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POTENTIAL COENZYME INHIBITORS—V[1]

THE SYNTHESIS AND SOME PROPERTIES OF 4-METHYLNICOTINAMIDE ADENINE DINUCLEOTIDE

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Abstract—The chemical synthesis of 4-methylnicotinamide adenine dinucleotide from 3-carbamoyl-1-(2,3-*O*-isopropylidine-D-ribofuranosyl)-4-methyl pyridinium chloride is presented, and some properties of the modified coenzyme are described with particular reference to its potential as a cofactor for the glycolytic sequence in neoplastic cells. The compound was found to be amenable to chemical 1,4-reduction, to be capable of binding to glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.12), and to act as a competitive inhibitor to nicotinamide adenine dinucleotide in the conversion of D-glyceraldehyde-3-phosphate to 1,3-diphospho-D-glycerate catalysed by this enzyme.

CANCER cells are often relatively deficient[2] in NAD^+ and many evince little or no glycerophosphate dehydrogenase (L-glycerol-3-phosphate: NAD oxidoreductase, EC 1.1.1.8) activity,[3] this enzyme being concerned, in normal tissue, in one of the routes for maintaining the level of NAD^+. Regeneration of the latter by the conversion of pyruvate to lactate therefore assumes particular importance in malignant tissue, and an agent which could affect the essential recycling of NAD^+ by inhibiting the step of glycolysis catalysed by lactate dehydrogenase, (L-lactate: NAD oxidoreductase, EC 1.1.1.27), would be of potential value for the inhibition of growth of such tissue.

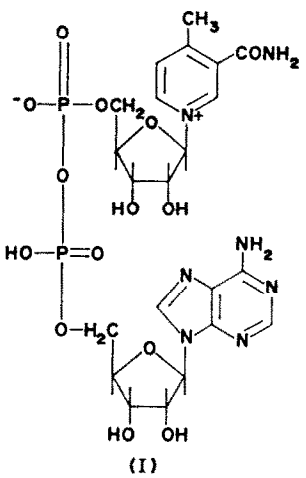
In previous papers in this series[1,4–6] the introduction into the pyridine ring of NAD^+ of a 4-substituent was proposed as a means whereby the aforementioned recycling process could be inhibited. The synthetic analogue must be able to bind to the glyceraldehyde-3-phosphate dehydrogenase apo-enzyme, competing favourably with the natural cofactor, and must accept a H-atom in the β-configuration, being thereafter released from the enzyme, in order not to interfere unduly with respiration in normal cells. The analogue would be unable, by virtue of the substituent, to transfer the α-hydrogen from its reduced form during the reduction of pyruvate to lactate.

The following experiments encompass the rigorous synthesis of 4-methylnicotinamide adenine dinucleotide (I) and investigation of its properties as a potential cofactor.

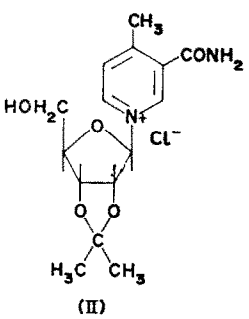
RESULTS AND DISCUSSION

The synthesis of 4-methylnicotinamide riboside and its conversion into an isopropylidene derivative (II) has been described.[7] Todd and his coworkers claimed selective phosphorylation of the 5′-position of unprotected nicotinamide riboside, using phosphorus oxychloride.[8] However, a poor yield was obtained, and it seemed

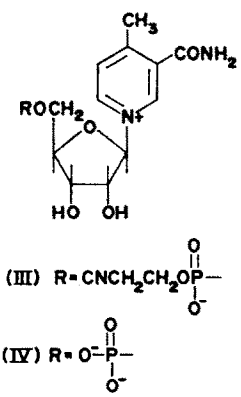
unlikely that high yields of the 5′-phosphate could be achieved without accompanying phosphorylation of the secondary hydroxyl groups. These workers were prevented from conducting the phosphorylation in pyridine by their observations that substantial exchange occurred between this reagent and nicotinamide. However when the 4-methyl derivative (II) was phosphorylated in this solvent, using 2-cyanoethyl phosphate and dicyclohexyl carbodiimide (DCC) no exchange products were detected. Even more surprisingly, the base-catalysed removal of the cyanoethyl group from the product (III) proceeded with only negligible cleavage of the base-labile N(1)–C(1′) linkage. Moreover, the removal was achieved under much milder conditions than are usual for the 2′,3′-protected purine and pyrimidine ribonucleotide analogues, which commonly require several hours in 3M aqueous ammonia at 50°.[9] Possibly, the



(I)



(II)



(III) R = $CNCH_2CH_2O\overset{O}{\overset{\|}{P}}-O^-$

(IV) R = $O\overset{O}{\overset{\|}{P}}-O^-$

positively charged nitrogen atom (N-1) tends to neutralise the negative charge of the phosphomonoester group in (III), which would otherwise hinder the attack of the hydroxyl ion on the cyanoethyl group. Consequently, the conditions required for the removal of this group from (III) would tend towards the extremely mild conditions (pH 8–9 at room temperature) required for its removal from neutral phosphate esters.[10]

The condensation between the phosphomonoester (IV) and adenosine-5′-phosphate using DCC in aqueous pyridine[11] gave the required 4-methyl NAD^+ (I) as the major product. The structure was confirmed by its electrophoretic mobility, identical[11] with NAD^+, and by its nuclear magnetic resonance (n.m.r.) spectra at 60 and 100 MHz, the 60 MHz spectrum containing all the C–H protons expected from the presence of the 4-methyl pyridinium[7] and adenine[12] attached to ribose moieties. Additionally, the 100 MHz spectrum contained both pairs of doublets ascribable to the anomeric protons [H(1′)] of the two ribose moieties, whereas these coalesced to a single doublet in the 60 MHz spectrum.

Dithionite reduction of pyridinium salts is known to produce 1,4-dihydropyridine derivatives.[13] Further, model experiments with 4-methylnicotinamide derivatives have established that reaction with dithionite leads to 1,4-addition,[5] just as in the reduction of NAD^+ by the same reagent which mimics the process catalysed by glyceraldehyde-3-phosphate dehydrogenase.[14] Walter and Kaplan[15] prepared 4-methyl NAD^+ enzymically by exchange of 4-methylnicotinamide for nicotinamide in NAD^+, catalysed

by pig-brain NADase, (NAD nucleosidase, EC 3.2.2.5) but were unable to demonstrate dithionite reduction of the product. Using a modification of the Lehninger method[16,17] we have been able to demonstrate the formation of an ultra-violet absorbing species, λ_{max} 335 nm, on treatment of 4-methyl NAD^+ with dithionite. The position of this maximum is consistent with the values obtained for the model 1,4-dihydro-compounds prepared from 4-methylnicotinamide derivatives[5] and is at a lower wavelength than the maximum obtained for the 1,6-dihydroderivative of 4-methyl NAD^+ (343 nm) by borohydride reduction.* This is in accord with the relative positions of the maxima of 1,4- and 1,6-dihydroderivatives of the model compounds.[5] The maximum of 335 nm can therefore be confidently assigned to the 1,4-dihydro derivative of 4-methyl NAD^+. The first requirement, see introduction, that the modified cofactor should be amenable to 1,4-reduction, has thus been satisfied.

The fluorescence of the reduced modified coenzyme (Fig. 1) resembles that of

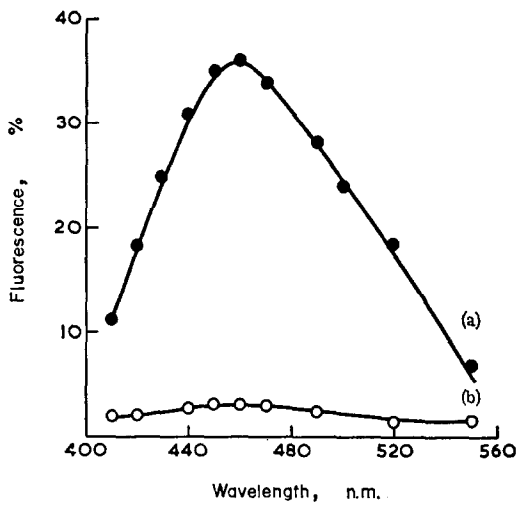


FIG. 1. Fluorescence spectra of 4-methyl NADH (a) Excitation at 340 nm. (b) Excitation at 260 nm.

NADH,[18] although it differs in that the intensity of fluorescence at 460 ± 5 nm produced by excitation at 260 nm is only one-tenth of that produced by excitation at 340 nm, suggesting that energy transfer between the adenine and modified nicotinamide ring in 4-methyl NADH is considerably less efficient than in NADH. (The intensities displayed are merely relative, being obtained under the same experimental conditions but no correction being applied to the emission spectra. Artefacts due to scattering were minimised by working at low concentrations.) Since evidence is accumulating that NADH is bound in an open configuration to lactate dehydrogenase[19,20] any difference in the proportion of folded conformation[21,22] of the modified coenzyme present, reflected in the extent of energy transfer, compared with that of the natural cofactor, is not necessarily significant in affecting the binding of the reduced modified coenzyme to lactate dehydrogenase, or, arguably, its release from glyceraldehyde-3-phosphate dehydrogenase.

* A. C. Lovesey, personal communication.

Velick[20] has titrated glyceraldehyde-3-phosphate dehydrogenase with NAD^+, following the decrease in protein fluorescence occasioned by the binding of the coenzyme to the apoenzyme. When a similar experiment was performed using charcoal-treated glyceraldehyde-3-phosphate dehydrogenase[23,24] and 4-methyl NAD^+, the results shown graphically in Fig. 2 were obtained. Stockell[24] defined an intrinsic dissociation constant

$$K_{NAD^+} = \frac{[E]\,[NAD^+]}{[E : NAD^+]}$$ where $[E]$ is concentration of

unbound enzyme sites, $[E{:}NAD^+]$ is concentration of bound enzyme sites, while Velick[20] stated that in principle the results of titrating a dehydrogenase with NAD^+ can be analysed by double reciprocal plots of $1/\Delta F$ (where ΔF is the change in fluorescence) versus $1/[NAD^+]$, similar to those used in extrapolation

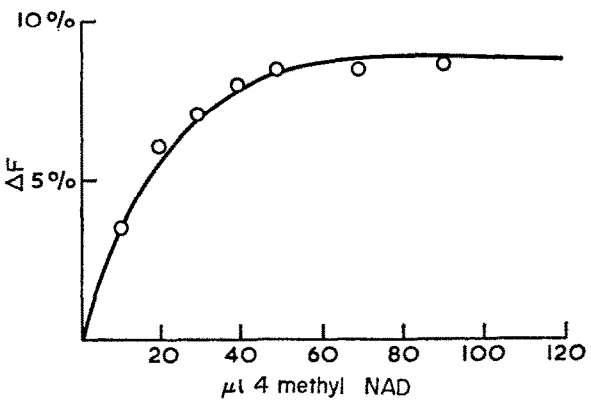


FIG. 2. Change in protein fluorescence of charcoal-treated glyceraldehyde-3-phosphate dehydrogenase on addition of 4-methyl NAD^+. (ΔF is the change in % fluorescence at 350 nm of a solution of apoenzyme irradiated at 300 nm on the addition of modified coenzyme.)

for Michaelis constants. If the results for 4-methyl NAD^+ are analysed in this way* (Fig. 3) a value $K_{4MeNAD^+} = 0{\cdot}8 \times 10^{-5}$ M is obtained. During the calculation several approximations are made: namely, that any trace of NAD^+ remaining after charcoal treatment is discounted, the enzyme is assumed to be of sufficient purity to justify linear proportionality of the decrease in fluorescence with the number of bound sites; the dissociation constant is unaffected by previous binding of 4-methyl NAD^+.

It is clear by analogy with Velick's work[20] that the modified coenzyme is capable of

* K_{NAD^+} is derived from the expression

$$K_{NAD^+} = \frac{[E_t - k'\Delta F]\,[NAD^+]}{[k'\Delta F]}$$

$$\frac{1}{k'\Delta F} = \frac{K_{NAD^+}}{[NAD^+]E_t} + \frac{1}{E_t}$$

from which it may be seen that if $1/\Delta F$ is plotted against $1/[NAD^+]$ the intercept on the $\frac{1}{[NAD^+]}$ axis when $\frac{1}{\Delta F} = 0$ is $\frac{-1}{K_{NAD^+}}$

where $k'\Delta F = [E{:}NAD^+]$, E_t is concentration of total enzyme sites (bound and unbound).

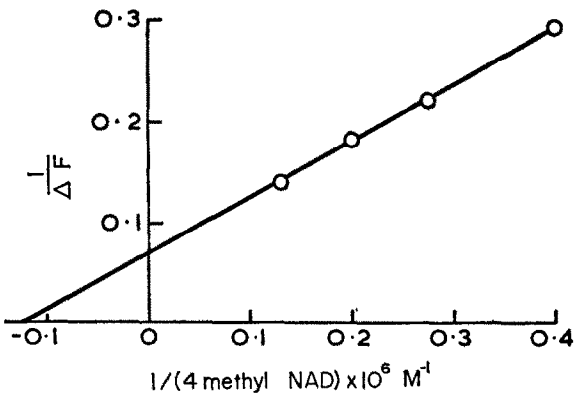


FIG. 3. Binding study of 4-methyl NAD^+ and glyceraldehyde-3-phosphate dehydrogenase.

binding to the glyceraldehyde-3-phosphate dehydrogenase apoenzyme (Fig. 2), so that the second criterion of a potential cofactor is satisfied. Whether or not the binding competes favourably with that of the natural cofactor should be possible to estimate by comparing the dissociation constant obtained with those available in the literature for NAD^+. There are 100-fold discrepancies between values quoted from kinetic studies and equilibrium dialysis or fluorimetric studies, the first lying in the range 10^{-5}M, the second 10^{-7}M.[20,24–27] According to Conway and Koshland,[27] these discrepancies may be resolved if the equilibrium constants of the four NAD^+ molecules binding to the enzyme K_1, K_2, K_3, K_4 are measured separately when it becomes apparent that the values in the 10^{-5}M range are those of K_4, while the values in the 10^{-7}M range correspond to K_3. Unless the conditions of pH and temperature are identical and the definition of K is, firstly, dependent on equivalent parameters and, secondly, if it includes assumptions, these assumptions are the same in both cases, direct comparison is misleading. None will therefore be attempted here: suffice it to declare that the modified coenzyme binds to the apoenzyme.

Since it had now been established that 4-methyl NAD^+ could be reduced to the 1,4-dihydropyridine derivative, under chemical conditions which mimic the enzyme-catalysed process, and could bind to glyceraldehyde-3-phosphate dehydrogenase, the remaining point was to check the activity of the enzyme using the analogue in place of the natural coenzyme in an *in vitro* system. When experiments were performed in which the development of absorbance at 335 nm was sought in conditions under which development of absorbance at 340 nm in the presence of enzyme, substrate and NAD^+ is readily detected, there was no observable increase in absorbance. When both NAD^+ and a constant level of 4-methyl NAD^+ were present, the results could be analysed by the Lineweaver–Burk method[28] and the analogue was shown to behave as does a competitive inhibitor to the natural coenzyme (Fig. 4). The value of $K_m = 3{\cdot}7 \times 10^5$M obtained for NAD^+ is close to that derived from a similar reciprocal plot by Cori, Slein and Cori,[29] ($3{\cdot}9 \times 10^{-5}$M), and of the same order of magnitude as those derived from kinetic studies, ($2{\cdot}6 \times 10^{-5}$M), and equilibrium dialysis studies, ($2{\cdot}0 \times 10^{-5}$M) by Conway and Koshland[27] or by Velick and Furfine.[25] The inhibitor constant $K_i = 1{\cdot}94 \times 10^{-4}$M.

It therefore appears that, although the 4-methyl derivative possesses chemical

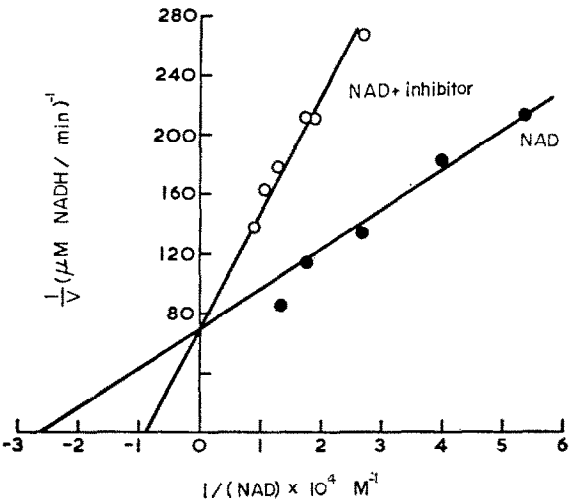


FIG. 4. Inhibition of glyceraldehyde-3-phosphate dehydrogenase by 4-methyl NAD^+.

properties which would make it potentially a cofactor for the *in vivo* reaction, the rate of enzymic reduction, if occurring at all, would be so lessened as to interfere seriously with the glyceraldehyde-3-phosphate oxidation. The rate of attack at the 4-position is dependent upon the electrophilicity at this position and any modification which can increase this while maintaining the properties already defined as necessary for a co-factor, should improve the ability of the analogue to undergo enzymic reduction. An estimated cyanide addition constant of 20–33 moles^{-1} for 3-acetyl-4-methyl NAD^+, derived from model compounds, which would be a substantial improvement on that for 4-methyl NAD^+ and reflects on increased anionic affinity at the 4-position[1] has encouraged the consideration of 3-acetyl-4-methyl NAD^+, which is currently under preparation in this laboratory, as a potential co-factor.

EXPERIMENTAL

Paper chromatograms were developed on Whatman No. 1 paper with *n*-butanol–acetic acid–water (5:2:3, by vol.) as solvent. Electrophoresis was carried out on Whatman No. 4 paper (10 V/cm, 1·75 hr, in 0·02M phosphate buffer, pH 8·0) with a Shandon Universal Electrophoresis Apparatus and Vokam power unit. Spots were detected by examination under ultraviolet light and by the molybdate spray reagent.[30] Nuclear magnetic resonance (n.m.r.) spectra were measured on a 60-MHz Perkin–Elmer R10 spectrometer on a 10% w/v solution in D_2O, using *t*-butanol as internal reference, and on a 100-MHz Varian spectrometer on a 0·5% w/v solution in D_2O using tetramethyl silane as an internal lock and 16 integrations. Ultra-violet measurements were made with a Unicam SP 800 spectrophotometer. Concentrations were performed under reduced pressure at 30°. Rates of enzymic reactions were measured at 30° using a Gilford multiple absorbance spectrophotometer. Fluorescence measurements were performed at 20° using an Aminco–Bowman spectro-photofluorometer. The commercial enzyme preparation (rabbit muscle enzyme, 10 mg/ml specific activity 70 U/mg) and the NAD^+ used were obtained from the Boehringer Corporation Ltd.

1-[5-(2-*cyanoethyl*)*phosphoryl*-D-*ribofuranosyl*]-4-*methylpyridinium*-3-*carboxamide*(III)

A solution of 3-carbamoyl-1-(2,3-0-isopropylidene-D-ribofuranosyl)-4-methylpyridinium chloride[7] (II; 1·035 g), dicyclohexylcarbodiimide (3·08 g) and pyridinium 2-cyanoethyl phosphate (from 2·04 g of the barium salt[31]) in dry (calcium hydride) pyridine (20 ml) was stirred at room temperature for 2 days. Water (1 ml) was added and the mixture left overnight. Dicyclohexylurea was filtered off and washed with aqueous pyridine (1 : 1, 2 × 5 ml). The filtrate and washings were concentrated to small volume, diluted with water to 25 ml and applied to a column (20 × 3 cm^2) of Dowex 1 × 8 (formate), 20–50 mesh. Elution with water (70 ml) gave material of the same R_f (0·75) as (II) but with a positive reaction to the phosphate spray.[30] The eluate was concentrated to 45 ml and treated with N hydrochloric acid (5 ml). After 4 days at room temperature, complete conversion into a single product had occurred. The solution was applied to a column (40 × 3 cm^2) of Dowex 50 (H^+) 200–400 mesh, and eluted with water (10 ml fractions) (cf. Ref. 8). Fractions beyond 16 were neutral. Fractions 31–39, which contained the required *product*, were concentrated to dryness. A boiling solution of the residue in methanol (10 ml) was slowly treated with ethanol to give a final volume of 25 ml. Large almost colourless cubic crystals (0·575 g, 48%) R_f 0·44 separated overnight at room temperature. (Found: C, 44·4%; H, 5·15%; N, 10·65%; P, 7·6%. Calc. for $C_{15}H_{20}N_3O_8P$: C, 44·9%; H, 5·0%; N, 10·45%; P, 7·7%.)

1-(5-*Phosphoryl*-D-*ribofuranosyl*)-4-*methylpyridinium*-3-*carboxamide* (IV)

A solution of the 2-cyanoethyl derivative (II) obtained on the scale described above, after elution from the Dowex 50(H^+) column but before crystallization, was diluted with water to 100 ml and treated with triethylamine (1 ml). After 6 hr at room temperature (27°)* the product (R_f 0·34) was accompanied by only traces of starting material and 4-methylnicotinamide (R_f 0·63). Triethylamine was extracted with chloroform (3 × 60 ml) and the aqueous solution was concentrated to dryness. Repeated concentration of ethanol from the *product* gave a straw-coloured powder (0·62 g). This was sufficiently pure for the subsequent preparation of 4-methyl NAD^+(I). A sample of the straw coloured product (28 mg) was taken for recrystallisation. The solid was dissolved in dry methanol (5 ml) and filtered. The methanolic solution was concentrated to dryness, redissolved in a mixture of methanol (10 ml) and *n*-butanol (5 ml) and reconcentrated. Final concentration from *n*-butanol (5 ml) yielded a white solid which was triturated with dry ether (5 ml) and transferred, after decantation of the ether, still damp with ether, to a desiccator over calcium chloride. The solid was dried overnight under vacuum. Yield 13 mg. (Found: C, 39·18%; H, 5·11%; N, 8·18%. Calc.: for $C_{12}H_{17}N_2O_8P$. H_2O C, 39·35%; H, 5·23%; N, 7·65%.)

4-*methylnicotinamide adenine dinucleotide* (4-*methyl NAD*$^+$), (I)

To a solution of the nucleotide (IV, 500 mg) and adenosine-5′-phosphate (free acid 1 g) in water (20 ml) was added pyridine (170 ml) and dicyclohexylcarbodiimide (10g). The mixture was kept at 0° and the addition of DCC was repeated three times at intervals of 24 hr. The mixture was poured into water (1 l.), and, after 2 hr, the dicyclohexyl urea was filtered off and washed with water (3 × 20 ml). The filtrate and washings were extracted with chloroform (3 × 150 ml) then concentrate was applied to a column

* Unlike other instances in the present preparative work, the exact value of "room temperature" must be noted and trial experiments run to find the optimum time for values different from the above.

(25 × 3 cm²) of Dowex 2-X (formate), 200–400 mesh. Elution with water (10 ml fractions, 500 ml) gave two fractions. Fractions 4–8 contained unreacted (IV), (electrophoretic mobility 5·6 cm) and the derived symmetrical pyrophosphate, P^1P^2 -di(4-methylnicotinamide nucleoside)-pyrophosphate (R_f 0·13, electrophoretic mobility −1·0 cm). Fractions 11–20 contained 4-methyl NAD^+ (I) (R_f 0·13, electrophoretic mobility 4·0 cm*) which was obtained as a white hygroscopic powder (0·16 g) by concentration of the solution to 2 ml and dropwise addition to acetone (30 ml) followed by centrifugation. Elution of the column with 0·01 N formic acid gave, in fractions 20–57, a component which, after concentration and precipitation from acetone as above (yield 0·14 g) was shown by chromatography, electrophoresis and n.m.r. spectroscopy to be identical with the component from the later aqueous fraction. The total yield of product was therefore 0·3 g (29% as dihydrate). The reason for this two-stage elution is unclear (it was not observed for NAD^+ by Todd and his coworkers[11]) but it was reproduced in a subsequent preparation. Moreover samples of the two products (5 mg) were eluted as one material in fractions 3–7 (5 ml fractions) on subsequent passage through a column (4·5 × 0·6 cm²) of Biorad AG 1 × 8 (formate), 200–400 mesh, the eluant being 0·05 M formic acid.† The fractions 3–7 were combined and freeze-dried, then dried under vacuum over phosphorus pentoxide at 60°, for an analysis sample. (Found: C, 36·60%; H, 4·68%; N, 13·21%; P, 8·49%. Calc. for $C_{22}H_{29}O_{14}N_7P_2.2H_2O$: C, 37·03%; H, 4·66%; N, 13·74%; P, 8·69%.)

The 60 MHz n.m.r. spectrum showed: τ 0·9 [1H, pyridine H (2)], 0·98 [1H, doublet, $J_{6,5}$ 6 cycles/sec, pyridine H(6)], 1·44 [1H, singlet, adenine H(8)], 1·60 [1H, singlet, adenine H(2)], 1·88 [1H, doublet, pyridine H(5)], 3·93 [2H, two coincident doublets $J_{1,2}$ *ca.* 6·0 cycles/sec, sugar H(1)], 7·30 [3H, singlet, Me].

The 100 MHz NMR spectrum was similar, except that the use of an external reference gave τ values shifted *ca.* 0·4 ppm downfield compared with the values in the 60 MHz spectrum. Additionally, the two sugar H(1) proton doublets were separated: τ 3·42 and τ 3·51, $J_{1,2}$ both *ca.* 5 cycles/sec. Owing to the small chemical shift between them it was uncertain to which ribose moiety each should be assigned.

Chemical reduction of 4-*methyl* NAD^+(I) *and fluorescence spectrum of product*

To a solution of 4-methyl NAD^+ (4 mg) in water (1 ml) was added a mixture of sodium bicarbonate (10 mg) and anhydrous sodium carbonate (10 mg). After the subsequent addition of sodium dithionite (10 mg) the stoppered solution was left to stand 1 hr at room temperature (22°) when the deep yellow colour initially produced had faded. The solution was concentrated to dryness and the residue extracted with AR ethanol (3 ml). The ethanolic solution was concentrated to dryness and the residue taken up in water (3 ml). The ultraviolet spectrum shows maxima at 260 nm (adenine) and 335 nm (ϵ 2760 based on adenine content[32]). The fluorescence spectra resulting from excitation of the aqueous solution of reduced 4-methylnicotinamide adenine dinucleotide at 260 nm and 340 nm are given in Fig. 1.

Binding study of 4-*methyl* NAD^+ *and glyceraldehyde*-3-*phosphate dehydrogenase*

Stock enzyme solution was prepared by diluting the commercial preparation of glyceraldehyde-3-phosphate dehydrogenase (10 mg/ml, 200 μl) in an ice-cold aqueous

* Other mobilities determined were: NAD^+ (4·0 cm), adenosine-5′-phosphate (8·0 cm).

† Modification of method recommended by Dr Grassl, The Boehringer Corporation (London) Ltd., for NAD^+ purification.

solution of 0·5 mM EDTA. The diluted enzyme solution (10 ml) was shaken with Darco 12 × 20 charcoal (500 mg) for 2 min at 0°, centrifuged at 2000 rev/min for 0·5 min, and the supernatant decanted. The recovery of enzyme, based on 280 nm absorption, was 60 per cent. The ratio of absorbances $A_{280/260}$ is 1·8 after charcoal treatment (1·4 prior to charcoal treatment), indicative of the removal of NAD^+ by the charcoal.[23] The aqueous solution of apoenzyme (3 ml) was irradiated at 300 nm after the addition of increasing amounts (10 μl) of an aqueous solution of 4-methyl NAD^+ (1 mg, 2 ml) and the % fluorescence was noted at 350 nm. Small but significant corrections were made for dilution by subtracting the readings obtained after the addition of increasing amounts of water (10 μl) to the aqueous solution of apoenzyme (3 ml). The results are expressed graphically in Figs. 2 and 3.

Competitive inhibition by 4-methyl NAD^+ in the conversion of D-*glyceraldehyde*-3-*phosphate to* 1,3-*diphospho*-D-*glycerate catalysed by glyceraldehyde*-3-*phosphate dehydrogenase*

DL-Glyceraldehyde phosphate was prepared from the diethyl acetal barium salt (Sigma Ltd.): Dowex $50H^+$ resin (150 mg) in water (6 ml) was boiled for 3 min with the diethyl acetal barium salt of DL-glyceraldehyde phosphate (10 mg) until the latter had dissolved. The hot solution was cooled in an ice-bath and filtered with resin, through a sinter funnel. The resin was washed with water and the final volume (10 ml) formed the stock glyceraldehyde solution. Stock aqueous solutions of NAD^+ (4·1 mg, 3 ml), 4-methyl NAD^+ (1·5 mg, 1 ml) and a buffered solution of enzyme (50 μl of commercial preparation of enzyme, 5 ml phosphate buffer pH 8·6) were prepared and kept in ice. [Phosphate buffer pH 8·6 was prepared as follows: Sodium arsenate (Na_2HAsO_4, 0·744 g), sodium pyrophosphate (22·3 g), EDTA disodium salt (0·372 g), cysteine hydrochloride (0·788 g) were dissolved in distilled water (1 l.)]. The initial velocities of reactions were measured by following the development of absorbance at 340 nm, using a constant level of enzyme solution (25 μl) and substrate (200 μl) with varying levels of NAD^+ (25–250 μl), the volume of buffer being such as to maintain a final volume of 2·875 ml. This experiment was repeated with no NAD^+ present but varying levels of 4-methyl NAD^+ (25–250 μl): no development of absorbance at 340 nm could be observed. The initial velocities were then redetermined using NAD^+ in the presence of a constant level of 4-methyl NAD^+ (50 μl), final volume 2·875 ml. The order of addition of the components to the reaction was buffer, enzyme, substrate, (inhibitor), NAD^+ to initiate reaction. The reciprocal of the initial velocity was plotted against the reciprocal of the concentration of NAD^+ to yield the results shown in Fig. 4.

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