The Requirement for Bivalent Cations in Formation of Nicotinamide-Adenine Dinucleotide by Nicotinamide Mononucleotide Adenylyltransferase of Pig-Liver Nuclei

By J. F. JACKSON AND M. R. ATKINSON*

Department of Agricultural Biochemistry, Waite Agricultural Research Institute, University of Adelaide, South Australia, and Department of Biochemistry, University of Cambridge

(Received 19 May 1966)

1. The requirement for bivalent cations in catalysis of NAD formation from ATP and NMN in the presence of NMN adenylyltransferase of pig-liver nuclei was studied. Rates of NAD formation in the presence of the activating cations Cd^{2+} , Mn^{2+} , Mg^{2+} , Zn^{2+} , Co^{2+} and Ni^{2+} were approximately a linear function of heats of hydration of the corresponding ions. Ba^{2+} , Sr^{2+} , Ca^{2+} , Cu^{2+} and Be^{2+} did not activate the enzyme; Be^{2+} inhibited the reaction in the presence of Mg^{2+} and, to a greater extent, in the presence of Ni^{2+} . 2. Michaelis constants for NAD formation, measured in a coupled assay with NMN adenylyltransferase and alcohol dehydrogenase at pH 8.0 and 25°, in the presence of 3 mM concentrations of the unvaried reactants, were $88 \pm 7 \mu\text{M}$ -ATP, $42 \pm 4 \mu\text{M}$ -NMN and $85 \pm 4 \mu\text{M}$ - Mg^{2+} . The results at this pH and at pH 7.5 were consistent with mechanisms in which Mg^{2+} -ATP complex is a reactant and free ATP a competitive inhibitor. 3. Formation of nicotinamide-hypoxanthine dinucleotide from NMN and ITP in the presence of the transferase was also more rapid with Ni^{2+} and Co^{2+} than with Mg^{2+} .

A requirement for Mg^{2+} or Mn^{2+} in catalysis of NAD formation by ATP-NMN adenylyltransferase (EC 2.7.7.1) from pig liver has been reported (Kornberg, 1950). This transferase, which is present in the nuclei of animal cells (Hogeboom & Schneider, 1952; Branster & Morton, 1956; Morton, 1961) and has been found in preparations of nuclear ribosomes (Traub, Kaufmann & Ginzburg-Tietz, 1964; Ginsburg-Tietz, Kaufmann & Traub, 1964), is released in a soluble form by salts or detergents (Traub *et al.* 1964; Atkinson, Jackson & Morton, 1964). The enzyme from liver catalyses reactions with nicotinate nucleotide (Preiss & Handler, 1958; Atkinson & Morton, 1960a) and a number of other pyridine nucleotides and purine nucleoside triphosphates (Atkinson, Jackson & Morton, 1961a). There is evidence that the reactions with both NMN and nicotinate nucleotide are involved in the biosynthesis of NAD (Ikeda *et al.* 1965; Dietrich, Fuller, Yero & Martinez, 1966).

From studies of the variation with pH of K_m for NMN it was concluded that this nucleotide reacts in its doubly-ionized form (Atkinson, Jackson & Morton, 1961b). To obtain more information on

the mechanism of the reaction, specificity of the transferase for bivalent cations has now been studied.

EXPERIMENTAL

Materials. NMN (Atkinson *et al.* 1961b) and NMN adenylyltransferase (Atkinson *et al.* 1964) were prepared as described before. Enzyme used in coupled assays with alcohol dehydrogenase was passed through Sephadex G-50 in 50 mM-tris (Cl^- , pH 8.0) before use. Bivalent metal salts were of A.R. grade. Sodium salts of ATP (C. F. Boehringer und Soehne, Mannheim, Germany, or Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.) and ITP (Sigma Chemical Co., St Louis, Mo., U.S.A.) were used.

Assay of NAD formation. (a) In most experiments NAD formation was measured with alcohol dehydrogenase after incubation at 37° and inactivation of transferase as described by Atkinson *et al.* (1961b). Details of buffer and substrate concentrations and pH are given in the Results section. Formation of nicotinamide-hypoxanthine dinucleotide from ITP was measured in the same way. Michaelis constants and extrapolated maximum velocities, together with their standard errors, were calculated by Wilkinson's (1961) method; a programme for calculation of these parameters on the IBM 1620 computer was written by Mrs Margaret Atkinson.

(b) In the coupled assay of NMN adenylyltransferase initial rates of increase of extinction at 340 m μ were measured at 25° in a 1 cm. cell. Reaction mixtures contained

* Present address: School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia.

7.5 μ moles of NMN, 7.5 μ moles of ATP, 7.5 μ moles of MgSO_4 , 250 μ moles of tris, 750 μ moles of ethanol, 0.2 mg. of yeast alcohol dehydrogenase and 0.25 mg. of transferase in a final volume of 2.5 ml. The pH was adjusted to 8.0 with H_2SO_4 . For calculation of Michaelis constants the concentration of either ATP, NMN or MgSO_4 was varied in the range 0.04–0.4 mM; for comparison of rates in the presence of different cations 0.5 μ mole of MgSO_4 , MnCl_2 , NiSO_4 , ZnSO_4 , CdSO_4 , SrCl_2 , BaCl_2 , CuSO_4 or CaCl_2 was used. Rates were constant for at least 1 min. and were not limited by inhibition of the dehydrogenase.

RESULTS

Comparative studies of specificity for bivalent cations in nucleotidyl transfer. Rates of NAD formation, relative to the rate with 0.2 mM- Mg^{2+} , in the coupled assay at pH 8.0, for a number of bivalent cations at total concentration of 0.2 mM (uncorrected for chelation by substrates or buffer) were: Co^{2+} , 1.29; Zn^{2+} , 0.88; Ni^{2+} , 0.48; Mn^{2+} , 0.39; Cd^{2+} , 0.03; Sr^{2+} , Ba^{2+} , Ca^{2+} and Cu^{2+} , < 0.01. When adenylyltransferase activity was measured in the batch assay for 4 min. at 37° with 4.5 mM-NMN–4 mM-ATP–0.2 M-glycylglycine (Na^+ , pH 7.6) and 20 mM-magnesium chloride, -nickel chloride, -cobalt chloride, -manganese chloride and -zinc sulphate, rates of NAD formation, relative to the rate with Mg^{2+} , were: Ni^{2+} , 3.70; Co^{2+} , 2.46; Mn^{2+} , 0.31; Zn^{2+} , 0.67. Slight precipitation took place during incubation with manganese chloride and zinc sulphate.

With 5 mM-ITP replacing ATP in these conditions the rate of nicotinamide-hypoxanthine dinucleotide formation in the presence of 20 mM-magnesium chloride was 3.5% of the rate of NAD formation from ATP. Rates of inosinyl transfer in the presence of 20 mM-nickel chloride, -cobalt chloride and -zinc sulphate, relative to the rate with magnesium chloride, were: Ni^{2+} , 3.20; Co^{2+} , 1.91; Zn^{2+} , 0.50. There was some precipitation in the assay with zinc sulphate.

To test for activation by Ni^{2+} with unpurified enzyme, NAD formation was measured for 5 min. at 37° in 4.5 mM-NMN–4 mM-ATP–0.2 M-glycylglycine (Na^+ , pH 7.6) after addition of 0.2 ml. of a 20% (w/v) homogenate of pig liver in 0.25 M-sucrose–3 mM-calcium chloride; the final assay volume was 1 ml. NAD formation in the presence of 20 mM-nickel chloride was 30.8 μ moles/min.; with 20 mM-magnesium chloride, 20 mM-magnesium chloride + 20 mM-nickel chloride, or no added bivalent cation, rates of NAD formation were 18.0, 20.6 and 11.3 μ moles/min. respectively.

Variation of rate of NAD formation with ATP, NMN and bivalent cation concentrations. In the coupled assay at pH 8.0 the apparent Michaelis constants for ATP, NMN and Mg^{2+} were $88 \pm 7 \mu\text{M}$, $42 \pm 4 \mu\text{M}$ and $85 \pm 4 \mu\text{M}$. The rate without added

magnesium chloride was 4% of the extrapolated maximum rate. From a plot of rate of NAD formation against magnesium chloride concentration this corresponds to an endogenous concentration of 3 μM -magnesium chloride, and concentrations of magnesium chloride were corrected by addition of this value before calculation of K_m .

Variation of rate of NAD formation with ATP and magnesium chloride concentrations was also studied in batch assays with 2.26 mM-NMN–0.1 M-glycylglycine (Na^+ , pH 7.5), 0.1–20 mM-magnesium chloride and 1.57–7.85 mM-ATP. Incubation was at 37° for 10 min., with 54 μg . of transferase/ml. of reactants. Concentrations of Mg^{2+} -ATP complex and free ATP (total of all ionic species) were calculated from published stability constants of Mg^{2+} -ATP complex and Mg^{2+} -glycylglycine complex (see the Discussion section). In Fig. 1 the reciprocal of the rate of NAD formation is plotted against the concentration of free ATP at three concentrations of Mg^{2+} -ATP complex (97 ± 3 , 194 ± 6 and $478 \pm 16 \mu\text{M}$) to test the possibility that Mg^{2+} -ATP complex is a substrate and free ATP a competitive inhibitor (cf. Dixon & Webb, 1964).

Rates of NAD formation were also measured in batch assays with 0.2 mM-, 2 mM- and 20 mM-magnesium chloride, -cobalt chloride, -nickel chloride, -manganese chloride and -zinc chloride in the presence of 2.26 mM-NMN, 3.02 mM-ATP and

Fig. 1. Plots of reciprocal of rate of NAD formation against concentration of free ATP for three ranges of Mg^{2+} -ATP complex concentration: ○, $97 \pm 3 \mu\text{M}$; ●, $194 \pm 6 \mu\text{M}$; Δ, $478 \pm 16 \mu\text{M}$. For details of reaction conditions and calculation of concentrations see the Results and Discussion sections.

Table 1. *Michaelis constants and extrapolated maximum rates of NAD formation by NMN adenylyltransferase when ATP or bivalent metal salt concentration was varied*

Reactions were carried out in 0.1M-glycylglycine (pH 8.0); for details see the Results section.

	Maximum rates of NAD formation (mμmoles/min.)		K_m (μM)	
	ATP varied	Cation varied	ATP varied	Cation varied
MgCl ₂	14.0 ± 0.1	16.9 ± 1.3	107 ± 5	172 ± 76
NiCl ₂	22.4 ± 0.2	*	197 ± 24	*
CoCl ₂	31.7 ± 1.6	33.2 ± 0.6	261 ± 51	1036 ± 85
ZnSO ₄	21.7 ± 0.4	21.8 ± 0.3	249 ± 20	1386 ± 89
MnCl ₂	5.9 ± 0.6	6.5†	145 ± 72	170†

* Deviates from the Michaelis relationship, with high apparent K_m ; for details see the Results section and Fig. 2.

† Precipitates formed during incubation; parameters were calculated from two values only.

0.2M-glycylglycine (Na⁺, pH 8.0). Assays were carried out at 37° for 3 min. with nickel chloride, 5 min. with cobalt chloride, 10 min. with magnesium chloride and zinc sulphate, and 16 min. with manganese chloride; 45 μg. of enzyme preparation (specific activity, 0.5 μmole of NAD formed/min./mg. of protein) was used in each assay. Apparent Michaelis constants and extrapolated maximum rates are shown in Table 1, together with the corresponding parameters calculated from experiments in which the ATP concentration was varied in the range 0.15–3 mM in 2.26 mM-NMN–0.2 M-glycylglycine (Na⁺, pH 8.0) with 4 mM-magnesium chloride, 20 mM-nickel chloride, 10 mM-cobalt chloride, 10 mM-zinc sulphate or 4 mM-manganese chloride. Precipitates formed during incubation at the higher [manganese chloride]/[ATP] ratios.

The high apparent Michaelis constant for nickel chloride indicated the possibility that much of the Ni²⁺ was chelated by buffer. Fig. 2 shows the variation of rate of NAD formation with the adenylyltransferase from 2.26 mM-NMN and 3.02 mM-ATP in the presence of 0.05 M-, 0.1 M- or 0.2 M-glycylglycine (Na⁺, pH 7.5) and nickel chloride (0.1–40 mM). In similar conditions (3 min. at 37°) but with 20 mM-magnesium chloride the rate of NAD synthesis was 17.2 mμmoles/min./ml.

Inhibition of the adenylyltransferase by EDTA. Formation of NAD from 2.26 mM-NMN and 3.02 mM-ATP in 0.2 M-glycylglycine (Na⁺, pH 8.0) during 10 min. at 37° was decreased to 10% of the control rate in the presence of 20 mM-magnesium chloride on addition of 40 mM-EDTA (Na⁺, pH 8.0). With 4 mM-magnesium chloride inhibition was complete in the presence of 40 mM-EDTA, and with 0.1 M-magnesium chloride 2 mM-EDTA gave complete inhibition, as did 40 mM-EDTA with 20 mM-nickel chloride.

Variation of rate of NAD formation with ATP concentration at high concentrations of magnesium

Fig. 2. Rates of NAD formation as a function of NiCl₂ concentration at various concentrations of glycylglycine (pH 7.5): Δ, 0.005M; ●, 0.1M; ○, 0.2M. For details of assay conditions see the Results section.

chloride, nickel chloride and cobalt chloride. To decrease interference by buffer ligands, NAD formation was measured with the adenylyltransferase (45 μg./ml.; specific activity, 0.5 μmole/min./mg. of protein) in 20 mM-magnesium chloride, -nickel chloride or -cobalt chloride. Reactions were carried out in 2.26 mM-NMN–0.1 M-glycylglycine (Na⁺, pH 7.5) at 37° for 5 min. (nickel chloride and cobalt chloride) or 10 min. (magnesium chloride) and the ATP concentration was varied in the range

Table 2. *Michaelis constants and extrapolated maximum rates of NAD formation with 20mm-bivalent salts when ATP was varied*

Reactions were carried out in 0.1M-glycylglycine (pH 7.5); for details see the Results section.

	Maximum rates of NAD formation (μ moles/min.)	K_m (μ M-ATP)
MgCl ₂	17.8 $\pm$ 0.1	163 $\pm$ 7
NiCl ₂	36.2 $\pm$ 0.3	121 $\pm$ 6
CoCl ₂	34.6 $\pm$ 0.3	135 $\pm$ 7

0.157–7.55mm. Table 2 lists apparent Michaelis constants and extrapolated maximum rates of NAD formation measured in these experiments. There was no evidence of deviation from hyperbolic relationships of rate to ATP concentration.

Inhibition of the adenylyltransferase by beryllium chloride. Beryllium chloride has been reported to decrease the specific activity of NMN adenylyltransferase in liver (Stirpe & Aldridge, 1961). In the presence of 16mm-magnesium chloride–4.52mm-NMN–4mm-ATP–0.2M-glycylglycine (Na⁺, pH 7.6) during 10min. at 37° the rate of NAD formation with NMN adenylyltransferase (9 μ g./ml.) was decreased from 1.01 to 0.78 μ moles/min./mg. of protein by 4mm-beryllium chloride. With 20mm-nickel chloride the rate was decreased from 3.11 to 0.09 μ moles/min./mg. of protein (97% inhibition). No NAD formation was observed when 4mm- or 20mm-beryllium chloride was the only bivalent salt in the assay system.

Absence of inhibition of NMN adenylyltransferase by fluoride. When adenylyltransferase (45 μ g./ml.; specific activity, 0.5 μ mole of NAD formed/min./mg. of protein) was incubated in 2.26mm-NMN–3.02mm-ATP–0.1M-glycylglycine (Na⁺, pH 7.5) for 5min. at 37° with 2mm-nickel chloride or -cobalt chloride or for 10min. at 37° with 2mm-magnesium chloride, -zinc sulphate or -manganese chloride, the rates of NAD formation (μ moles/min.) were: Mg²⁺, 13.6; Ni²⁺, 18.3; Co²⁺, 26.2; Zn²⁺, 21.6; Mn²⁺, 5.3. In the presence of 50mm-sodium fluoride the corresponding rates were: Mg²⁺, 14.3; Ni²⁺, 17.4; Co²⁺, 26.8; Zn²⁺, 22.6; Mn²⁺, 4.7.

DISCUSSION

Schulman & Sternlicht (1965) concluded from nuclear-magnetic-resonance studies of Co²⁺-ATP, Ni²⁺-ATP, Mn²⁺-ATP and Co²⁺-ITP complexes that the metal ion in these complexes interacts with acidic functions on P^(α), P^(β) and P^(γ) and with the purine; the only species for which evidence exists resemble Szent-Györgi's (1957) model, with the polyphosphate chain bound to the purine through

Fig. 3. Relationship of rates of NAD formation with NMN adenylyltransferase (relative to the rate with MgCl₂ in the same conditions) to heats of hydration of the corresponding bivalent cations: O, rates extrapolated to saturating ATP concentrations at pH 7.5 (Table 2); Δ, rates extrapolated to saturating ATP concentrations at pH 8.0 (Table 1); ●, rates measured in the continuous assay at pH 8.0 (cf. the Results section).

the metal ion (for other references see Atkinson & Morton, 1960b). It is probable that enzymic formation of NAD from NMN involves a nucleophilic attack by the ionic species (ribosylnicotinamide)-5'-O-PO₃²⁻ on P^(α) of the metal ion-ATP complex at the active centre of the transferase (Atkinson *et al.* 1961b). The similarity of Michaelis constants for [ATP]_{total} and [Mg]_{total} and their difference from the Michaelis constant for NMN (cf. the Results section) are consistent with mechanisms in which Mg²⁺-ATP complex is the substrate (Dixon & Webb, 1964). This model is also supported by the agreement between observed rates of NAD formation and those calculated on the assumption that Mg²⁺-ATP complex is a substrate and free ATP a competitive inhibitor of the transferase (Fig. 1). For this calculation the stability constant of Mg²⁺-ATP complex was taken as 24.5mm⁻¹ (Nanninga, 1961) and that of Mg²⁺-glycylglycine complex as 0.0115mm⁻¹ (Chemical Society, London, 1957). From the results, which are plotted according to Dixon's (1953) method, K_m for Mg²⁺-ATP complex is 0.12mm and free ATP is a competitive inhibitor with K_i 2.0mm (calculated as the sum of all ionic species). Interaction with buffer ligands has prevented an extension of this type of analysis to the more firmly-bound metal ions such as Ni²⁺ (Fig. 2).

As shown in Fig. 3, there is an approximately linear increase in rate of NAD formation with decrease in heat of hydration of activating ions. Heats of hydration are those listed by Williams

(1954), except that for Be^{2+} (cf. Conway & Bockris, 1954). The heat of hydration of an ion can be correlated with its coefficient of polarization or polarizability (Guerdjikov, 1961). If, as seems probable, polarizability is inversely related to the polarizing effect of a metal ion on adjacent ligands (for a discussion of this relationship see Glasstone, 1946), the higher rates of adenylyl transfer are associated with those bivalent cations with a greater tendency to polarize P-O bonds in metal ion-ATP complexes. A similar activation has been proposed by Hammes & Kochavi (1962) for hexokinase. Rates of non-enzymic transfer of a phosphoryl group from ATP to acetate in the presence of bivalent cations (Lowenstein & Schatz, 1961) also increase in the sequence: Mn^{2+} ; Zn^{2+} ; Co^{2+} ; Ni^{2+} . Activation of phosphorus in metal ion-ATP complexes is probably related to τ_m , the time of interaction of metal with each phosphorus. Schulman & Sternlicht (1965) found in nuclear-magnetic-resonance studies with metal ion-ATP complexes that this 'metal-sticking time' increased in the sequence: Mn^{2+} , $5\mu\text{sec.}$; Co^{2+} , $20\mu\text{sec.}$; Ni^{2+} , $700\mu\text{sec.}$

Though many nucleotidyltransferases are known to be activated by Mg^{2+} and Mn^{2+} , little is known of the ability of other bivalent cations to participate in these reactions. Co^{2+} is an activator in a number of cases [e.g. several UTP-sugar phosphate uridylyltransferases (Neufeld, Ginsburg, Putman, Fanshier & Hassid, 1957), CTP-glycerol phosphate cytidylyltransferase (Shaw, 1962) and UTP-glucose phosphate uridylyltransferase (Turner & Turner, 1958)]. In the last case Ni^{2+} was also reported as an activator. No reports of activation of nucleotidyltransferases by Zn^{2+} have been found, but this ion, together with all the effective bivalent ions discussed in the present paper, are known as activators of enzymic phosphoryl transfer from ATP. Ni^{2+} , Co^{2+} , Zn^{2+} , Mg^{2+} , Mn^{2+} and Cd^{2+} activate phosphorylation of fructose by ATP in the presence of hexokinase (Brintzinger & Fallab, 1960) and hydrolysis of ATP by the cold-labile adenosine triphosphatase of mitochondria (Selwyn, 1962).

The similar patterns of activation in reactions involving nucleophilic attack at $\text{P}^{(\alpha)}$ or $\text{P}^{(\gamma)}$ of ATP may be related to Todd's (1962) suggestion that if adenylyl transfer involves an adenoside 5'-metaphosphate intermediate the metal ion may block acidic functions on $\text{P}^{(\beta)}$ and $\text{P}^{(\gamma)}$, whereas if phosphoryl transfer involves monomeric metaphosphate its generation would be favoured by interaction of the bivalent metal ion with acidic functions on $\text{P}^{(\alpha)}$ and $\text{P}^{(\beta)}$. The recent observation of equivalent interactions of metals with -O- on $\text{P}^{(\alpha)}$, $\text{P}^{(\beta)}$ or $\text{P}^{(\gamma)}$ of ATP (Schulman & Sternlicht, 1965) is consistent with these proposals.

Thomas & Aldridge (1966) have discussed inhibi-

tion of enzymes by Be^{2+} and only find evidence for direct action on the enzyme with alkaline phosphatase and phosphoglucomutase. Other inhibitions are attributed to combination of Be^{2+} with the substrate, as with yeast hexokinase (Gutfreund & Hammond, 1963), where there is also evidence that Mg^{2+} -ATP complex is a substrate and free ATP a competitive inhibitor. A greater effect of Be^{2+} with Ni^{2+} -ATP complex than with Mg^{2+} -ATP complex was observed in the present studies of NAD formation, but the mechanism of inhibition is not known.

The award of a Nuffield Foundation Dominion Travelling Fellowship (to M. R. A.) and an Australian Dairy Produce Board Senior Post-Graduate Studentship (to J. F. J.) is gratefully acknowledged. We thank Professor Malcolm Dixon, F.R.S., and Professor F. G. Young, F.R.S., for provision of research facilities in the Biochemistry Department, University of Cambridge; we also record our indebtedness to the late Professor R. K. Morton for his interest and support in this work.

REFERENCES

- Atkinson, M. R., Jackson, J. F. & Morton, R. K. (1961a). *Nature, Lond.*, **192**, 946.
- Atkinson, M. R., Jackson, J. F. & Morton, R. K. (1961b). *Biochem. J.* **80**, 318.
- Atkinson, M. R., Jackson, J. F. & Morton, R. K. (1964). *Biochem. J.* **90**, 433.
- Atkinson, M. R. & Morton, R. K. (1960a). *Nature, Lond.*, **188**, 58.
- Atkinson, M. R. & Morton, R. K. (1960b). In *Comparative Biochemistry*, vol. 2, p. 1. Ed. by Florkin, M. & Mason, H. S. New York: Academic Press Inc.
- Branster, M. V. & Morton, R. K. (1956). *Biochem. J.* **63**, 640.
- Brintzinger, H. & Fallab, S. (1960). *Helv. chim. Acta*, **43**, 43.
- Chemical Society, London (1957). *Special Publication no. 6: Stability Constants Part I—Organic Ligands*. London: Chemical Society.
- Conway, B. E. & Bockris, J. O'M. (1954). *Modern Aspects of Electrochemistry*, p. 52. London: Butterworths Scientific Publications.
- Dietrich, L. S., Fuller, L., Yero, I. L. & Martinez, L. (1966). *J. biol. Chem.* **241**, 188.
- Dixon, M. (1953). *Biochem. J.* **55**, 170.
- Dixon, M. & Webb, E. C. (1964). *Enzymes*, 2nd ed., p. 429. London: Longmans, Green and Co. Ltd.
- Ginsburg-Tietz, Y., Kaufmann, E. & Traub, A. (1964). *Exp. Cell Res.* **34**, 384.
- Glasstone, S. (1946). *Textbook of Physical Chemistry*, 2nd ed., p. 541. London: Macmillan and Co. Ltd.
- Guerdjikov, D. C. (1961). *C.R. Acad. bulg. Sci.* **14**, 471.
- Gutfreund, H. & Hammond, B. R. (1963). *Nature, Lond.*, **198**, 667.
- Hammes, G. G. & Kochavi, D. (1962). *J. Amer. chem. Soc.* **84**, 2076.
- Hogebloom, G. H. & Schneider, W. C. (1952). *J. biol. Chem.* **197**, 611.

- Ikeda, M., Tsuji, H., Nakamura, S., Ichiyama, A., Nishezuka, Y. & Hayaishi, O. (1965). *J. biol. Chem.* **240**, 1395.
- Kornberg, A. (1950). *J. biol. Chem.* **182**, 779.
- Lowenstein, J. M. & Schatz, M. N. (1961). *J. biol. Chem.* **236**, 305.
- Morton, R. K. (1961). *Aust. J. Sci.* **24**, 260.
- Nanninga, L. B. (1961). *Biochim. biophys. Acta*, **54**, 338.
- Neufeld, E. F., Ginsburg, V., Putman, E. W., Fanshier, D. & Hassid, W. Z. (1957). *Arch. Biochem. Biophys.* **69**, 602.
- Preiss, J. & Handler, P. (1958). *J. biol. Chem.* **233**, 493.
- Schulman, R. G. & Sternlicht, H. (1965). *J. molec. Biol.* **13**, 952.
- Selwyn, M. J. (1962). *Biochem. J.* **84**, 62P.
- Shaw, D. R. D. (1962). *Biochem. J.* **82**, 297.
- Stirpe, F. & Aldridge, W. N. (1961). *Biochem. J.* **80**, 481.
- Szent-Györgi, A. (1957). *Bioenergetics*, p. 70. New York: Academic Press Inc.
- Thomas, M. & Aldridge, W. N. (1966). *Biochem. J.* **98**, 94.
- Todd (Lord) (1962). *Proc. chem. Soc., Lond.*, p. 199.
- Traub, A., Kaufmann, E. & Ginzburg-Tietz, Y. (1964). *Exp. Cell Res.* **34**, 371.
- Turner, D. H. & Turner, J. F. (1958). *Biochem. J.* **69**, 448.
- Wilkinson, G. N. (1961). *Biochem. J.* **80**, 324.
- Williams, R. J. P. (1954). *J. phys. Chem.* **58**, 121.