dried, and photographed. *Above*: The 6 μl of solution used for each spot contained A. 0.05 μM of dithionite-free, sulfite-free Y, prepared essentially as described in Table II. (A control solution, DPN omitted, was prepared simultaneously for use at spots C and D.); B. 0.05 μM of dithionite-free, sulfite-free Y, 2 μM of formaldehyde; C. 0.05 μM of sodium hydroxymethyl sulfinate, 2 μM of formaldehyde in control solution; D. 2 μM of formaldehyde in control solution. The solutions were allowed to incubate for an hour at room temperature (23°) before application to the paper. *Below*: The 10 μl of solution used for each spot contained A. 0.1 μM of sodium amino imino methane sulfinate; B. 2 μM of formaldehyde; C. 0.1 μM of sodium amino imino methane sulfinate, 2 μM of formaldehyde; D. 0.1 μM of sodium amino imino methane sulfinate, 0.1 μM of sodium hydroxymethyl sulfinate. The solutions were allowed to incubate for an hour at room temperature (25°) before application to the paper. The paper was dried sufficiently well before spraying so that no formaldehyde spot appears here. Compare with the upper photograph.

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hydroxymethyl sulfinate (Fig. 5 [above*]). The formaldehyde-treated Y produces two reducing spots. One of these, near the origin, corresponds to formaldehyde. The other is identical in mobility, appearance, and size with a spot obtained from the appropriate concentration of commercial sodium hydroxymethyl sulfinate**. We take this to support strongly the view that Y is a sulfinyl derivative of DPNH.

Reaction E may be regarded as a sulfinate transfer in which DPN participates as either the acceptor or the donor. To establish that reactions of this general type can and do occur, a transfer of the sulfinate radical from amino imino methane sulfinate to formaldehyde was demonstrated:

(F) $$HN{:}C(NH_2)SO_2^- + CH_2O + H_2O \rightleftharpoons CH_2(OH)SO_2^- + (NH_2)_2C{:}O$$

This was accomplished by showing that the product of Reaction F has the same electrophoretic mobility and capacity to reduce silver ion as commercial hydroxymethyl sulfinate (Fig. 5 [below]).

Incorporation of deuterium in the conversion of Y to DPNH

Direct hydrolysis of sulfinyl DPNH (Equation C) could give rise to DPNH and bisulfite. This mechanism for the conversion of Y to DPNH was tested by the two reciprocal experiments of Table III, which were designed to demonstrate that in the reduction process deuterium from heavy water may be incorporated into the final product (DPN_{red}) without being incorporated into the intermediate (Y). In each experiment Y was made at pH 11*** and, after oxygenation, brought to pH 7 anaerobically so as to form DPN_{red}.

The DPN_{red} to be assayed for deuterium content was reoxidized with neutral ferricyanide. This procedure removes less deuterium than does reoxidation with ADH[22]. The resulting DPN_{ox} was split with DPNase to yield nicotinamide, which was isolated by passing the solution through an anion exchange resin. The effluent and washings were assayed for nicotinamide and then diluted by a known factor with unlabeled carrier nicotinamide. Exchangeable deuterium was removed by alternately diluting with normal water and drying. The nicotinamide was crystallized from benzene, dried, and analyzed for deuterium.

The data show that in the reduction process the only step which incorporates the deuterium of heavy water into the product is the conversion of Y to DPN_{red}, in accord with the proposed reaction mechanism.

* Untreated Y appears to be oxidized by air, perhaps in the course of the partial drying to which the paper was subjected before development of the spots.

** The term "formaldehyde sulfoxylate", originally applied to this compound[20] and still commonly used, appears to be a misnomer in view of the evidence favoring the sulfinate structure[15–18]. The interpretation that Y represents the sulfoxylate of DPN, *i.e.*, DPN–OSO^-, previously reported by us[8,9], was based on incomplete information concerning the structure of the so-called sulfoxylates. It introduces unnecessary difficulties especially as regards the hydrolytic conversion of Y to DPNH.

*** When Y was made under more alkaline conditions (0.15 milliequivalents of NaOH per ml of deuterium oxide) and neutralized anaerobically in a relatively large volume of normal water, the resulting DPN_{red} was found to contain an amount of unexchangeable deuterium comparable to the amount incorporated in the reciprocal experiment. This result may be due to an enolization of the sulfinate group of Y at high pH values, rendering the hydrogen atom at the 4-position of the nicotinamide ring susceptible to exchange with the medium. Recently, SAN PIETRO showed the DPN-cyanide addition compound to be, like Y, susceptible to proton exchange with the medium under conditions sufficiently alkaline to cause enolization[21]. The proton which can undergo exchange was found to be the one at the 4-position.

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TABLE III

INCORPORATION OF DEUTERIUM IN THE REDUCTION OF DPN

Equal volumes of 0.6 *M* L-proline buffer and 0.03 *M* DPN were mixed and twice as many moles of solid sodium dithionite as DPN present were added. After two minutes the yellow solution was oxygenated for eight minutes and 1 to 3 ml transferred to the bulb of a Thunberg tube which was fitted onto a Thunberg tube containing 9 volumes of 0.1 *M* potassium phosphate buffer, pH 6.8. In the first two experiments the DPN and proline buffer (pH 10.8) were made up in deuterium oxide. In the second two experiments the DPN and proline buffer (pH 10.7) were made up in normal water and the phosphate buffer in deuterium oxide. The tubes were evacuated, sealed, tipped, and incubated at 37° for 30 minutes. Their contents (pH 7.0) were then assayed for DPN_{red} and prepared for deuterium analysis as indicated under MATERIALS AND METHODS.

Procedure	*Per cent yield of DPN_{red} from DPN_{ox}*	*Atom per cent excess deuterium measured*	*Atoms D per molecule of DPN_{red}**
$DPN_{ox} \xrightarrow{D_2O\ /\ H_2O = 1/9} Y \rightarrow DPN_{red}$	79	0.037	0.05
	73	0.030	0.05
$DPN_{ox} \xrightarrow{D_2O\ /\ H_2O = 9/1} Y \rightarrow DPN_{red}$	72	0.245	0.43
	62	0.345	0.65

* Basis of calculations: The atom per cent excess of deuterium in the nicotinamide was converted to deuterium atoms per molecule of DPN_{red} with the aid of an appropriate conversion factor. In the determination of this factor the following assumptions were made: (a) The DPN unaccounted for as DPN_{red}, which was not separated from the latter, was assumed to contribute unlabeled nicotinamide. (b) Ferricyanide oxidation of deuterium-labeled DPN_{red} formed with dithionite in deuterium oxide was assumed to leave the nicotinamide ring with 54% of the unexchangeable deuterium[22].

DISCUSSION

The use of dithionite for the reduction of the pyridine nucleotides was introduced by WARBURG[23,24]. Shortly thereafter the yellow intermediates which occur in the dithionite reduction of DPN[1] and N^1-methyl nicotinamide iodide[3] were investigated. According to KARRER AND BENZ[3] the formation of such intermediates appears to be a necessary stage in the dithionite reduction of pyridinium compounds.

Simultaneously and independently the intermediates which occur in the dithionite reduction of DPN[2–4] and N^1-methyl nicotinamide iodide[5] were interpreted as representing free radicals because of such properties as reducing power (in both the kinetic and thermodynamic sense), instability and color. At best, this evidence was only suggestive. The observation of KARRER *et al.*[25] that the ferricyanide oxidation of N^1-methyl dihydronicotinamide proceeds with the intermediate appearance of a strongly negative potential remains to be explained, but appears to bear no relation to the intermediate produced in the dithionite reduction of N^1-methyl nicotinamide.

Arguments of a more general nature have been advanced in support of the existence of a DPN free radical and of the biological importance of free radical forms of the coenzymes. Particularly influential has been MICHAELIS' concept of obligatory one-electron transfer[26] as well as analogies between enzyme-catalyzed reactions and free radical chain reactions[7,27–29]. Compulsory one-electron transfer no longer appears to be an inviolable rule in the oxidation-reduction reactions of either organic or

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inorganic chemistry[30]. Further, although some enzymes may act so as to initiate free radical chain reactions (*e.g.*, lipoxidases[31]), and although there is perhaps an additional case of an enzyme stabilizing a free radical form of its prosthetic group[32], to date there is no convincing evidence that free radicals of the pyridine nucleotides participate in enzymic reactions[30], despite contrary arguments offered by MACKINNON AND WATERS[28].

Recently, authors have accepted or noted without criticism the hypothesis that the yellow intermediates formed upon the reduction of the pyridine nucleotides with dithionite are half-reduced forms of the coenzymes[33,34]. While there was formerly reason to believe it unlikely, the free radical hypothesis has now been rendered untenable by several lines of evidence. Whereas Y can be prepared from DPN by the action of certain closely related reductants possessing the sulfinate grouping, no such compound appears on reducing DPN with borohydride[35,36] or at a mercury electrode* (although yellow compounds clearly unrelated to Y appear as byproducts in both cases). For a free radical, Y in strongly alkaline solution is remarkably resistant to further reduction by dithionite, by amalgams of sodium or zinc[2], by X-irradiation or by hydrogenation[14]. The practically stoichiometric conversion of Y to DPNH in the absence of reductant is additional evidence against the free radical hypothesis. By microwave spectroscopy it has not been possible to detect the presence of any free radicals during the dithionite reduction of N^1-propyl nicotinamide iodide[37]. Another recent investigation[14] shows that solutions of Y lack the paramagnetic susceptibility required if Y were a free radical.

The nearly stoichiometric release of sulfite from Y under mild treatment suggests that Y is an addition compound containing one sulfur atom per molecule**. Although it was clear in 1936 that the reaction which produces Y modifies, in some way, the nicotinamide ring[1], specification of the site of attachment of the sulfur-containing residue of Y has had to await the recent demonstration that dithionite reduces DPN at the 4-position of the nicotinamide ring[22,39] ([40,41] compare also). The dithionite ion may be expected to attack the 4-position of the nicotinamide ring and react there as it is believed to react with aldehydes, namely, to form a sulfinate. The aldehydic character of DPN is also shown by the existence of reactions of the nucleotide with bisulfite[12], cyanide[10,12], carbonyl compounds[11], and hydroxylamine[42]. In the case of cyanide it is now established[21] that this addition is also at the 4-position, *i.e.*, gamma to the ring nitrogen.

The hypothesis that Y represents sulfinyl DPNH is consistent with the proposal[16,18] and recent proof*** that dithionite possesses the symmetrical dimeric stucture

* An alkaline solution of DPN (Coenzyme "65", Sigma) has been reduced electrolytically at a mercury cathode with an automatic control to maintain the impressed potential constant. The instrument was made available to us through the kindness of Dr. ALSOPH CORWIN. The impressed potential was varied gradually from 0.10 V to —1.75 V with respect to the standard calomel electrode. At least 30% of the DPN originally present was converted to enzymically active DPNH. The product was contaminated with a yellow material stable at neutrality.

** Y is not a member of the class of sulfite or dithionite addition compounds reported by KARRER *et al.*[38] to arise secondarily upon dithionite reduction of certain N-alkyl pyridinium salts (but not of N^1-alkyl nicotinamide salts). The derivatives described by KARRER's group are pictured as the result of sulfite (or dithionite) addition onto the already reduced pyridine ring. Neither sulfite nor dithionite treatment of DPNH results in Y formation.

*** J. D. DUNITZ, personal communication, has determined the structure of anhydrous sodium dithionite by X-ray analysis. The molecule consists of two SO_2^- radicals held together by a rather weak S–S bond.

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which the name should denote. The ability of a number of sulfinates to substitute for dithionite in the reaction which forms Y, makes it clear that Y does not represent an addition of the entire dithionite ion to DPN. The sulfinate hypothesis is strongly supported by the identification of hydroxymethyl sulfinate as the product of the reaction between Y and formaldehyde and by the demonstration that sulfinate transfer can occur readily. The observation that Y decomposes largely to DPN and sulfite on aerobic neutralization is in keeping with the ease of air oxidation of certain sulfinates. Although sulfinate oxidation usually results in a stable sulfonic acid, in the case of sulfinyl DPNH the sulfonate oxidation product would presumably be identical with the DPN-bisulfite addition product which would be expected to dissociate immediately into DPN and sulfite. The sulfur-containing group of Y may be oxidized by substances other than oxygen. Y can reduce a dye such as methylene blue, a metallic ion such as silver[1,2], or sulfite (to dithionite)[2,4], reductions which are equally well effected by certain sulfinates*[17,44]. With respect to lability to heat[1,2] and stability towards cyanide and alkali Y again behaves like a typical sulfinate derivative of a carbonyl compound[45].

In support of the view that DPNH formation from Y can proceed by hydrolysis of the sulfinate, an analogous reaction may be cited, namely the spontaneous hydrolysis of the sulfinate of pyruvic acid with the liberation of sulfite[46]. The experiments with heavy water are also consistent with the proposed reaction mechanism. Thus the bulk of the evidence appears to support the hypothesis that the reduction of DPN by dithionite, or by certain organic sulfinates, proceeds *via* the sulfinate of DPNH.

The mechanism of DPN reduction illustrated here may bear some relation to the enzymic reduction of DPN by D-glyceraldehyde 3-phosphate dehydrogenase[47]. RACKER AND KRIMSKY[48] have suggested that this enzyme forms an addition compound with DPN, the two being connected through a sulfur bridge. According to these authors DPNH is formed by aldehydolysis of the DPN-S-enzyme compound in much the same way as we picture hydrolysis to release DPNH from sulfinyl DPNH.

MATERIALS AND METHODS

Reagents

Sodium dithionite was obtained from Eimer and Amend as sodium hydrosulfite, pure, low in iron. It was assayed for purity by the method of SMITH[49] and found to be 70 to 81 % pure, depending on the period of storage of the opened bottle. Sodium hydroxymethyl sulfinate was "sodium formaldehyde sulfoxylate", practical grade, purchased from the Eastman Kodak Company. Amino imino methane sulfinic acid was prepared by oxidation of thiocarbamide (Eastman Kodak, practical grade) according to the method of BARNETT[43]. Heavy water was purchased from the Stuart Oxygen Company on allocation from the U.S. Atomic Energy Commission and was more than 99.5 % D_2O. NMN was prepared by the action of snake venom pyrophosphatase* on DPN. Purity was determined by reaction with cyanide[10]. The DPN was Cozymase "90", Sigma, unless otherwise indicated. As a routine DPN_{ox} and DPN_{red} were assayed enzymically with ADH prepared according to RACKER[50], by measuring the change in optical density at 340 mμ on the addition of ethanol at pH 10 or acetaldehyde at pH 8, respectively. An extinction coefficient for DPN_{red} of $6.3 \cdot 10^6$ cm^2 $mole^{-1}$ [51,52] was used in calculating concentrations. In certain experiments DPN was assayed by spectrophotometric measurement of the DPN-cyanide complex.

* Although BARNETT's observation[43] that amino imino methane sulfinic *acid* does not reduce metal salts may be correct, the same is not true of the sulfinate. We find that in ammoniacal solution the sulfinate reduces silver nitrate instantaneously, just as does Y. The failure to obtain reducing spots with either Y or amino imino methane sulfinate (Fig. 5, positions A) must be due to enhancement of the lability of these compounds under the treatment they receive.

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Preparation of the yellow compounds

For the determination of the absorption spectrum in Fig. 1, Y was formed in a relatively concentrated solution of final volume 1 ml, 0.05 N in NaOH, and containing 0.5 μM of DPN and 5 μM of sodium dithionite. The latter was dissolved in the alkali immediately prior to the addition of DPN. After allowing three minutes for the formation of Y to be completed, the solution was made up to the volume appropriate for the spectral measurements using 0.05 N NaOH. A stream of oxygen was passed through the yellow solution in the cuvette until the excess dithionite (determined spectrophotometrically at 313 mμ[4]) was completely destroyed; one minute sufficed. Essentially the same procedure for preparing Y was used in the remaining experiments.

Dithionite decomposed by oxygenation in alkaline solutions yields, as the major product, sulfite[53,54]. Addition of excess $BaCl_2$ in solution, followed by centrifugation in the cold (25,000 g, 5 minutes) quantitatively removes sulfite without loss of Y. The excess barium may be removed by precipitation with Na_2SO_4 in a similar manner.

Y′ is formed by the action of hydroxymethyl sulfinate on DPN in alkaline solution. The hydroxymethyl sulfinate is not readily autoxidized and no attempt was made to remove the excess. Conveniently, 0.1 M sodium hydroxymethyl sulfinate in 0.05 N NaOH exhibits no light absorption above 300 mμ. For the determination of the far ultraviolet portion of the absorption spectrum of Y′ (Fig. 1) 98% conversion of DPN to Y′ was obtained by adding 0.1 ml of 5 mM DPN to 0.5 ml of 100 mM sodium hydroxymethyl sulfinate in 0.05 N NaOH, and diluting to 15 ml with 0.05 N NaOH after three minutes.

Sulfite determinations

Sulfite was determined by the micromethod of GRANT[55]. The test is based on a reaction of sulfur dioxide with fuchsin and formaldehyde to form a red chromogen. Calculation of sulfite concentrations in the unknowns was based on comparison with internal standards.

Determination of deuterium incorporated into DPN_{red}

The procedures for chemical oxidation of DPN_{red}, cleavage of the oxidized product, isolation and combustion of the resulting nicotinamide, and mass spectrometric analysis were essentially the same as those employed by PULLMAN, SAN PIETRO, AND COLOWICK[22].

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SUMMARY

1. Evidence is presented that the reduction of the pyridine nucleotides by sodium dithionite occurs *via* a sulfinate, the added group being attached to the 4-position of the pyridine ring. It is suggested that hydrolysis of the sulfinate yields reduced nucleotide and sulfite.

2. The intermediate is identified with a yellow stage which has often been regarded as a half-reduced, free radical form of the coenzyme.

3. Two organic sulfinates have been shown to be capable of reducing DPN to enzymically active DPNH *via* the same yellow intermediate.

4. A possible similarity between DPN reduction *via* a sulfinate and its reduction through the action of D-glyceraldehyde 3-phosphate dehydrogenase is suggested.

* The enzyme was purified by the procedure of L. ASTRACHAN, T. P. WANG AND N. O. KAPLAN, to be published.

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REFERENCES

1 H. VON EULER, E. ADLER AND H. HELLSTRÖM, *Z. physiol. Chem.*, 241 (1936) 239.
2 E. ADLER, H. HELLSTRÖM AND H. VON EULER, *Z. physiol. Chem.*, 242 (1936) 225.
3 P. KARRER AND F. BENZ, *Helv. Chim. Acta*, 19 (1936) 1028.
4 H. HELLSTRÖM, *Z. physiol. Chem.*, 246 (1937) 155.
5 F. SCHLENK, H. HELLSTRÖM AND H. VON EULER, *Ber.*, 71 (1938) 1471.
6 F. SCHLENK in J. SUMNER AND K. MYRBÄCK, *The Enzymes*, Vol. II, Pt. 1, Academic Press, Inc., New York, 1951, p. 250.
7 W. A. WATERS, *The Chemistry of Free Radicals*, Oxford University Press, London, 1946, Chap. 12.
8 M. YARMOLINSKY AND S. P. COLOWICK, *Federation Proc.*, 13 (1954) 327.
9 S. P. COLOWICK in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 353.
10 S. P. COLOWICK, N. O. KAPLAN AND M. M. CIOTTI, *J. Biol. Chem.*, 191 (1951) 447.
11 R. M. BURTON AND N. O. KAPLAN, *J. Biol. Chem.*, 206 (1954) 283.
12 O. MEYERHOF, P. OHLMEYER AND W. MOHLE, *Biochem. Z.*, 297 (1938) 113.
13 N. O. KAPLAN, S. P. COLOWICK AND A. NASON, *J. Biol. Chem.*, 191 (1951) 473.
14 A. J. SWALLOW, *Biochem. J.*, 60 (1955) 443.
15 F. RASCHIG, *Ber.*, 59 (1926) 859.
16 M. BAZLEN, *Ber.*, 60 (1927) 1470.
17 M. GOEHRING, *Z. anorg. Chem.*, 253 (1947) 313.
18 A. SIMON AND H. KÜCHLER, *Z. anorg. Chem.*, 260 (1949) 161.
19 R. MARKHAM AND J. D. SMITH, *Biochem. J.*, 52 (1952) 552.
20 K. REINKING, E. DEHNEL AND H. LABHARDT, *Ber.*, 38 (1905) 1069.
21 A. SAN PIETRO, *J. Biol. Chem.*, 217 (1955) 579.
22 M. E. PULLMAN, A. SAN PIETRO AND S. P. COLOWICK, *J. Biol. Chem.*, 206 (1954) 129.
23 O. WARBURG, W. CHRISTIAN AND A. GRIESE, *Biochem. Z.*, 282 (1935) 157.
24 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 285 (1936) 156.
25 P. KARRER, G. SCHWARTZENBACH, F. BENZ AND U. SOLMSSEN, *Helv. Chim. Acta*, 19 (1936) 811.
26 L. MICHAELIS in J. SUMNER AND K. MYRBÄCK, *The Enzymes*, Vol. II. Pt. 1, Academic Press, Inc., New York, 1951, p. 1.
27 F. HABER AND R. WILLSTÄTTER, *Ber.*, 64B (1931) 2844.
28 D. J. MACKINNON AND W. A. WATERS, *J. Chem. Soc.*, (1953) 323.
29 S. J. LEACH, *Advances in Enzymol.*, 15 (1954) 1.
30 F. H. WESTHEIMER in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, p. 321.
31 S. BERGSTRÖM AND R. T. HOLMAN, *Advances in Enzymol.*, 8 (1948) 425.
32 E. HAAS, *Biochem. Z.*, 290 (1937) 291.
33 S. J. LEACH, J. H. BAXENDALE AND M. G. EVANS, *Australian J. Chem.*, 6 (1953) 395.
34 T. P. SINGER AND E. B. KEARNEY, *Advances in Enzymol.*, 15 (1954) 79.
35 M. B. MATHEWS, *J. Biol. Chem.*, 176 (1948) 229.
36 M. B. MATHEWS AND E. E. CONN, *J. Am. Chem. Soc.*, 75 (1953) 5428.
37 F. H. WESTHEIMER in W. D. MCELROY AND B. GLASS, *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore, 1954, 356.
38 P. KARRER, F. W. KAHNT, R. EPSTEIN, W. JAFFÉ AND T. ISHII, *Helv. Chim. Acta*, 21 (1938) 223.
39 F. A. LOEWUS, B. VENNESLAND AND D. L. HARRIS, *J. Am. Chem. Soc.*, 77 (1955) 3391.
40 D. MAUZERALL AND F. H. WESTHEIMER, *J. Am. Chem. Soc.*, 77 (1955) 2261.
41 G. W. RAFTER AND S. P. COLOWICK, *J. Biol. Chem.*, 209 (1954) 773.
42 R. M. BURTON AND N. O. KAPLAN, *J. Biol. Chem.*, 211 (1954) 447.
43 E. DE B. BARNETT, *J. Chem. Soc.*, 97 (1910) 63.
44 M. BAZLEN, *Ber.*, 38 (1905) 1057.
45 P. PASCAL *et al.*, *Traité de chimie minérale*, Masson et Cie, Paris, 1932, Vol. 2, p. 151.
46 E. B. KEARNEY AND T. P. SINGER, *Biochim. Biophys. Acta*, 8 (1952) 698.
47 E. RACKER, *Physiol. Revs.*, 35 (1955) 1.
48 E. RACKER AND I. KRIMSKY, *J. Biol. Chem.*, 198 (1952) 731.
49 J. H. SMITH, *J. Am. Chem. Soc.*, 43 (1921) 1307.
50 E. RACKER, *J. Biol. Chem.*, 184 (1950) 313.
51 P. OHLMEYER, *Biochem. Z.*, 297 (1938) 66.
52 B. L. HORECKER AND A. KORNBERG, *J. Biol. Chem.*, 175 (1948) 385.
53 J. N. FRIEND, *A Text-Book of Inorganic Chemistry*, Vol. VII, Pt. 2, Griffin, London, 1931, p. 225.
54 N. V. SIDGWICK, *The Chemical Elements and Their Compounds*, Vol. II, Oxford Univ. Press, London, 1950, p. 906.
55 W. M. GRANT, *Ind. Eng. Chem., Anal. Ed.*, 19 (1947) 345.

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