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COENZYME ANALOGS

II. PHOTOCHEMICAL INACTIVATION OF REDUCED THIONICOTINAMIDE ADENINE DINUCLEOTIDE*

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SUMMARY

1. Reduced thionicotinamide adenine dinucleotide was inactivated by exposure to visible light. The dinucleotide photoproduct was a competitive inhibitor of dehydrogenase-catalyzed oxidation of NADH.

2. The photochemical reaction appeared to take place in two consecutive steps resulting in the formation of a single final product.

3. Amperometric titration of reduced thionicotinamide adenine dinucleotide demonstrated that the thiocarbonyl group was not as readily available for complexation with silver ions as in 1-methyl-1,4-dihydrothionicotinamide.

INTRODUCTION

The inactivation of NAD^+ by ultraviolet irradiation[3] was suggested to result from rupture of the nucleoside and nucleotide linkages as well as from changes in the pyridinium moiety of the molecule[4]. The photosensitivity of reduced thionicotinamide adenine dinucleotide has already been reported[1,2] although the coenzymic properties of the photoproduct were not thoroughly investigated. This paper reports on the examination of the coenzymic properties of the photoproduct, some of its physical and chemical characteristics, as well as the photochemical steps leading to its formation.

MATERIALS AND METHODS

Coenzymes

NAD^+ and its thionicotinamide analog were purchased from Pabst Laboratories. NAD^+ could not be detected in the thionicotinamide adenine dinucleotide as examined by paper chromatography using ammonium acetate (pH 3.5) –ethanol (2:5) as the solvent system[5]. Polyvinylpyrrolidone was kindly supplied by Dr. R. RESNICK and dextran was the gift of Dr. P. T. MORA.

Enzymes

Crystalline horse-liver alcohol dehydrogenase and beef-heart lactic dehydrogenase were purchased from Boehringer and Soehne.

* Preliminary reports of the photosensitivity of reduced thionicotinamide adenine dinucleotide have appeared[1,2].

Photoproduct of reduced analog

Thionicotinamide adenine dinucleotide (75 mg) was dissolved in 100 ml of distilled water adjusted to pH 9.5 with NaOH. Enzymic reduction by liver alcohol dehydrogenase and ethanol was followed spectrophotometrically at 397.5 mμ. After equilibrium was reached, the reduced analog was diluted to 1500 ml with water and placed in the sunlight. The progress of the photochemical reaction was followed at 338.5 mμ. When the absorbancy reached its maximum value, the solution was concentrated by freeze-drying and the product was examined by paper chromatography.

Enzymic activity

The rate of enzyme-catalyzed reactions was followed spectrophotometrically at the longest absorption maximum; *i.e.*, 397.5 mμ for the thionicotinamide analog and 340 mμ for NADH. The temperature was maintained at 25.5 ± 0.2° in a thermostated cell compartment. Enzymic reduction of the coenzyme was carried out at pH 9.2 using either 0.1 M Na_2HPO_4 or 0.1 M glycine–NaOH buffer. The reverse reaction was carried out at pH 7.2 in 0.1 M phosphate buffer.

Sequence of photochemical changes

Solutions of the reduced analog were irradiated with a 375-W lamp at a distance of 1 ft from the sample for specified time intervals. The spectrum was scanned from 500 to 220 mμ within 10 sec after the light source was turned off and again 10 min after the first scan.

Argentimetric titrations

Titration of the analog and the photoproduct was carried out amperometrically and spectrophotometrically in 0.1 M Tris–nitrate buffer (pH 7.4) by the method described in the previous paper[6].

Absorption spectra

Absorption spectra were examined on a Cary Recording Spectrophotometer, Model 14, having a thermostated cell compartment.

Electrophoresis

Electrophoretic mobility was measured on Whatman 3mm paper in 0.05 M glycine–NaOH buffer (pH 9.1 or 10.2), using a water-cooled flat-bed electrophoresis apparatus. Spots were detected under ultraviolet light.

RESULTS

Absorption spectra

A comparison of the absorption spectra of $4.37 \cdot 10^{-5}$ M solutions of thionicotinamide adenine dinucleotide, its enzymically reduced form, and the photoproduct from the reduced analog in 0.1 M glycine buffer (pH 9.5) is shown in Fig. 1. The broad absorption band in the 290–300 mμ region of thionicotinamide adenine dinucleotide and the reduced analog is absent in NAD^+ and NADH and has been shown to be contributed by the thionamide group.

The absorption spectrum of the dinucleotide photoproduct is very similar to that

of the photoproduct of 1-methyl-1,4-dihydrothionicotinamide except for a shift of the maximum from 346 to 338.5 mμ in going from the model to the dinucleotide.

The position of the absorption maximum of the dinucleotide photoproduct varied with pH; thus at pH 10.1, λ_{max} was 338.5 mμ and at pH 7.4 it was 325 mμ.

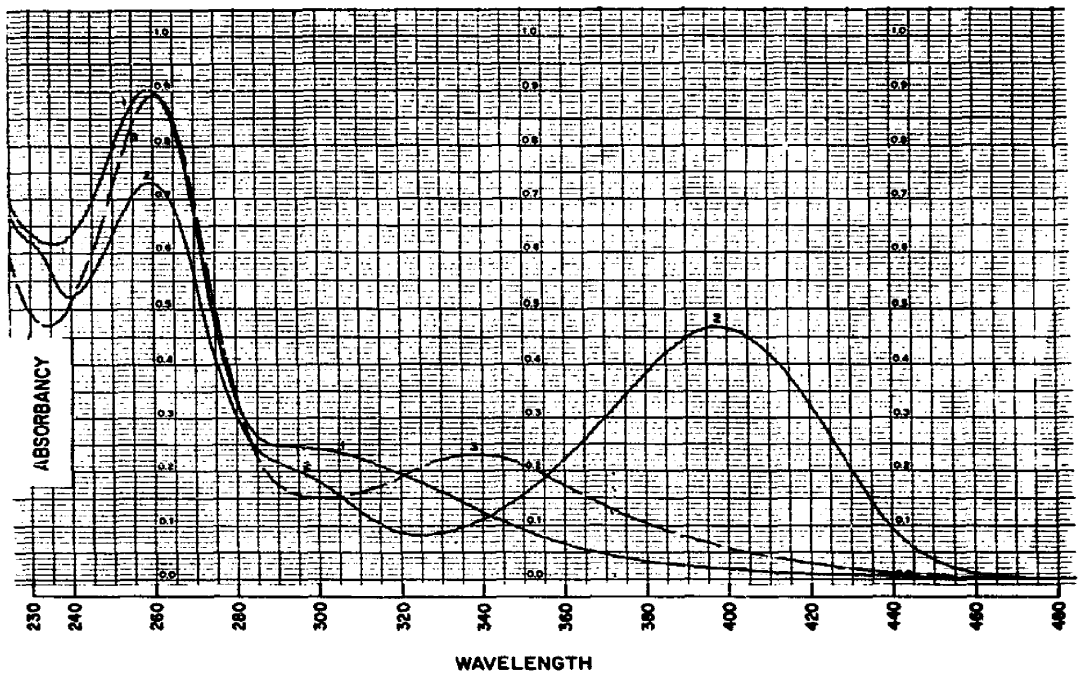

Fig. 1. Absorption spectrum of the thionicotinamide analog in 0.1 M glycine (pH 9.5). Curve 1, a $4.37 \cdot 10^{-5}$ M solution of thionicotinamide adenine dinucleotide; Curve 2, a $4.37 \cdot 10^{-5}$ M solution of reduced thionicotinamide adenine dinucleotide obtained by alcohol dehydrogenase-catalyzed reduction; Curve 3, solution from Curve 2 exposed to a 375-W lamp for 20 min.

Properties of the dinucleotide photoproduct

Electrophoresis: The mobility of the dinucleotide photoproduct was measured on Whatman 3mm paper in 0.05 M glycine buffer (pH 9.1 and 10.2). Its mobility was compared with the thionicotinamide adenine dinucleotide, with its reduced form and with NADH. The dinucleotide photoproduct travelled the same distance toward the anode as the reduced nucleotides and twice as far as the oxidized analog. It is concluded that under the pH conditions of the electrophoresis, the dinucleotide photoproduct had a net charge of — 2.

Enzymic activity: The participation of the dinucleotide photoproduct in enzyme-catalyzed oxidations and reductions was examined spectrophotometrically at 338.5 mμ. Horse-liver alcohol dehydrogenase and beef-heart lactic dehydrogenase were used with their appropriate substrates in this study. There were no changes in the absorbancy at 338.5 mμ under oxidizing or reducing conditions where the unilluminated samples of the analog were very active.

Although the dinucleotide photoproduct did not participate directly in the enzymic reactions, its ability to bind to the enzyme was determined by its efficiency

as an inhibitor. The assumption was made that the molecular weight of the photoproduct had not changed significantly from the unilluminated analog.

The enzyme-catalyzed reduction of NAD^+ was not inhibited by the dinucleotide photoproduct under conditions in which NADH decreased the rate by 32 %. However, the photoproduct was found to be a very efficient inhibitor of the enzymic oxidation of NADH. A comparison of reduced thionicotinamide adenine dinucleotide and its photoproduct as inhibitors of lactic dehydrogenase-catalyzed oxidation of NADH is shown in Table I. The photoproduct was nearly 40 % more effective than the unilluminated reduced analog in inhibiting the oxidation.

TABLE I

INHIBITION OF NADH OXIDATION BY REDUCED THIONICOTINAMIDE ADENINE DINUCLEOTIDE AND THE DINUCLEOTIDE PHOTOPRODUCT

The reaction mixture consisted of 0.28 ml of a $1.86 \cdot 10^{-3}$ M solution of NADH, incubated with 0.14 ml or 0.28 ml of a $1.25 \cdot 10^{-3}$ M solution of reduced thionicotinamide adenine dinucleotide (Expt. 2) or the same volume of a $1.44 \cdot 10^{-3}$ M solution of the dinucleotide photoproduct (Expt. 3) and 0.005 ml of a stock solution of beef-heart lactic dehydrogenase. The volume was brought to 3.0 ml with 0.1 M phosphate buffer (pH 7.2) and the mixture was incubated at 37° for 5 min prior to the addition of 0.1 ml of 10^{-2} M pyruvate. Phosphate buffer, 0.1 ml, instead of pyruvate, was added to the blank which contained all of the reactants. The absorbancy at 340 mμ was followed every 15 sec for 4 min.

Expt. No.	*Additions to reaction mixture*	*Final concentration ($\times 10^6$ M)*	*ΔA/min*	*Inhibition (%)*
1	None	1.69 (NADH)	0.302	0
2	Reduced thionicotinamide adenine dinucleotide	0.57	0.267	12
		1.14	0.188	38
3	Dinucleotide photoproduct	0.65	0.173	43
		1.31	0.087	71

The inhibitor properties of the photoproduct were further studied by varying the concentrations of NADH and the photoproduct in competition experiments. A plot of $1/v$ *versus* the dinucleotide photoproduct concentration, I, is shown in Fig. 2. The inhibition was found to be of the competitive type[7] with a K_i of $1.4 \cdot 10^{-5}$M.

Rate of bleaching and formation of the dinucleotide photoproduct

Effect of pH: The range of pH in which the photochemical reaction could be carried out was limited by the stability of the reduced analog. Due to acid decomposition of the reduced thionicotinamide, the rate of decrease in absorbancy at 397.5 mμ followed between pH 6.1 and 10.5. The rate was not dependent upon pH within experimental error (± 5 %).

Effect of concentration: The rate of bleaching was greater the more dilute the solution presumably because the incident light was more effectively absorbed by the dilute solution. Illumination of concentrated solutions of the reduced thionicotinamide analog resulted in the formation of a product with a long absorption tail extending to 530 mμ. Since the composition of the resulting solution was not known, the dinucleotide photoproduct used during the course of these investigations was prepared from dilute solutions.

Effect of other additions: The rate of bleaching was unaffected by additions of potassium iodide, dichlorophenol indophenol and varying concentrations of polyvinylpyrrolidone and dextran.

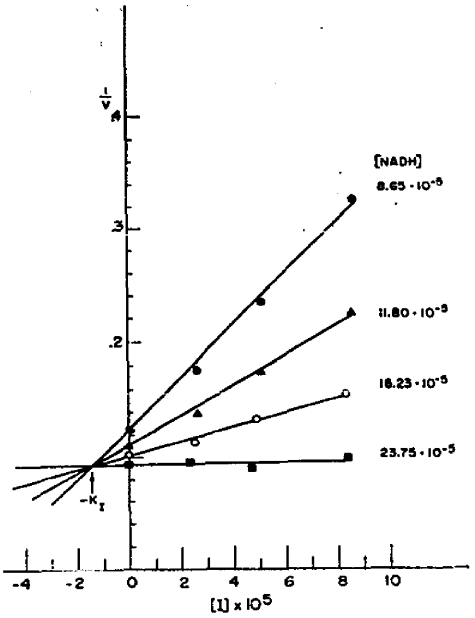

Fig. 2. A plot of $1/v$ *versus* the final concentration of dinucleotide photoproduct, *I*. The final concentration of NADH is given opposite the appropriate curve. Velocity is expressed as mμmoles of NADH oxidized in 3 min.

Mechanism of photoproduct formation

In an attempt to determine the sequence of events in the photochemical reaction, continuous spectra of solutions of reduced thionicotinamide adenine dinucleotide were recorded from 500 to 220 mμ after brief periods of exposure to a 375-W lamp. The recordings are shown in the upper and lower portions of Fig. 3. The change in absorption properties as a function of time may be followed most easily by decreasing absorbancy at 397.5 mμ. In the upper portion of Fig. 3, illumination was for 0, 10 and 25 sec, respectively. Longer periods of illumination were required for significant changes in the spectra as shown in the lower portion of the figure where times of 1, 2, 3, 4, 6 and 10 min respectively, were measured. The formation of a single product is suggested by the isosbestic point at 313 mμ.

In order to follow the absorbancy changes at several wavelengths as a function of period of illumination more effectively, the absorbancy at 397.5, 340 and 295 mμ are plotted graphically in Fig. 4. The biphasic reaction is quite evident: within the first minute of illumination the absorbancy at 295 and 259 mμ increased while the 397.5-mμ band decreased more than 50% (upper portion of Fig. 3). After the first minute of illumination, the absorbancy at 338.5 mμ started to increase while there was a simultaneous decrease at 295 mμ (lower portion of Fig. 3).

If the 295-mμ band is indeed indicative of the presence of the thiocarbonyl group as had been suggested[6], there should be a difference in the availability of the sulfur

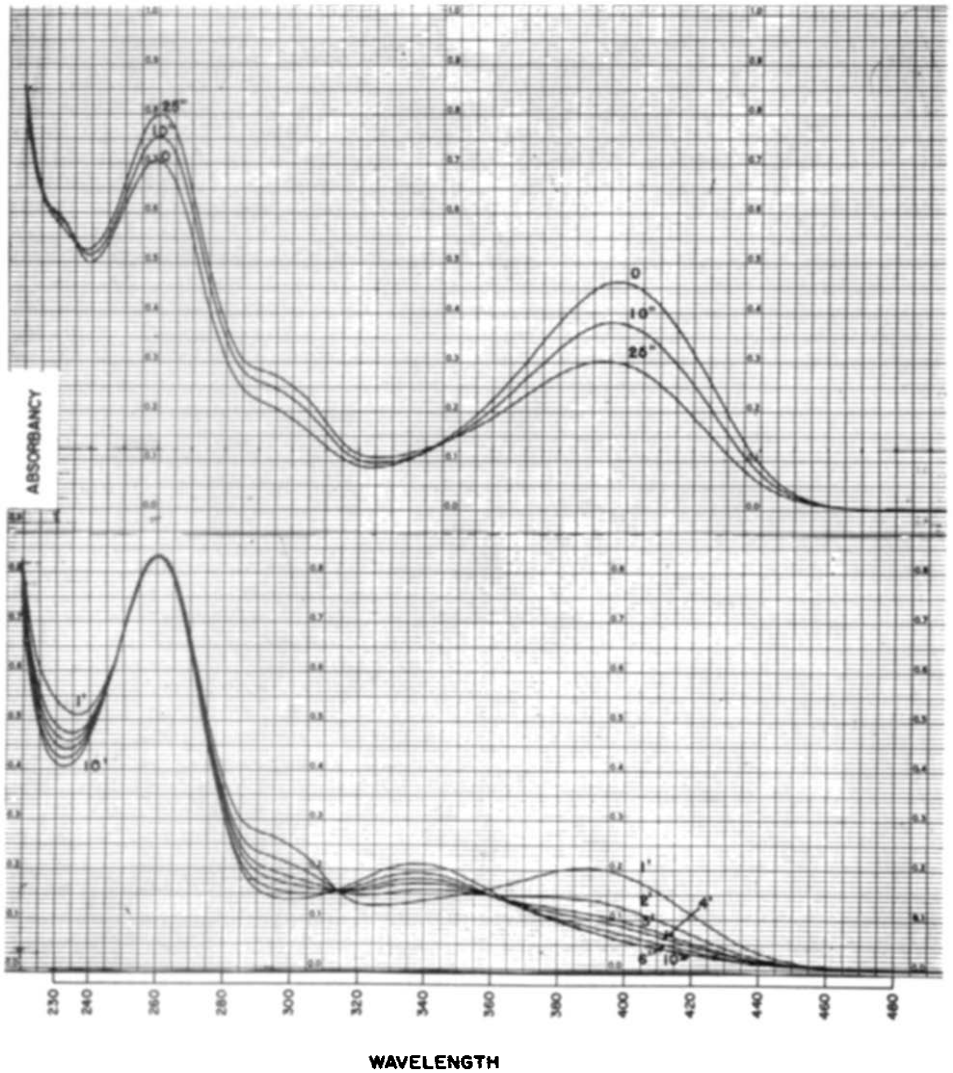

Fig. 3. Photochemical transformation of reduced thionicotinamide adenine dinucleotide. Upper portion, absorption spectrum of a $4.37 \cdot 10^{-5}$ M solution with decreasing absorbancy at 397.5 mμ from 0, 10 and 25 sec illumination. Lower portion, consecutive scans of the spectrum at 1, 2, 3, 4, 6 and 10 min illumination, respectively.

for complexation with silver nitrate between the unilluminated reduced analog and the dinucleotide photoproduct.

Reactions with silver nitrat.

1 μmole of thionicotinamide adenine dinucleotide was titrated with silver nitrate in Tris–nitrate buffer (pH 7.4) and complex formation followed amperometrically. The current remained constant through the addition of 1.45 μmoles of silver nitrate after which the current increased linearly with silver nitrate addition.

Argentimetric titration of reduced thionicotinamide adenine dinucleotide, however, resulted in an unusual titration curve as shown in Fig. 5. Initially it appeared that the reduced analog was not combining with the silver ion. The slope of the curve gradually decreased, however, and after the addition of 1.45 μmoles of silver nitrate there was a sudden increase in current.

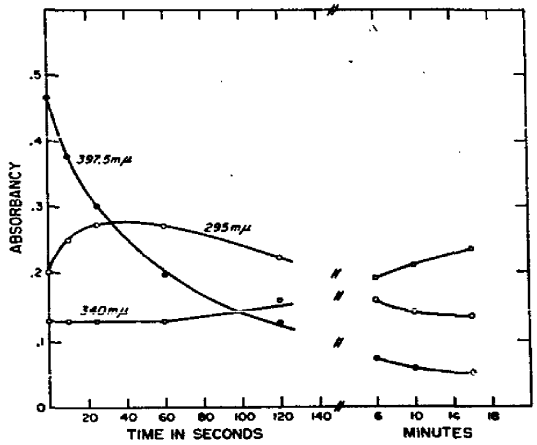

Fig. 4. Graphical representation of absorbancy changes at 397.5, 340 and 295 mμ as a function of period of exposure to light.

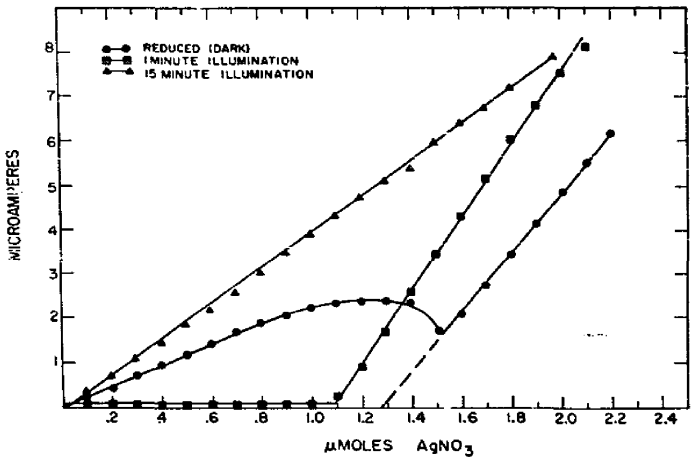

Fig. 5. Argentimetric titration of reduced thionicotinamide adenine dinucleotide and the products obtained on 1 and 15 min illumination followed amperometrically. 1 μmole of reduced coenzyme analog in Tris–nitrate buffer (pH 7.4) titrated with 10^{-2} N silver nitrate. Illumination was with a 375-W lamp at a distance of 1 ft.

1 min illumination of the reduced analog, corresponding to the maximum absorbancy at 295 mμ, eliminated the resistance to silver-ion binding found with the unilluminated material, and the equivalence point was at 1.08 μmoles. The dinucleotide photoproduct, obtained by illumination of the reduced analog for 15 min, did not complex with silver ion at all.

Elemental analysis of the dinucleotide photoproduct showed the same compo-

sition of sulfur as was present in thionicotinamide adenine dinucleotide. Therefore, although sulfur is still present, it is not available for complexing with the metal ion. Complexation of the thiocarbonyl of the reduced analog with silver nitrate was also examined spectrophotometrically by following the decrease at 325 mμ in Tris–nitrate buffer (pH 7.4). The absorption maximum of the silver complex with the reduced analog was 325 mμ. The equivalence point in the titration was at 1.08 μmoles of silver nitrate.

DISCUSSION

Incorporation of thionicotinamide into the coenzyme molecule did not eliminate the photosensitivity of the reduced compound. As a matter of fact, the presence of the dinucleotide reduces the number of photoproducts obtained on illumination of the dihydrothionicotinamide so that only a single dinucleotide photoproduct could be detected.

While the absorption spectrum of the photoproduct did not change under conditions in which NAD^+ would be reduced and NADH would be oxidized, it was found to be an effective inhibitor of the lactic dehydrogenase-catalyzed oxidation of NADH. The competitive nature of the inhibition might suggest some structural similarities between the dinucleotide photoproduct and NADH. While the electrophoretic mobility of the photoproduct supports the similarity with NADH as far as net charge is concerned, there is no conclusive evidence for the structure of the photoproduct.

The thione function of 1-methyl-1,4-dihydrothionicotinamide had been reported[6] to complex 2 μmoles of silver nitrate per μmole of sulfur as followed amperometrically. A high value for the equivalence point in argentimetric titration of the oxidized and reduced coenzyme analog was also observed. However, the most outstanding feature of the titration of reduced thionicotinamide adenine dinucleotide with silver nitrate was the apparent absence of a binding site until a 50 % excess was added. The suggestion that the thione may be involved in bonding to the adenine moiety[1] could explain the results of the amperometric titration. In addition to other structural changes, illumination of the reduced analog for 1 min removes the resistance to complex formation with silver. Continued illumination completely removed the silver-binding site presumably due to changes in the nature of the group in which the sulfur atom is now located.

It had been suggested that the photochemical reaction occurs *via* a free-radical form[6] which was prompted by the generalization that thiones exist to a small extent as free radicals. The absence of the dependence of rate of bleaching on pH, although the study was made within a limited pH range, further suggests the free-radical nature of the reaction. The photochemical reaction appeared to occur in two consecutive steps. In the first, the 259- and 295-mμ bands increase and the sulfur is readily titratable with silver ion. The product of the first reaction then participates in another reaction in which the 295-mμ absorbancy slowly decreases and a new absorption band appears at 338.5 mμ. There is also removal of the silver-ion binding site during the course of the second step of the reaction.

The absorption of visible light by reduced thionicotinamide adenine dinucleotide and the photosensitivity of this molecule prompt caution in the use of the analog in routine studies with dehydrogenases. The isolation of an inhibitor of dehydrogenases

formed from NADH which also appears to be a reduced pyridine nucleotide with absorption maxima at 340 and 260 mμ has been reported recently by KAPLAN *et al.*[8,9]. However, the absorption properties of the photoproduct differs from the NADH inhibitor obtained from frozen solutions of the reduced coenzyme at acid and alkaline pH.

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