4.74. Fraction b, m.p. 185–186°: C, 50.48; H, 7.94; N, 3.93.

2-(2-Ethylpiperidyl)-cyclohexanol (III).—The reduction of 2-(2-ethylpyridyl)-cyclohexanone was carried out as in the preparation of IV above, but at room temperature (50 hours). The reduction mixture was decanted from the catalyst, made basic with aqueous alkali, and extracted with chloroform. The chloroform was dried, evaporated, and the residue distilled, giving 57% of a thick, colorless liquid, b.p. 145–148° (2 mm.). This was crystallized from Skellysolve B, the crystals taken up in boiling ether, and set at 0° for 2 days. A small amount of IV was filtered off, and the ether filtrate evaporated. The residue was recrystallized from Skellysolve B, giving a 40% yield of white solid, m.p. 83–88°.

Anal. Calcd. for $C_{13}H_{25}NO$: C, 73.88; H, 11.92; N, 6.63. Found: C, 73.83; H, 12.14; N, 6.91.

The *p*-nitrobenzoate (N-*p*-nitrobenzoyl), prepared in the usual manner, crystallized from ethanol in pale yellow prisms, m.p. 158–160°.

Anal. Calcd. for $C_{27}H_{31}N_3O_7$: C, 63.64; H, 6.13; N, 8.25. Found: C, 63.96; H, 6.32; N, 8.30.

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Carotenoids in *Phycomyces*

BY G. MACKINNEY, C. O. CHICHESTER AND PATRICIA S. WONG

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A marked effect on β-carotene production in *Phycomyces* may be shown by addition of β-ionone to cultures.[1] Recent experiments with purified α-ionone indicate that this isomer also enhances β-carotene production. By contrast, we reported that citral and pseudoionone had a slight effect on lycopene and that methylheptenone had no effect. This requires revision. Control and ionone-treated cultures, harvested 60 to 100 hours after inoculation on a glucose–yeast autolysate medium give extracts whose carotenoid spectrum is essentially that of β-carotene. Methylheptenone-treated cultures show weak pigmentation, when compared with controls, but the carotenoid spectrum is radically different. After chromatography of the crude petroleum ether extracts on MgO–SiO_2 columns and spectrophotometric estimation of the components, the following effects of methylheptenone may be shown: production of β-carotene is halved; the phytofluene content is increased 6- to 15-fold; ζ-carotene which is not demonstrable in control cultures grown under our conditions is found in significant amount, neurosporene is detected, and the lycopene content is also increased.

Culture conditions and procedures have already been described.[1] Methylheptenone, 20 μl., (Fritzsche Bros.) was applied to each culture at times varying from 6 to 27 hours after inoculation. To minimize adverse growth effects, the methylheptenone should be applied 12 to 24 hours after inoculation. If applied immediately germination of the spores is unduly delayed.

The following results are typical of several independent runs. Figures for the heptenone-treated cultures precede values for the controls, in μg. carotenoid per g. of dry mycelium, each value representing five plates: (1) cultures treated 24 hr. after inoculation, harvested in 60 hr., *vs.* controls; dry weights, 0.400, 0.573 g.; phytofluene 46, 3.7; β-carotene 118, 253; ζ-carotene 60, not detected. (2) Cultures treated 17.5 hr. after inoculation, harvested in 112 hr., *vs.* controls; dry weights 0.555, 0.582 g.; phytofluene 88.5, 5.8; β-carotene 195, 392; ζ-carotene 43.3, trace.

In no case was neurosporene detected in the controls, though present in the treated cultures. The lycopene zone was definitely more prominent in the treated cultures, but at best was still a minor component, not exceeding 5 to 10 μg./g.

The striking effects of methylheptenone are therefore three: reduction of the β-carotene, a marked increase in phytofluene and the appearance of ζ-carotene as a major constituent. Re-examination of the absorption curves from extracts of citral-treated cultures makes it apparent that they are intermediate between controls and methylheptenone-treated cultures. Loss in β-carotene is not so marked, nor is production of phytofluene so enhanced, under comparable conditions.[2] These qualitative interpretations are supported also by the observation that the fluorescence of the citral-culture extracts is intermediate in intensity. We cannot as yet comment on possible additional effects ascribable to pseudoionone.

We have hitherto been puzzled by failure to detect numerous minor components observed by Goodwin.[3] He obtained phytofluene yields of 70 to 109 μg. per 250 ml. culture solution (Table 9, ref. 2) after 9 days growth on a 3% glucose–0.2% asparagine medium, in the presence of diphenylamine. Our 5 plates, collectively containing 100 ml. medium, produced 49 μg. of phytofluene (run 2), from 0.555 g. of dry matter, in a total of 112 hr., when treated, compared with 3.4 μg. for the controls. It is clear that great variation may be anticipated in the proportions of the different carotenoids comprising the mixture.

(1) G. Mackinney, T. Nakayama, C. O. Chichester and C. D. Buss, THIS JOURNAL, **74**, 3456 (1952); **75**, 236 (1953).

(2) The culture response to citral is affected more by the mode of application than is the response to ionone.

(3) T. W. Goodwin, *Biochem. J.*, **50**, 550 (1952).

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The Reaction of Diphosphopyridine Nucleotide with Sodium Borohydride—A Correction and Extension

BY MARTIN B. MATHEWS AND ERIC E. CONN

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It was previously observed[1] that diphosphopyridine nucleotide (DPN) was quantitatively reduced by sodium borohydride, in agreement with values obtained by reduction with sodium

(1) M. B. Mathews, *J. Biol. Chem.*, **176**, 229 (1948).

hydrosulfite and with formic dehydrogenase[2] and that the reduced products were only 50% enzymatically oxidizable. Subsequent results obtained with other preparations of the same reactants disagreed in part with the original findings. The reaction was thoroughly reinvestigated and the new data are reported in the present communication.

Experimental

Materials and Methods.—Sodium borohydride, potassium borohydride and sodium trimethoxyborohydride ($NaBH(OCH_3)_3$) were purchased from Metal Hydrides, Inc. DPN was obtained from various commercial sources. The enzymes and other chemicals were standard preparations suitably identified or purchased as C.P. reagents.

Most of the methods used have been described previously.[1] Chromatographic purification of the nucleotides was carried out using Dowex-1 resin.[3]

Assay of DPN with Sodium Borohydride.—The observation that a sample of DPN assayed with sodium borohydride gave considerably less than the value expected from enzymatic assay prompted the investigation of this effect with other preparations of DPN, as well as with some preparations of triphosphopyridine nucleotide (TPN). The results obtained are shown in Table I. The average obtained by assay of the first nine samples of DPN with sodium borohydride is 66% of the value obtained by enzyme assay. A probable error of 3% indicates the degree of consistency obtained with samples ranging in purity from 30 to 90%.

TABLE I

Sample	Purity by assay by enzyme,[a] %	Assay[b] by sodium boro-hydride, % of enzyme value	Assay[b] by sodium hydro-sulfite, % of enzyme value	Enzymatic reoxidation[c] of samples reduced with sodium borohydride % of optical density at 340 mμ
DPN, NB-1[d]	61	68		
DPN, SG 11-38[e]	62	60		
DPN, S 4903[f]	42	72		
DPN, SA[f]	63	60		
DPN, SG 51-30[e]	65	71		
DPN, SB[f]	30	68		
DPN, SC[f]	40	66		
DPN, CPA[g]	90	63	100	49
DPN, CPB[g]	80	66	100	49
DPN, SGX[e]	40	100	138	36
DPN, SGX 30[h]	90	69		50
DPN, SGX 20[h]	0	+	+	
TPN, PA[i]	90	71	100	
TPN, AM[j]	40	65		

[a] Crystalline alcohol dehydrogenase prepared according to Racker[4] used for DPN samples; Zwischenferment was used for TPN samples.[5] [b] Previously described.[1] [c] With lactic dehydrogenase. [d] Nutritional biochemicals product. [e] Sigma chemical product. [f] Schwartz Laboratories product. [g] Chromatographically purified preparation. [h] Chromatographic fractions of DPN, SGX. [i] Chromatographically purified. [j] Armour and Co. product from bovine liver.

The highly purified samples of DPN, CPA and CPB (see Table I), obtained by chromatography of commercial DPN were assayed with sodium hydrosulfite, and yielded values equal to those obtained with enzyme. The reduction products obtained by the action of sodium borohydride on these samples were reoxidized enzymatically on the average only 49% (a result in agreement with previous findings[1]).

Of all samples examined only sample SGX assayed 100% of the enzyme value with sodium borohydride. However, when sample SGX was assayed with sodium hydrosulfite, a value of 138% of the enzyme value was found. This result suggested contamination with compounds capable of reduction with absorption in the 340 mμ region (*e.g.*, nicotinamide nucleoside and nicotinamide nucleotide). The contaminants were isolated, in part, by chromatography of sample SGX, and designated SGX20. SGX20 was reduced by both sodium borohydride and sodium hydrosulfite with resulting absorption at 340 mμ, but was not reduced by enzyme. Sample SGX30, also obtained by chromatography from SGX, had a DPN purity of 90% and reacted with sodium borohydride as did the other preparations of DPN listed. It thus appeared that the unusual results obtained with sample SGX were due to reducible impurities. Consistent with this view were the facts that the assay by sodium borohydride was 72% of the assay by sodium hydrosulfite and that after correction for reducible impurities, the extent of enzymatic reoxidation was 50% of the optical density at 340 mμ due to reduction of DPN by sodium borohydride. The presence of *d*-ribose, adenosine-5-monophosphate, or nicotinamide at concentrations of 2×10^{-4} *M* had no measurable influence upon the formation of the band at 340 mμ. In the reaction with DPN, potassium borohydride or sodium trimethoxyborohydride were quantitatively equivalent to sodium borohydride.

Reoxidation of Reduction Products.—It was noted above that the products of reduction of DPN by sodium borohydride were reoxidized enzymatically only to the extent of 50%. With the reduction products obtained by the action of sodium borohydride on highly purified DPN, it was noted that addition of iodine caused essentially complete removal of absorption at 340 mμ. Also, as was previously reported,[1] the amount of iodine absorbed in titration of the products of reaction between DPN and sodium borohydride correspond, within experimental error, to that required for quantitative oxidation of an amount of reduced DPN which could be calculated from the optical density at 340 mμ.

Although the oxidation by iodine is apparently a stoichiometric reaction, oxidized DPN is not a product since subsequent addition of sodium borohydride fails to increase the optical density at 340 mμ. If, however, potassium ferricyanide in slight excess is used in place of iodine and sodium borohydride subsequently added, the increase in optical density at 340 mμ is found to be, on the average, 66% of that present before oxidation by ferricyanide.[6] It appears probable therefore that the original reduction products formed by action of sodium borohydride on DPN are restored by ferricyanide to their original state, at least so far as concerns that portion of the DPN molecule responsible for the band at 340 mμ.

Discussion

For a variety of DPN samples it has been found that reaction with sodium borohydride produces only about $^2/_3$ of the absorption at 340 mμ corresponding to complete reduction of the DPN by enzyme. This result disagrees with a previous finding of complete reduction by sodium borohydride.[1] No ready explanation of the discrepancy appears, although it is conceivable that some yet unknown contaminant in the first crude DPN preparations was responsible for the original results.

It has been confirmed that the spectrophotometrically measurable products of the reaction of DPN and sodium borohydride can be reoxidized enzymatically to the extent of close to 50%. This fact, taken in conjunction with the observation of close to $^2/_3$ apparent reduction of DPN by sodium borohydride, suggests the possibility that this reaction may involve reduction of the nicotinamide ring of DPN at the 2-, 4- and 6-positions.

(2) M. B. Mathews and B. Vennesland, *J. Biol. Chem.*, **186**, 667 (1950).

(3) Chromatography was accomplished according to an unpublished procedure of A. Kornberg and B. L. Horecker.

(4) E. Racker, *J. Biol. Chem.*, **184**, 313 (1950).

(5) E. Kornberg, *ibid.*, **182**, 805 (1950).

(6) Ferricyanide oxidizes reduced DPN and is itself reduced to ferrocyanide. Subsequent addition of sodium borohydride reduces any remaining excess of ferricyanide to ferrocyanide. Thus, the absorption of this solution at a given wave length less the absorption of a solution containing the same concentration of ferrocyanide yields the absorption due to the products of reaction of sodium borohydride with oxidized DPN.

The products of reduction at the 2- and the 6-positions may absorb less strongly in the 340 mμ region than does the product of reduction at the 4-position. Only the latter product would be expected to show enzymatic activity.[7]

(7) M. E. Pullman, *Federation Proc.*, **12**, 255 (1953).

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Preparation of Highly Compressed Samples for Adsorption Studies[1]

BY S. V. R. MASTRANGELO,[2] R. J. TYKODI AND J. G. ASTON

RECEIVED JULY 13, 1953

While determining the B.E.T.[3] surface area of a highly compressed sample of TiO_2, it was discovered that equilibrium time for coverages between $0.5V_m$ and saturation was very much greater than usual, 8–10 hours as compared to 1–2 hours in less compact samples. When the B.E.T. surface area plot was made a non-linear curve was obtained as shown by the dotted curve through the circles with a left bar in Fig. 1. The surface area determined

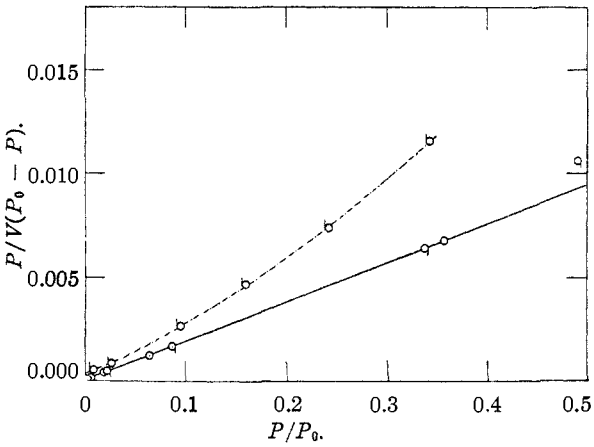

Fig. 1.—B.E.T. plot for nitrogen on TiO_2: o, points for loosely packed test sample; b, points for highly compressed samples; q, points for highly compressed samples after vibration.

by means of a straight line through the low pressure points is 134.5 m.²/g. However, a portion of the same sample had already been run in a small testing chamber, and this sample gave an excellent linear B.E.T. plot as shown by the plain circles in Fig. 1. This test sample was loosely packed relative to the first mentioned sample, and equilibrium was reached in 1–2 hours. The surface area obtained for the test sample is 230.0 m.²/g.

From these results it was concluded that some TiO_2 was not available to the gas in the first sample and that the slow equilibration was caused by gas leaking through solid cakes resulting from high compression. The packed calorimeter was vibrated by means of an ordinary electric vibrating machine. The calorimeter was clamped above the vibrator with a rubber stopper placed between to absorb most of the shock. It was not necessary to use the vibrating mechanism; the vibration of the body of the machine was apparently sufficient to loosen the packing. In order to prevent the machine from getting too hot, the sample was vibrated periodically for about 8 hours.

After the treatment described above the B.E.T. surface area determination was made again with nitrogen. This time, equilibrium was attained in less than 1 hour and in 0.5 hour for some points, all above coverages of $0.5V_m$. The points below $0.5V_m$ were, as usual, slow. The B.E.T. plot obtained for this run is not only linear, but falls on the same plot as for the test sample. These data are shown by the circles with a right bar in Fig. 1.

The authors wish to recommend this technique for preparing highly compressed samples used in adsorption studies at very low temperatures where the dead space corrections are large.

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(1) This Research was carried out under Contract N6ONR, Task Order X, of the Office of Naval Research.

(2) Postdoctoral Fellow, 1950–1951. Barrett Division of Allied Chemical and Dye Corporation, Genolden, Pa.

(3) S. Brunauer, P. H. Emmett and E. Teller, THIS JOURNAL, **60**, 309 (1938).

The Reactions of *p*-Arsanilic Acid and 4-Hydroxyphenylarsonic Acid with Brominated Fatty Acids[1,2]

BY ROBERT L. MCGEACHIN AND MARVIN GREENWALD

RECEIVED JULY 13, 1953

In the course of a study of the preparation of homologs and analogs of Tryparsamide, the condensations of a number of brominated fatty acids with *p*-arsanilic acid and 4-hydroxyphenylarsonic acid were attempted. Standard conditions known to give satisfactory results in the preparation of N-4-arsonophenylglycine[3] and several variations from these conditions (including the use of sodium iodide as a catalyst) all failed to bring about the condensation of α-bromobutyric, α-bromoisobutyric, α-bromovaleric and α-bromoisovaleric acids with *p*-arsanilic acid. However, condensations of *p*-arsanilic acid with α-bromopropionic and β-bromopropionic acids were successful under the standard conditions[3] giving N-(4-arsonophenyl)-α-aminopropionic acid (I) and N-(4-arsonophenyl)-β-aminopropionic acid (II). These products did not recrystallize from hot water as readily as the lower homolog, N-4-arsonophenylglycine, making their isolations somewhat more difficult and resulting in lower yields.

Preparation of α-(4-arsonophenoxy)-propionic acid (III) and β-(4-arsonophenoxy)-propionic acid (IV) were carried out following conditions outlined[4] for 4-arsonophenoxyacetic acid but the yield for the β-isomer was very low. Here again recrystallization and purification were more difficult than with the lower homolog.

The ethyl esters of I, II and III were prepared using the method given by Jacobs and Heidelberger[5] for the ethyl and methyl esters of N-4-arsonophenylglycine. The amide of I (a higher

(1) This work was aided by a grant to the University of Louisville from the Kentucky State Medical Research Commission.

(2) From the M.S. Thesis of Marvin Greenwald, 1952.

(3) German Patent 204,644.

(4) H. Gilman and A. H. Blatt, "Organic Syntheses," Coll. Vol. I, John Wiley and Sons, Inc., New York, N. Y., 1947, p. 75.

(5) W. A. Jacobs and M. Heidelberger, THIS JOURNAL, **41**, 1950 (1919).