2. Another glycolipid, obtained from a strain of *Mycobacterium bovis*, contained 2-O-methylrhamnose alone.

3. A scheme for the tentative identification of small amounts of O-methylated 6-deoxyhexoses is described.

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REFERENCES

- Allen, S., Bonner, T. G., Bourne, E. J. & Saville, N. M. (1958). *Chem. & Ind.* p. 630.
- Andrews, P. & Hough, L. (1958). *J. chem. Soc.* p. 4476.
- Andrews, P., Hough, L. & Jones, J. K. N. (1955). *J. Amer. chem. Soc.* **77**, 125.
- Asselineau, J., Choucrour, N. & Lederer, E. (1950). *Biochim. biophys. Acta*, **5**, 197.
- Bergey's *Manual of Determinative Bacteriology* (1957). 7th ed., p. 706. Ed. by Breed, R. S., Murray, E. G. D. & Smith, N. R. Baltimore: The Williams and Wilkins Co.
- Brown, F., Hough, L. & Jones, J. K. N. (1950). *J. chem. Soc.* p. 1125.
- Brown, M., Yeadon, D. A., Goldblatt, L. A. & Dieckert, J. W. (1957). *Analyt. Chem.* **29**, 30.
- Bush, I. E. (1952). *Biochem. J.* **50**, 370.
- Butenandt, A. & Stodola, F. H. (1939). *Liebigs Ann.* **539**, 40.
- Butler, K., Lloyd, P. F. & Stacey, M. (1955). *J. chem. Soc.* p. 1531.
- Cifonelli, J. A. & Smith, F. (1954). *Analyt. Chem.* **26**, 1132.
- Crowder, J. A., Stodola, F. H. & Anderson, R. J. (1936). *J. biol. Chem.* **114**, 431.
- Dieckert, J. W. & Reiser, R. (1956). *J. Amer. Oil Chem. Soc.* **33**, 123.
- Dische, Z. & Shettles, L. B. (1948). *J. biol. Chem.* **175**, 595.
- Foster, A. B. (1952). *Chem. & Ind.* p. 1050.
- Gardiner, J. G. & Percival, E. (1958). *J. chem. Soc.* p. 1414.
- Garrod, A. R. N. & Jones, J. K. N. (1954). *J. chem. Soc.* p. 2522.
- Gibbons, M. N. (1955). *Analyst*, **80**, 268.
- Godin, P. (1954). *Nature, Lond.*, **174**, 134.
- Hauser, G. & Karnovsky, M. L. (1957). *J. biol. Chem.* **224**, 91.
- Hough, L., Jones, J. K. N. & Wadman, W. H. (1950). *J. chem. Soc.* p. 1702.
- Isherwood, F. A. & Jermyn, M. A. (1951). *Biochem. J.* **48**, 515.
- Jarvis, F. G. & Johnson, M. J. (1949). *J. Amer. chem. Soc.* **71**, 4124.
- Krauss, M. T., Jäger, H., Schindler, O. & Reichstein, T. (1960). *J. Chromat.* **3**, 63.
- Kubica, G. P., Randall, H. M. & Smith, D. W. (1956). *Amer. Rev. Tuberc.* **73**, 529.
- Lambou, M. G. (1957). *Analyt. Chem.* **29**, 1449.
- MacLennan, A. P., Randall, H. M. & Smith, D. W. (1959). *Analyt. Chem.* **31**, 2020.
- MacLennan, A. P., Smith, D. W. & Randall, H. M. (1960). *Biochem. J.* **74**, 3 p.
- Noll, H. (1957). *J. biol. Chem.* **224**, 149.
- Noll, H. & Bloch, H. (1955). *J. biol. Chem.* **214**, 251.
- Partridge, S. M. (1946). *Nature, Lond.*, **164**, 443.
- Randall, H. M. & Smith, D. W. (1953). *J. opt. Soc. Amer.* **43**, 1086.
- Runyon, E. H. (1955). *Amer. Rev. Tuberc.* **72**, 866.
- Runyon, E. H. (1959). *Med. Clin. N. Amer.* **43**, 273.
- Smith, D. W., Harrell, W. K. & Randall, H. M. (1954). *Amer. Rev. Tuberc.* **69**, 505.
- Smith, D. W., Randall, H. M., Gastambide-Odier, M. M. & Koevoet, A. L. (1957). *Ann. N.Y. Acad. Sci.* **69**, 145.
- Smith, D. W., Randall, H. M., MacLennan, A. P. & Lederer, E. (1960a). *Nature, Lond.*, **186**, 887.
- Smith, D. W., Randall, H. M., MacLennan, A. P., Putney, R. K. & Rao, S. V. (1960b). *J. Bact.* **79**, 217.
- Trevelyan, W. E., Procter, D. P. & Harrison, J. S. (1950). *Nature, Lond.*, **166**, 444.
- Uyei, N. & Anderson, R. J. (1931). *J. biol. Chem.* **94**, 653.
- Wallenfels, K. (1950). *Naturwissenschaften*, **37**, 491.
- Wilson, D. M. & Ajl, S. (1957). *J. Bact.* **73**, 410.
- Wong, S. & Weinzierl, J. (1936). *Amer. Rev. Tuberc.* **33**, 577.

Biochem. J. (1961) **80**, 318

Nicotinamide Mononucleotide Adenylyltransferase of Pig-Liver Nuclei

THE EFFECTS OF NICOTINAMIDE MONONUCLEOTIDE CONCENTRATION AND pH ON DINUCLEOTIDE SYNTHESIS

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Kornberg (1948, 1950) found that extracts of pig liver catalysed the reaction:

owing to the presence of nicotinamide mononucleotide adenylyltransferase (nicotinamide-adenine dinucleotide pyrophosphorylase). Hogeboom & Schneider (1952) and Branster & Morton (1956) found that this activity was associated with the

nuclear fraction of liver homogenates, and Branster (1958) showed that the activity per nucleus was essentially the same in intact liver cells as in nuclei isolated from the same tissue. Preiss & Handler (1958) found that liver nuclei also catalysed the reaction

Nicotinamide mononucleotide and nicotinic acid mononucleotide are both substrates of purified nicotinamide mononucleotide adenylyltransferase from pig liver; nicotinic acid mononucleotide is also a competitive inhibitor of the formation of nicotinamide-adenine dinucleotide (Atkinson, Jackson & Morton, 1960; Atkinson & Morton, 1960).

Competitive inhibition of this enzyme by analogues of nicotinamide mononucleotide or nicotinic acid mononucleotide affords a possible means of control of pyridine nucleotide coenzyme levels in tissues (Morton, 1958) and therefore the interaction between the enzyme and its substrates has been studied. This paper describes the effect of pH and of concentration of nicotinamide mononucleotide on the rate of formation of nicotinamide-adenine dinucleotide and on K_m for nicotinamide mononucleotide. Preliminary accounts of this work have been given (Atkinson, Jackson & Morton, 1958; Atkinson *et al.* 1960; Jackson, 1960).

EXPERIMENTAL

Abbreviations. ATP, adenosine triphosphate; NAD, nicotinamide-adenine dinucleotide (α -isomer, α -NAD); NMN, nicotinamide mononucleotide.

Materials

Nicotinamide mononucleotide adenylyltransferase. The nuclear fraction of pig liver was prepared essentially as described by Allfrey, Mirsky & Osawa (1957) for thymus nuclei. The nuclei were extracted with 0.25 M-sucrose-0.15 M-NaCl-0.01 M-sodium phosphate (pH 6.5) for 15 min. at 2° and centrifuged for 20 min. at 900 g. The washed nuclei from 400 g. of fresh liver were diluted to 1.2 l. in a solution containing 0.2 M- Na_2HPO_4 -0.1 M-ethylenediaminetetra-acetate, stirred for 30 min. at 20° and centrifuged at 3000 g for 60 min. The combined supernatant (2.9 l.) from several batches of nuclei was brought successively to 5, 15, 30 and 45% (v/v) of acetone at -2° to -10°. The precipitate obtained between 15 and 30% of acetone was dissolved in water and dialysed against 0.1 M-ethylenediaminetetra-acetate (pH 7) at 2°. The supernatant (420 ml.) obtained after centrifuging at 20 000 g for 20 min. at 0-2° contained 69 units (see Methods) of NMN adenylyltransferase. The solution was adjusted to pH 7.4 and the enzyme was adsorbed by two successive additions (30 ml.) of calcium phosphate gel (30 mg./ml.). The gel was washed twice with 0.02 M-sodium phosphate, pH 7.0 (200 ml.), and the enzyme was eluted with 0.5 M- K_2HPO_4

(180 ml.) by stirring for 30 min. at 2°. The eluate (188 ml.) contained 50 units of NMN adenylyltransferase; it was brought successively to 0.25, 0.45 and 0.60 saturation with ammonium sulphate at 4°. Material which was precipitated between 0.25 and 0.45 saturation was dissolved in water and dialysed for 18 hr. against water. After removal of insoluble material at 10 000 g for 20 min. at 2°, the solution (19 ml.) contained 22 units of NMN adenylyltransferase and 67 mg. of protein. Little activity was lost during several months at 2° or -15°. For kinetic studies the enzyme was diluted with water just before use.

Nucleotide pyrophosphatase. This was prepared from potatoes essentially as described by Branster & Morton (1956) up to the first ethanol precipitate, which was dissolved in water, adjusted to pH 5.0 and centrifuged at 5000 g for 30 min. at 0°. The supernatant was stored at 2°; there was little decline in activity during several months.

Myosin-adenosine triphosphatase. This was purified from rabbit skeletal muscle as described by Perry (1955).

Nicotinamide-adenine dinucleotide nucleosidase. This was prepared from *Neurospora crassa* (wild type) according to Kaplan (1955); at pH 7.2 and 37° the product hydrolysed 12 μ moles of NAD/min./mg. of protein and did not hydrolyse α -NAD.

Alcohol dehydrogenase. Recrystallized yeast enzyme (Sigma Chemical Co.) was dissolved in water to 1% (w/v) of protein before use.

Adenosine triphosphate. The sodium salt (Sigma Chemical Co.) was the same material found by Atkinson, Burton & Morton (1961) to be at least 98% pure (based on extinction at 259 m μ) by enzymic and electrophoretic analyses.

Nicotinamide-adenine dinucleotide. The material from Sigma Chemical Co. contained less than 2% of α -isomer, as determined by enzymic analysis. About 96% of the extinction at 259 m μ was due to the β -isomer, estimated with alcohol dehydrogenase. The main nucleotide contaminant was adenosine diphosphate ribose. Throughout this work, NAD concentration was calculated from the increase in extinction at 340 m μ ($\Delta\epsilon = 6.2 \times 10^3$; Horecker & Kornberg, 1948) on reduction with ethanol and alcohol dehydrogenase (Racker, 1950).

α -Nicotinamide-adenine dinucleotide. A preparation containing α -NAD, NAD and NMN in molar proportions 48:40:12 was generously provided by Dr J. M. Siegel of Pabst Laboratories. NAD was removed by hydrolysis with the specific nucleosidase.

Nicotinamide mononucleotide. (a) NAD was hydrolysed with nucleotide pyrophosphatase (Kornberg & Pricer, 1950) until no more alkali was needed to keep the reactants at pH 7. The nucleotide was purified by passage through Dowex 1 acetate and Dowex 50 (H^+) or Zeo-Karb 225 (H^+) (Plaut & Plaut, 1954; Haynes, Hughes, Kenner & Todd, 1957). Dowex 1 chloride was used instead of the acetate in purification of NMN used for determining the pK.

(b) NAD and pyrophosphate were converted into NMN and ATP with NAD adenylyltransferase (Kornberg, 1950; Klenow & Andersen, 1957); ATP was removed with added myosin-adenosine triphosphatase. After purification as in (a) NMN was obtained as a single component which absorbed light at 254 m μ after chromatography (R_f 0.8) in ammonium sulphate-0.1 M-sodium phosphate (pH 6.8)-propan-1-ol (60:100:2, w/v/v) or electrophoresis in 0.04 M-sodium citrate (zero net charge at pH 4.2; 0.1 cm./v hr./cm. at pH 4.6). The nucleotide contained phosphorus and

pentose in molar proportions 1.00:0.96 (see Methods). In 0.01M-sodium phosphate, pH 7.0, the nucleotide had maximum absorption at 265.5 m μ [ϵ , $(4.6 \pm 0.1) \times 10^3$ based on phosphorus content] and inflexions at 260 and 272 m μ . In M-KCN, 4 min. after mixing the material had maximum absorption at 325 m μ [ϵ , $(6.3 \pm 0.1) \times 10^3$]. On hydrogenation in 0.1M-2-amino-2-hydroxymethylpropane-1,3-diol at 37° and at atmospheric pressure with a Pd-Ba(OH)₂ catalyst, this material took up 2.95 mol.prop. of hydrogen. An aqueous solution (c, 3.0) had $[\alpha]_D^{25} - 42^\circ$, $[\alpha]_D^{35} - 42^\circ$. Dr P. T. Gilham (personal communication) has found $[\alpha]_{300m}^{20} - (4.4 \times 10^3)^\circ$. Kaplan, Ciotti, Stolzenbach & Bachur (1955) found $[\alpha]_D^{23} - 38^\circ$ for nucleotide derived from yeast, and Haynes *et al.* (1957) found $[\alpha]_D^{20} - 24^\circ$ for material prepared from ribose. NMN prepared as in (a) or (b) was converted to the same extent into reduced NAD with ethanol in the presence of alcohol dehydrogenase and NMN adenylyltransferase at pH 9.4. This coupled assay is specific for the β -anomer of NAD, as shown by tests with purified α -NAD, and indicates the presence of no more than 2% of α -anomer in the NMN.

Buffers and inorganic reagents. These were A.R. grade (British Drug Houses Ltd.) except glycylglycine, which was laboratory-reagent grade.

Methods

Apparent pK of nicotinamide mononucleotide. NMN (20 ml., 17 mM) was titrated at 25° with 0.2N-NaOH (free of CO₂) from pH 2.2 to pH 8.0 and with 0.1N-HCl from pH 8.0 to pH 3.5. The apparent pK was 6.2 ± 0.05 .

Phosphorus in nicotinamide mononucleotide. Samples containing about 20 μ g. of phosphorus were digested with a mixture of H₂SO₄, HNO₃ and HClO₄. After evaporation of the HNO₃ and HClO₄, the digest was diluted with water and boiled for 1 min. to hydrolyse pyrophosphate. After addition of *p*-nitrophenol as indicator, conc. aq. NH₃ was added until the solution was yellow and then 4N-HCl until the yellow colour just disappeared. Orthophosphate in this solution was determined by the molybdate-hydrazine procedure (Boltz & Mellon, 1947).

Pentose in nicotinamide mononucleotide. With Militzer's (1946) orcinol method, low values of ribose were found with NMN. However, when solutions of NMN containing 5–50 μ g. of bound pentose in 0.2 ml. were mixed with 0.025 ml. of about 4M-KCN, subsequent treatment with Militzer's (1946) reagent gave about 97% of the expected absorption at 680 m μ by comparison with arabinose standards. Cyanide adducts of nicotinamide nucleotides are hydrolysed more readily by acid than are the corresponding nucleotides.

Assay of nicotinamide-adenine dinucleotide adenylyltransferase. (a) *Assay for enzyme preparation.* Enzyme (0.6 ml.) was added to 0.4 ml. of solution at pH 7.5 and at 37° containing 4 μ moles of ATP, 4 μ moles of NMN, 15 μ moles of MgCl₂ and 150 μ moles of glycylglycine. After 10 min., 1.5 ml. of 0.5N-trichloroacetic acid was added; any precipitate which formed was removed by centrifuging at 2°. Then 2.0 ml. of the supernatant was mixed in a cell of 4 cm. light-path with 3.0 ml. of solution containing 94 mg. of glycine, 50 mg. of NaOH and 0.15 ml. of ethanol; the pH was about 9.5. The concentration of NAD was estimated from the increased absorption at 340 m μ (corrected for the blank) after addition of 10 μ l. of alcohol dehydrogenase. One unit of NMN adenylyltransferase catalysed synthesis

of 1 μ mole of NAD/min. under these conditions. Whereas this procedure gave true rates and sufficed for estimation of enzymic activity, at lower concentrations of NMN the rate of NAD formation decreased with time. With 3.3 mM- and 1.3 mM-NMN, the apparent reaction rates estimated from NAD formed after 5 min. were 97% and 87% respectively of the corresponding apparent rates at 2 min. For the kinetic studies described here, the procedure (b), in which there was a lower proportional conversion of NMN into NAD, was used.

(b) *Assay for kinetic studies.* A composite buffer (0.8 ml.) containing 208 μ moles each of acetic acid, succinic acid, glycylglycine and glycine adjusted to a suitable pH with KOH was mixed with 0.75 ml. of a solution containing 12 μ moles of ATP and 45 μ moles of MgCl₂ adjusted to pH 6.6 and with 1.2 ml. of NMN. These reactants (in a tube, 120 mm. $\times$ 15 mm., covered with a polythene sheet) were mixed and brought to 25.0°. After addition of 0.25 ml. of enzyme, the tubes were inverted three times within 5 sec. and kept at 25.0° for the assay period (usually 4 min.). The reaction was stopped with 1.0 ml. of 1.2N-trichloroacetic acid; the tubes were inverted as before, cooled in ice for several minutes and centrifuged at 2°. Then 3.5 ml. of the supernatant was mixed with 1.5 ml. of a solution containing 164 mg. of glycine, 87.5 mg. of NaOH and 0.15 ml. of ethanol in a 4 cm. cell; the pH was about 9.5. NAD formed during the reaction period was determined as in (a).

The pH was measured with a glass electrode at 25.0° in duplicates of all reaction mixtures used in kinetic studies of NAD formation with the adenylyltransferase. Variation of pH with concentration of NMN and variation of pH during the reaction period were less than 0.02 pH unit. Recovery of added NAD in this assay system was $100 \pm 2\%$.

With this procedure, the rate of formation of NAD did not vary with period of incubation by more than 1% at substrate concentrations between 0.14 and 1.4 mM-NMN in the range pH 4.97–10.55.

Rates of NAD formation (v , μ moles/min./mg. of protein) were measured as in (b) above at 5 or 6 concentrations of NMN (s , M) at each of 17 values in the range pH 4.97–10.55. There was a linear relationship between $1/v$ and $1/s$ when s was less than 1.5×10^{-3} ; at higher concentrations there was inhibition by NMN, resulting in deviation from linearity. To correct for the slight decrease in activity of the adenylyltransferase preparation during the period of several weeks necessary for these studies, an assay of activity at pH 7.6 and at 1.4 mM-NMN was included with each set of reaction mixtures. The observed rates were then multiplied by the appropriate factor to permit expression of all rates on the same basis.

RESULTS AND DISCUSSION

Determination of K_m and V'

The linear relationship between NAD formation and reaction time even at extremes of pH and at low substrate concentrations permitted estimation of true initial rates in all conditions used. In replicate determinations at a single pH and substrate concentration the standard error was about 3% of the mean.

Table 1. *Kinetic parameters of the reaction of nicotinamide mononucleotide with magnesium-adenosine triphosphate catalysed by nicotinamide mononucleotide adenylyltransferase at 25°*

For other experimental details, see Methods section. Standard errors (s.e.) are calculated from a combined estimate of internal variance of reaction rate based on 66 degrees of freedom; $S^2 = 2.09 \times 10^{-5}$.

pH	K_m (mm)	Coeff. of variation of K_m	$-\log K_m \pm \text{s.e.}$	V' ($\mu\text{moles/min./}$ mg. of protein)	Coeff. of variation of V'	$-\log V' \pm \text{s.e.}$
4.95	0.594	0.11	3.226 ± 0.047	0.230	0.05	0.638 ± 0.022
5.15	0.450	0.09	3.347 ± 0.038	0.267	0.04	0.573 ± 0.016
5.53	0.187	0.09	3.728 ± 0.040	0.275	0.03	0.561 ± 0.014
5.85	0.139	0.07	3.857 ± 0.028	0.303	0.02	0.519 ± 0.007
6.21	0.105	0.06	3.979 ± 0.027	0.334	0.01	0.476 ± 0.006
6.66	0.108	0.06	3.967 ± 0.027	0.341	0.01	0.467 ± 0.006
7.16	0.0879	0.10	4.056 ± 0.041	0.328	0.02	0.484 ± 0.011
7.63	0.0877	0.09	4.057 ± 0.040	0.341	0.02	0.467 ± 0.010
7.95	0.0752	0.10	4.124 ± 0.043	0.335	0.02	0.475 ± 0.010
8.44	0.0791	0.09	4.102 ± 0.041	0.342	0.02	0.466 ± 0.010
8.76	0.0719	0.07	4.143 ± 0.032	0.361	0.02	0.442 ± 0.007
9.40	0.1129	0.09	3.947 ± 0.040	0.344	0.03	0.463 ± 0.013
9.55	0.106	0.10	3.975 ± 0.045	0.308	0.03	0.511 ± 0.014
9.77	0.201*	0.09	3.697 ± 0.037	0.336	0.04	0.474 ± 0.017
10.00	0.180	0.12	3.745 ± 0.050	0.277	0.05	0.558 ± 0.021
10.35	0.298	0.16	3.526 ± 0.068	0.242	0.08	0.616 ± 0.036
10.55	0.336	0.18	3.474 ± 0.078	0.228	0.10	0.642 ± 0.043

* This value was omitted in the calculation of K_1 , K_2 and K_m (cf. Wilkinson, 1961).

It was found that $1/v$ was a linear function of $1/s$ between pH 4.97 and 10.55 and for s between 0.14×10^{-3} and 1.4×10^{-3} . Initially, K_m and V' were determined graphically from the expression (Lineweaver & Burk, 1934; cf. Dixon & Webb, 1958):

$$\frac{1}{v} = \frac{K_m}{V'} \left(\frac{1}{s} + \frac{1}{V'} \right).$$

To avoid possible errors arising from subjective fitting of the line in the graphical method, a more reliable procedure for determination of these parameters was sought. As described in the next paper (Wilkinson, 1961), least-squares fitting of the relationship $v = V'/(1 + K_m/s)$ gives the best estimate of both K_m and V' and the standard errors of these values.

Table 1 shows the results obtained with the statistical method of Wilkinson (1961) for each of the 17 pH values.

In the present study, there was a minimum value of K_m near pH 8, and at higher or lower pH values there was a continuous increase of the type which has been attributed to changes in ionic species of enzyme or substrate or enzyme-substrate complex.

Dixon (1953), Waley (1953) and Laidler (1955*a, b*) have discussed the quantitative relationship between K_m and $[H^+]$, and have shown that this relationship has the form of the Michaelis (1914) pH functions from which the apparent dissociation constants (K_1 and K_2) of the dissociating groups may be calculated. The appropriate relationship here is:

$$K_m = \tilde{K}_m \left(1 + \frac{[H^+]}{K_1} \right) \left(1 + \frac{K_2}{[H^+]} \right),$$

Fig. 1*A*. Plot of pK_m against pH for the adenylation of nicotinamide mononucleotide by adenosine triphosphate catalysed by NMN adenylyltransferase; for details, see text. The circles are experimentally determined values. The solid line was derived from the relationship

$$K_m = \tilde{K}_m \left(1 + \frac{[H^+]}{K_1} \right) \left(1 + \frac{K_2}{[H^+]} \right)$$

(see text). The line at Y shows the average 95% fiducial range for the individual K_m values. The broken lines are the asymptotes to the curve; as shown, these intercept at the values of pK_1 and pK_2 (cf. Dixon & Webb, 1958).

B. Plot of $\log V'$ against pH for the same reaction; the circles are experimentally determined values and the solid line is fitted by inspection.

where $\tilde{K}_m$ is the minimum value of K_m , attained when both enzyme and substrate are entirely in their effective ionic forms. From the values of K_m

and $[H^+]$ obtained from Table 1, $\bar{K}_m$, K_1 and K_2 , together with their standard errors, were obtained by a least-squares fitting of the relationship above (Wilkinson, 1961). In the logarithmic form, these values were: $p\bar{K}_m$, 4.10 ± 0.019 ; pK_1 , 5.76 ± 0.048 ; pK_2 , 9.97 ± 0.069 . Fig. 1A shows the variation of pK_m with pH; the theoretical relationship for these values of $p\bar{K}_m$, pK_1 and pK_2 is shown by the line.

Dixon (1953) and Dixon & Webb (1958) have shown that whereas such changes of pK_m with pH may be due to dissociation of groups in the enzyme, the substrate or the enzyme-substrate complex, only the last of these causes a unit change of the slope in the line relating $\log V'$ to pH. It is evident from Fig. 1B that no such unit change occurs between pH 4.95 and 10.55 in the interaction of NMN and NAD adenyllyltransferase. Hence the apparent pK values of pH 5.76 and 9.97 must be those of groups in the enzyme, in the enzyme-ATP-magnesium complex or in the NMN.

Reactive groups which influence interaction of nicotinamide mononucleotide with the enzyme

(a) *The group with apparent pK 5.76.* The apparent pK of the second dissociation of the phosphoryl group of NMN was 6.2 ± 0.05 (see Methods). Unless the ionic species of NMN with singly dissociated phosphoryl group ($R \cdot O \cdot PO_3H^-$) and that with doubly dissociated phosphoryl group ($R \cdot O \cdot PO_3^{2-}$) participate to the same extent in NAD formation, a change of slope corresponding to this pK would be expected in the pK_m -pH relationship (Dixon, 1953). NAD formation almost certainly involves nucleophilic attack on the α -phosphorus of ATP (or its magnesium complex); the doubly dissociated form of NMN, having a greater availability of electrons, is clearly a more effective reactant in such a system. The pK of 5.76 ± 0.05 is therefore allocated to the phosphoryl group of NMN. As pointed out by Danielli & Davies (1951) and by McLaren & Estermann (1957), the effective hydrogen-ion concentration close to a charged surface differs from that measured in the bulk of a solution with a glass electrode. Although nothing is known yet of the charge distribution in the vicinity of the reactive site of NMN adenyllyltransferase, our unpublished studies indicate that the isoelectric point is close to pH 7. If there is a net positive charge near the reactive site of the enzyme at pH 5.8 repulsion of hydrogen ions would result in a lower pH at the electrode than at the reactive site. The discrepancy of 0.4 pH between the pK of NMN and its apparent pK at the enzyme surface may well be due to this effect. This source of discrepancy seems to have received little attention in published studies of pK_m -pH relationships.

If the phosphoryl group of NMN in the enzyme-substrate complex was free to associate with a second proton, the pK of this association would be expected to be near pH 6. A corresponding unit change of slope about pH 6 should therefore appear in the plot of $\log V'$ against pH. The absence of such a change of slope (Fig. 1B) indicates that the phosphoryl group in the NMN-enzyme complex is not free to associate with a second proton. Hence it seems likely that the phosphoryl oxygen is bound either to magnesium or directly to the α -phosphorus atom of the enzyme-ATP-magnesium complex. Analogous reactions have been discussed by Lowenstein (1960) for the non-enzymic displacement of pyrophosphate from ATP by orthophosphate, and by Callaghan & Weber (1959) for the attack of adenylate on the adenylate kinase-ATP-magnesium complex.

The results given here do not allow of distinction between the S_N2 and S_N1 displacement mechanisms; it is not certain whether the sp^3d intermediate (cf. Van Wazer, 1958) in the former case would have sufficient kinetic stability to account for the absence of the phosphoryl dissociation. In the latter case, adenosine metaphosphate, $(R \cdot O \cdot PO_2)_n$, would be attacked by the nucleophilic phosphoryl oxygen of NMN; here, a preliminary attachment of this phosphoryl group to a bound Mg^{2+} ion is possible. Metaphosphoric acid derivatives have recently been implicated in phosphoryl-transfer reactions (Todd, 1960), and it seems likely that such derivatives may be intermediates in enzymic reactions involving ATP.

The concentrations of ATP and Mg^{2+} ions used in these studies were considerably greater than the corresponding K_m values at pH 7.4 (ATP, 0.46 mM; Mg^{2+} , 0.2 mM) reported by Kornberg (1950). Since the maximum rates reported here were calculated for infinite concentration of NMN, but not for infinite concentrations of ATP and Mg^{2+} ion, these rates have been designated V' rather than V , which has been reserved for the limiting rate calculated for infinite concentrations of all substrates. The slight decrease of $\log V'$ at the extremes of pH (Fig. 1B) is probably due to the concentration of the effective ATP-magnesium complex falling below that necessary to saturate the enzyme. At these extremes of pH there is competition for the components of the complex by H^+ or OH^- ion.

(b) *The group with apparent pK 9.97.* As NMN has no dissociation near pH 9.97, the unit change of slope about this pH (Fig. 1A) may be due to association of an OH^- ion with a group in the enzyme or in its ATP-magnesium complex. Alternatively the change of slope may result from the dissociation of a proton from a group in the enzyme or in the enzyme-ATP-magnesium com-

plex. Only the ionic species of this group predominating below pH 9.97 is effective in the catalytic process. The ϵ -ammonium group of lysyl residues, the hydroxyl of tyrosyl residues, and sulphhydryl groups have pK values in this pH region. Involvement of the cationic ammonium group seems unlikely as the affinity of NMN adenylyltransferase for NMN is about three times that for nicotinic acid mononucleotide (Atkinson *et al.* 1960; Atkinson & Morton, 1960), which has an additional negative charge at this pH. The possibility that NMN is bound to the enzyme by a hydrogen bond between its amide group and a tyrosyl hydroxyl group is at present being examined.

SUMMARY

1. Nicotinamide mononucleotide was prepared from nicotinamide-adenine nucleotide by hydrolysis with nucleotide pyrophosphatase or by pyrophosphorolysis with nicotinamide mononucleotide adenylyltransferase from pig-liver nuclei. The product was characterized by polarimetry, spectrophotometry and potentiometric titration.

2. The rate of formation of nicotinamide-adenine dinucleotide from adenosine triphosphate (4 mM) and nicotinamide mononucleotide (at 5 or 6 concentrations from 0.14 to 1.4 mM) in the presence of the adenylyltransferase and of magnesium chloride (15 mM) was measured at 25° and at 17 pH values from 4.97 to 10.55. With the assay system used, the rate of formation of nicotinamide-adenine dinucleotide did not vary during the period of incubation by more than 1%.

3. A statistical method was used to evaluate the Michaelis constants (K_m) and the apparent maximum rates (V' , at infinite nicotinamide mononucleotide concentration) for each pH.

4. A further statistical analysis of the variation of K_m with pH indicated the presence of dissociating groups, with pK values 5.76 ± 0.048 and 9.97 ± 0.069 , which participated in the interaction of nicotinamide mononucleotide with the enzyme-adenosine triphosphate-magnesium complex. For the effective ionic species, pK_m was 4.10 ± 0.019 .

5. From a consideration of the variation of pK_m with pH and of $\log V'$ with pH, possible mechanisms of the synthesis of nicotinamide-adenine dinucleotide have been formulated.

REFERENCES

- Allfrey, V. G., Mirsky, A. E. & Osawa, S. (1957). *J. gen. Physiol.* **40**, 451.
- Atkinson, M. R., Burton, R. M. & Morton, R. K. (1961). *Biochem. J.* **78**, 813.
- Atkinson, M. R., Jackson, J. F. & Morton, R. K. (1958). *Aust. J. Sci.* **21**, 119.
- Atkinson, M. R., Jackson, J. F. & Morton, R. K. (1960). *Aust. J. Sci.* **22**, 414.
- Atkinson, M. R. & Morton, R. K. (1960). *Nature, Lond.*, **188**, 58.
- Boltz, D. F. & Mellon, M. G. (1947). *Analyt. Chem.* **19**, 873.
- Brinster, M. V. (1958). Ph.D. Thesis: University of Melbourne.
- Brinster, M. V. & Morton, R. K. (1956). *Biochem. J.* **63**, 640.
- Callaghan, O. H. & Weber, G. (1959). *Biochem. J.* **73**, 473.
- Danielli, J. F. & Davies, J. T. (1951). *Advanc. Enzymol.* **11**, 35.
- Dixon, M. (1953). *Biochem. J.* **55**, 161.
- Dixon, M. & Webb, E. C. (1958). *Enzymes*. London: Longmans, Green and Co. Ltd.
- Haynes, L. J., Hughes, N. A., Kenner, G. W. & Todd, A. (1957). *J. chem. Soc.* p. 3727.
- Hogebom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.
- Horecker, B. L. & Kornberg, A. (1948). *J. biol. Chem.* **175**, 385.
- Jackson, J. F. (1960). M.Sc. Thesis: University of Adelaide.
- Kaplan, N. O. (1955). In *Methods in Enzymology*, vol. 2, p. 664. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Kaplan, N. O., Ciotti, M. M., Stolzenbach, F. E. & Bachur, N. R. (1955). *J. Amer. chem. Soc.* **77**, 815.
- Klenow, H. & Andersen, B. (1957). *Biochim. biophys. Acta*, **23**, 92.
- Kornberg, A. (1948). *J. biol. Chem.* **176**, 1475.
- Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
- Kornberg, A. & Pricer, W. E. (1950). *J. biol. Chem.* **182**, 763.
- Laidler, K. J. (1955a). *Trans. Faraday Soc.* **51**, 528.
- Laidler, K. J. (1955b). *Trans. Faraday Soc.* **51**, 550.
- Lineweaver, H. & Burk, D. (1934). *J. Amer. chem. Soc.* **56**, 658.
- Lowenstein, J. M. (1960). *Biochem. J.* **75**, 269.
- McLaren, A. D. & Estermann, E. F. (1957). *Arch. Biochem. Biophys.* **68**, 157.
- Michaelis, L. (1914). *Biochem. Z.* **60**, 91.
- Militzer, W. E. (1946). *Arch. Biochem.* **9**, 85.
- Morton, R. K. (1958). *Nature, Lond.*, **181**, 540.
- Perry, S. V. (1955). In *Methods in Enzymology*, vol. 2, p. 582. Ed. by Colowick, S. P. & Kaplan, N. O. New York: Academic Press Inc.
- Plaut, G. W. E. & Plaut, K. A. (1954). *Arch. Biochem. Biophys.* **48**, 189.
- Preiss, J. & Handler, P. (1958). *J. biol. Chem.* **233**, 493.
- Racker, E. (1950). *J. biol. Chem.* **184**, 313.
- Todd, A. (1960). *Nature, Lond.*, **187**, 819.
- Van Wazer, J. R. (1958). *Phosphorus and its Compounds*, vol. 1, chapt. 3. New York: Interscience Publishers Inc.
- Waley, S. G. (1953). *Biochim. biophys. Acta*, **10**, 27.
- Wilkinson, G. (1961). *Biochem. J.* **80**, 324.