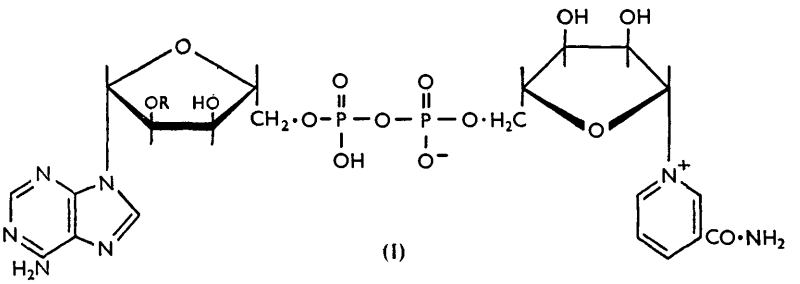
735. *Codehydrogenases. Part III.* A Synthesis of Diphosphopyridine Nucleotide (Cozymase), and Some Observations on the Synthesis of Triphosphopyridine Nucleotide.*

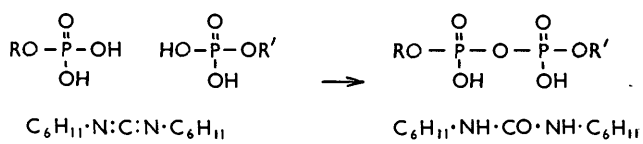
By N. A. Hughes, G. W. Kenner, and Sir Alexander Todd.

Condensation of nicotinamide nucleotide [1] and adenosine-5′ phosphate, dissolved in aqueous pyridine, has been accomplished by adding a large excess of di*cyclo*hexylcarbodi-imide. Only a small amount of the nicotinamide nucleotide is converted into the symmetrical pyrophosphate, and some 50% of it condenses to yield diphosphopyridine nucleotide, which can be separated by ion-exchange chromatography. This coenzyme fraction, like the nicotinamide nucleotide used in its preparation, is a mixture of α- and β-forms. The pure β-form has been isolated by reduction with alcohol and alcohol dehydrogenase, alkaline destruction of the unreduced α-isomer, and enzymic regeneration from the dihydro-form. Preliminary experiments on the condensation of nicotinamide nucleotide with a mixture of adenosine-2′ : 5′ and -3′ : 5′ diphosphate [2] have given a product containing triphosphopyridine nucleotide (TPN), which has been obtained by ion-exchange chromatography as a mixture of isomers.†

Although diphosphopyridine nucleotide (cozymase, DPN, coenzyme I, codehydrogenase I) (I; R = H) [4,5] is the longest-known coenzyme, comparatively little progress towards its synthesis has been hitherto recorded. While the present study was under way, it was reported [6] that this coenzyme can be detected enzymically in the mixture produced from nicotinamide nucleotide (derived from natural sources) and adenosine-5′ phosphate



(I)



by the action of trifluoroacetic anhydride.[7] Having developed a relatively convenient synthesis of nicotinamide nucleotide,[1] we were in a position to examine thoroughly this type of condensation, which forms the final step in the scheme of synthesis envisaged in Part I.[8] Various methods of condensation have been used successfully by us in the

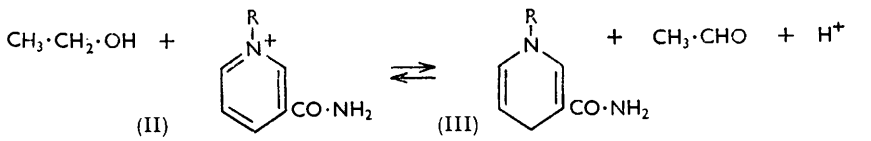
* Part II, preceding paper.

† A preliminary announcement of these results was made in a lecture given to the Basle Chemical Society in July 1955 which has since been published.[3]

[1] Haynes, Hughes, Kenner, and Todd, preceding paper.
[2] Cramer, Kenner, Hughes, and Todd, *J.*, 1957, 3297.
[3] Todd, *Stoll Festschrift*, Birkhauser, Basle, 1957, p. 508.
[4] Lythgoe, *Ann. Reports*, 1945, **42**, 180.
[5] Singer and Kearney, *Adv. Enzymol.*, 1954, **15**, 79.
[6] Shuster, Kaplan, and Stolzenbach, *J. Biol. Chem.*, 1955, **215**, 195.
[7] Cf. Christie, Elmore, Kenner, Todd, and Weymouth, *J.*, 1953, 2947.
[8] Haynes and Todd, *J.*, 1950, 303.

coenzyme field.[3,9] The carbodi-imide method[10] (see formulæ) seemed most suitable for initial study on account of its simplicity and because of the dipolar-ionic character and reducibility of nicotinamide nucleotides. In fact, it was successful to an unexpected degree, and consequently none of the other methods, which may also be equally applicable, has been tried.

Nicotinamide nucleotide is insoluble in anhydrous pyridine or dimethylformamide, the solvents used in our previous applications of the carbodi-imide method to the synthesis of nucleotide pyrophosphates.[7,11] However, Khorana has shown that aqueous pyridine can be used satisfactorily, for example in the self-condensation of adenosine-5′ phosphate, provided that a vast excess of the carbodi-imide is added.[12] This technique was applied to a mixture of nicotinamide nucleotide and adenosine-5′ phosphate; the latter compound, being much the more accessible, was used in excess. Examination of the reaction mixture by paper chromatography and electrophoresis showed the presence of all the expected products, *viz.*, diphosphopyridine nucleotide and the two symmetrical pyrophosphates, together with some of the starting materials. On anion-exchange chromatography, neither the nicotinamide nucleotide nor the symmetrical pyrophosphate derived from it—both effectively neutral—was retained by the resin, while the diphosphopyridine nucleotide, adenosine-5′ phosphate, and P^1P^2-diadenosine-5′ pyrophosphate were successively eluted with increasing strengths of formic acid.[13] A partial separation of nicotinamide nucleotide from the corresponding symmetrical pyrophosphate was achieved by cation-exchange chromatography and thus the proportions of all the components of the reaction mixture could be estimated. Almost half the nicotinamide nucleotide had been converted into diphosphopyridine nucleotide and only about a tenth into the symmetrical P^1P^2-di-(nicotinamide nucleoside) pyrophosphate. Since the latter compound is stable under the conditions of reaction there is evidently some hindrance to its formation. A similar phenomenon has been observed in the condensation of choline phosphate and cytidine-5′ phosphate effected by di*cyclo*hexylcarbodi-imide in aqueous pyridine:[14] none of the



expected P^1P^2-dicholine pyrophosphate could be detected. There would be little purpose in speculating here about the way in which the interaction of charges in a dipolar ion may affect participation in a condensation caused by a carbodi-imide; it suffices to note that such cases appear to be particularly favourable for application of the carbodi-imide method. Apparently the trifluoroacetic anhydride method does not share this useful selectivity.[6]

The synthetic diphosphopyridine nucleotide was isolated after ion-exchange chromatography as an amorphous powder, which had the correct elementary composition and a coenzyme content of about 70% according to spectroscopic assay with alcohol dehydrogenase.[15] This value was of the expected order because the synthesis had started from nicotinamide nucleotide containing the β- and the α-anomer in approximately 80 : 20 ratio and the optical rotation stood in the same relation to the published values[16] for the β- and the α-form of the coenzyme obtained from natural sources. Careful elution of the synthetic material from an anion-exchange column also suggested the presence of

[9] Todd, *Proc. Roy. Soc.*, 1954, *A*, **226**, 70.
[10] Khorana and Todd, *J.*, 1953, 2257.
[11] Kenner, Todd, and Webb, *J.*, 1954, 2843.
[12] Khorana, *J. Amer. Chem. Soc.*, 1954, **76**, 3517.
[13] Cf. Kornberg and Pricer, *Biochemical Preparations*, 1953, **3**, 20.
[14] Kennedy, *J. Biol. Chem.*, 1956, **222**, 185.
[15] Racker, *ibid.*, 1950, **184**, 313.
[16] Kaplan, Ciotti, Stolzenbach, and Bachur, *J. Amer. Chem. Soc.*, 1955, **77**, 815.

two components, and comparison with natural material showed that the β-isomer was more easily eluted, as would be expected from the greater proximity of the positive and negative charges. But there seemed little hope of a practical separation by this means and there was no sign at all of separation by paper chromatography or electrophoresis even in presence of borate ions. However, a simple and efficient way of purifying the β-isomer was offered by its selective reduction with alcohol dehydrogenase. It is well known [5] that quaternary nicotinamide nucleotides (II) are easily decomposed by alkali, whereas their dihydro-derivatives (III) are stable to alkali although easily hydrolysed by acid. Consequently the α-isomer should, in principle, be completely removed by successive enzymic reduction, alkaline treatment, enzymic oxidation, and ion-exchange chromatography. In practice, the material recovered after this series of operations, which again had the correct elementary analysis, was identical with the natural coenzyme in paper chromatographic, electrophoretic, and ion-exchange behaviour, and appeared to be 90% pure as judged by both optical rotation and enzymic assay with alcohol dehydrogenase. Our tests were made by two observers over a period of several months and gave values diminishing steadily with time from 93 to 85%; commercial "95—100% cozymase" gave in our hands a value of 75% by the same method. Independent tests of our synthetic material with alcohol, lactate, and α-glycerophosphate dehydrogenases, which were very kindly carried out by Dr. M. Dixon in Cambridge and Dr. D. E. Hughes in Oxford, gave values about 65%. The discrepancy in these results is not as remarkable as it may appear at first sight. All the assays depend on measuring the absorption of light at 340 mμ, generated by enzymic action and associated with dihydro-coenzyme (III). A published survey [17] shows the wide variation in the extinction coefficients ascribed by various workers to the dihydro-coenzyme. Expressed in another way, these variations would correspond to different estimates of purity, if the same extinction coefficient was used. Throughout the present series of tests the value, 6220, which has been established by the ingenious work of Horecker and Kornberg,[18] was adopted. Perhaps the best identification of diphosphopyridine nucleotide is the absorption spectrum of its dihydro-derivative, which is compounded from those of the adenine and dihydronicotinamide chromophores. (The spectrum of the coenzyme itself is not particularly characteristic because the absorptions of the two chromophores are superimposed.) Unfortunately, and very surprisingly, so far as we have been able to discover, an absorption spectrum with the extinction coefficient 6220 at 340 mμ has never been recorded. Ohlmeyer [19] reported the isolation of dihydrocozymase with extinction coefficient 6270, but the actual spectrum was not recorded. Our Figure shows the close correspondence between the absorption curve of our material and those reproduced in standard works.[20, 21] We conclude that our synthetic diphosphopyridine nucleotide is at least comparable in purity with the best samples from natural sources.

The synthesis of diphosphopyridine nucleotide here described could be held to constitute also a synthesis of triphosphopyridine nucleotide (TPN, coenzyme II, codehydrogenase II), because treatment with phosphoryl chloride produces material with the appropriate coenzyme activity.[22] However, the purification of this material has not been described and therefore it seemed worth while to examine a more rational scheme of synthesis. The structure of triphosphopyridine nucleotide has been established as (I; R = PO_3H_2) by Kornberg and Pricer, who degraded the coenzyme enzymically to nicotinamide nucleotide and adenosine-2′ : 5′ diphosphate.[23] As the mixed adenosine-2′ : 5′ and -3′ : 5′

[17] Drabkin, *J. Biol. Chem.*, 1945, **157**, 563.
[18] Horecker and Kornberg, *ibid.*, 1948, **175**, 385.
[19] Ohlmeyer, *Biochem. Z.*, 1938, **297**, 66.
[20] Schlenk, "The Enzymes" (ed. Sumner and Myrbäck), Academic Press, New York, 1951, Vol. II, p. 267.
[21] Hasse, "Moderne Methoden der Pflanzenanalyse" (ed. Paech and Tracey), Springer, Berlin, 1955, Vol. IV, p. 322.
[22] Schlenk, *Naturwiss.*, 1937, **25**, 668.
[23] Kornberg and Pricer, *J. Biol. Chem.*, 1950, **186**, 557.

diphosphate can be made readily from adenosine,[2] it was an obvious step to try the reversal of this hydrolysis with di*cyclo*hexylcarbodi-imide in aqueous pyridine. The product appeared on paper chromatography and electrophoresis to be the expected mixture, and the triphosphopyridine nucleotide fraction was separated in about 25% yield by anion-exchange chromatography.[24] Although the amorphous powder had the correct elementary analysis and ultraviolet absorption, small amounts of nicotinamide nucleotide and adenosine diphosphate were detected in it by paper electrophoresis. We believe that these arose, through decomposition after the ion-exchange separation, from those isomers of the coenzyme carrying the phosphate at position 3'. Such decomposition is easy to imagine, and it is worthy of note that coenzyme A, which has an analogous structure in the sense that it too bears a 3'-phosphate residue in the adenosine portion of the molecule, is relatively unstable. The coenzyme activity in the glucose 6-phosphate dehydrogenase system was kindly examined by Dr. M. Dixon, who found about 14% of the theoretical value. This value is in the expected region, since the material is presumably a mixture of four isomers. Enzymic purification of the β, 2'-isomer should be feasible, although there is not such a convenient enzyme for this purpose as in the case of the cozymase isomers.

Although this experiment undoubtedly yielded synthetic triphosphopyridine nucleotide, it is clearly only of a preliminary nature and further work is required.

Experimental

Condensation of Nicotinamide Nucleotide and Adenosine-5' Phosphate.—Pyridine (325 c.c.) and then di*cyclo*hexylcarbodi-imide (18 c.c.) were added to a solution of nicotinamide nucleotide (1·0 g., 3 mmoles) and adenosine-5' phosphate (2·0 g., 5·8 mmoles) in water (75 c.c.). The solution was kept overnight at 0° and then filtered from *s*-di*cyclo*hexylurea, which was extracted with hot water (10 c.c.). A second equal portion of the carbodi-imide was added to the combined filtrate and extract, which was kept at 0° for a further 24 hr. This process was repeated thrice so that in all 90 c.c. of carbodi-imide were used. Finally the solution was poured into water (2 l.). After 2 hr. the solution was filtered, washed with chloroform (3 × 350 c.c.), and concentrated (to 50 c.c.) under reduced pressure. The solution (pH 6·0) was put on a column (14 cm. × 8 cm.²) of Dowex-2 resin (formate form), which was washed with water (400 c.c.). The aqueous washings (A) contained all the nicotinamide nucleotide and P^1P^2-di(nicotinamide nucleoside) pyrophosphate while the diphosphopyridine nucleotide and other products remained adsorbed on the resin (B).

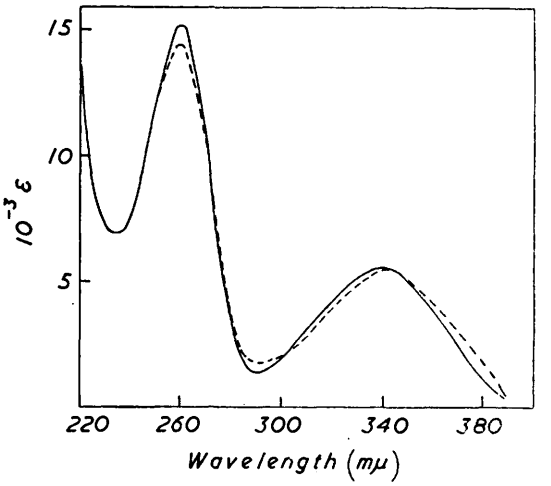
(A) The gum, obtained by evaporating the aqueous eluate, was dissolved in 0·1N-formic acid (10 c.c.) and added to a column (17 cm. × 2 cm.²) of Dowex-50 resin (hydrogen form). When the column was developed with 0·1N-formic acid and 10 c.c. fractions were collected, the peak of the pyrophosphate band was found in tube 16 and the peak of mononucleotide band in tube 22. The separation was incomplete both in this experiment and in subsequent ones with longer columns.

(B) The Dowex-2 column was washed with 0·001N-formic acid (1 l.) before the diphosphopyridine nucleotide was eluted with 0·01N-formic acid (1·5 l.), the adenosine-5' phosphate with 0·1N-formic acid (1·5 l.), and the P^1P^2-diadenosine-5' pyrophosphate with N-formic acid (1·5 l.). The amounts of material eluted from the two columns were estimated from the light absorption at 260 mμ (the molar extinction coefficients given in parentheses) as follows: diphosphopyridine nucleotide (18,000) 1·40 mmoles; adenosine-5' phosphate (14,000) 1·64 mmoles; P^1P^2-di-adenosine-5' pyrophosphate (28,000) 0·95 mmole; nicotinamide nucleotide (5000) 0·63 mmole; P^1P^2-di(nicotinamide nucleoside) pyrophosphate (10,000) 0·17 mmole. The gum left after evaporation of the 0·01N-formic acid eluate was dissolved in water (20 c.c.) and freed from traces of cupric ion (from the distilled water) by passage through a column (2 cm. × 6 cm.²) of Dowex-50 resin (hydrogen form), which was washed with water (400 c.c.). The combined solution and washings were concentrated to 30 c.c. before being added dropwise to " AnalaR " acetone (400 c.c.). The flocculent colourless precipitate was kept for several hours before removal by centrifugation and washing with acetone (200 c.c.). At this stage the diphosphopyridine nucleotide (0·935 g., 1·4 mmoles) had $[\alpha]_D^{20}$ −24·5° (±0·5°) (*c* 5·5 in H_2O) (Found, in

[24] Cf. Kornberg and Horecker, *Biochemical Preparations*, 1953, **3**, 24.

material dried at 20°/10^{-3} mm.: C, 36·9; H, 4·5; N, 14·2; P, 9·0. Calc. for $C_{21}H_{27}O_{14}N_7P_2,H_2O$: C, 37·0; H, 4·3; N, 14·4; P, 9·1%).

Enzymic Purification of Diphosphopyridine Nucleotide.—A suspension (0·1 c.c. containing 3 mg. of crystalline enzyme; Boehringer G.m.B.H., Mannheim) of alcohol dehydrogenase and ethanol (30 c.c.) were added to a solution of the foregoing material (0·200 g.) in 0·01M-glycine buffer (500 c.c.; pH 9·0). After 3 hr. the optical density at 340 mμ had become constant and 10% sodium hydroxide solution (20 c.c.) was added. The mixture was kept during 12 hr. at 20° and its pH was then adjusted to 9·0 with 3N-hydrochloric acid. The solution was concentrated to 250 c.c. and then diluted with water to 500 c.c. A second portion (0·1 c.c.) of alcohol dehydrogenase solution, and acetaldehyde (0·5 c.c.), were added; in 10 min. the light absorption at 340 mμ had vanished. The solution was brought to pH 3 with 3N-hydrochloric acid before being concentrated to 150 c.c. It was then added to a column (10 cm. × 20 $cm.^2$) of Dowex-50 resin (lithium form), which was washed with water (300 c.c.). The aqueous eluate was concentrated to 2 c.c. and then diluted with a mixture of acetone (150 c.c.) and ethanol (50 c.c.). The precipitate was collected by centrifugation, washed with a second portion of ethanolic acetone, and dissolved in water (100 c.c.). The solution was concentrated to 50 c.c. and then added to a column (3 cm. × 12 $cm.^2$) of Dowex-2 resin (formate form). The column was washed with 0·001N-formic acid (1 l.) before the coenzyme was eluted with 0·01N-formic



Absorption spectrum of synthetic diphosphopyridine nucleotide (—) *after reduction with alcohol dehydrogenase; and absorption spectrum of the natural dihydrocoenzyme* (– – – –).[20]

acid (1·5 l.). The coenzyme was recovered as a gum by evaporation of the formic acid, and it was then precipitated by " AnalaR " acetone (200 c.c.) from water (2 c.c.) and washed with acetone (100 c.c.). The product (0·091 g.) had $[\alpha]_D^{20}$ −31·5° (±0·5°) (*c* 1·2 in H_2O) {Kaplan *et al.*[16] give $[\alpha]_D^{23}$ −34·8° (*c* 1 in H_2O)}, light absorption $\varepsilon_{max.}$ 18,100 (*M* 681) at 260 mμ (Found, in material dried at 20°/10^{-3} mm.: C, 37·5; H, 4·9; N, 14·1; P, 9·0. $C_{21}H_{27}O_{14}N_7P_2,H_2O$ requires C, 37·0; H, 4·3; N, 14·4; P, 9·1%). The behaviour of samples (about 1 mg.) of the synthetic and the natural material on a column (8 cm. × 1 $cm.^2$) of Dowex-2 resin (acetate form) was compared. Elution of the two materials by 0·1N-acetic acid followed a very similar pattern, with the peak at about 170 c.c. On the other hand a very broad peak centred about 310 c.c. was produced by some synthetic diphosphopyridine nucleotide which contained only 30% of coenzyme; it had been prepared from nicotinamide nucleotide which was mainly the α-anomer and it had not been enzymically purified.

Determination of Coenzyme Activity with Alcohol Dehydrogenase.—The following experiment is typical; concordant results were obtained by slightly different techniques, for example with 0·1M-disodium hydrogen phosphate instead of the glycine buffer. Ethanol (1 c.c.) and synthetic diphosphopyridine nucleotide (1·212 mg.) were made up to 25·0 c.c. with 0·01M-glycine–sodium glycine buffer (pH 9·0). The optical density of this solution was measured in a 1 cm. cell at 260 mμ (1·29) and 340 mμ (0·007) against a cell filled with ethanolic buffer. A small quantity of alcohol dehydrogenase was then added to each cell. In 15 min. the optical density at 340 mμ had become constant (0·400), and the complete spectrum was determined (see Figure). The

increase (0·393) in optical density at 340 mμ was 88·5% of that expected for the dihydrocoenzyme (ε 6220 [18]) produced from hydrated diphosphopyridine nucleotide (*M* 681). In other experiments it was shown that the light absorption at 340 mμ was destroyed by addition of excess of acetaldehyde.

Condensation of Nicotinamide Nucleotide with a Mixture of Adenosine-2′ : 5′ and -3′ : 5′ Diphosphate.—Pyridine (75 c.c.) and then di*cyclo*hexylcarbodi-imide (6 c.c.) were added to a solution of nicotinamide nucleotide (0·30 g.) and the mixed adenosine diphosphates [2] (0·80 g.) in water (15 c.c.). The mixture was kept at 20° and three more equal portions of carbodi-imide were added at intervals of 2 days. After 8 days in all, water (700 c.c.) was added and the *s*-di*cyclo*hexylurea was removed. The filtrate was washed with ether (2 × 300 c.c.), concentrated to 30 c.c., and treated with N-hydrochloric acid (8 c.c.). After 4 hr. the precipitated di*cyclo*hexylurea was removed and the solution was neutralised (pH 7·0) with lithium hydroxide. The solution was evaporated to a syrup which was shaken with alcohol (25 c.c.) and acetone (75 c.c.). The resulting brown precipitate was washed with a second portion of ethanolic acetone before being dissolved in water (25 c.c.) and added to a column (8 cm. × 5 cm.^{2}) of Dowex-2 resin (chloride form). Most of the nicotinamide nucleotide and P^1P^2-di(nicotinamide nucleoside) pyrophosphate were removed by washing the column with water (500 c.c.), but small amounts were contained in the first fractions during the main elution. This was carried out with 0·1N-formic acid–0·1M-sodium formate, 50 c.c. fractions being collected. The triphosphopyridine nucleotide was contained in fractions 10—50 (peak at fraction 22), which were combined and evaporated to dryness. The residue was dissolved in water (300 c.c.), one-third of which was added to a column (7 cm. × 20 cm.^{2}) of Dowex-50 resin (hydrogen form). The column was washed with water (500 c.c.) until no more light-absorbing material was removed. The other two-thirds were then treated in the same way and the combined washings were evaporated to dryness. The residue was precipitated from water (5 c.c.) by " AnalaR " acetone (200 c.c.) and washed at a centrifuge with a second portion of acetone before being dried (0·154 g.). It had maximum light absorption at 260 mμ (ε 18,150 calc. with *M* 797) (Found, in material dried at 20°/10^{-3} mm.: C, 31·7; H, 4·8; N, 11·7. Calc. for $C_{21}H_{28}O_{17}N_7P_3,3H_2O$: C, 31·7; H, 4·3; N, 12·3%). Dr. M. Dixon reported that, when this material was treated with glucose 6-phosphate and glucose 6-phosphate dehydrogenase in phosphate buffer (pH 7·4), the light absorption developed at 340 mμ was 14% of that expected for pure anhydrous triphosphopyridine nucleotide. Paper electrophoresis showed that it contained, in addition to material travelling at the same speed as the natural coenzyme, small amounts of adenosine-2′ : 5′ or -3′ : 5′ diphosphate and nicotinamide nucleotide.

Paper Electrophoresis and Paper Chromatography of the Nucleotides.—Electrophoresis was much the more useful technique (see Table); it was carried out for 6 hr. on Whatman no. 54 paper in 0·05M-disodium hydrogen phosphate (pH 8·0) at a potential gradient of about 5 v/cm. (220 v applied). Whatman no. 1 paper was used for ascending chromatography with butan-1-ol–acetic acid–water (5 : 2 : 3) (system A) or *iso*butyric acid–N-ammonia containing ethylenediaminetetra-acetic acid (5 : 3) (system B).[25]

Substance	Migration (cm.)	R_F in system A	R_F in system B
Nicotinamide nucleotide	3·4	0·18	0·41
P^1P^2-Di(nicotinamide nucleoside)pyrophosphate	−1·0	0·08	—
Adenosine-5′ phosphate	6·2	0·17	0·52
Adenosine-2′ : 5′ or -3′ : 5′-diphosphate	10·5	0·09	0·30
P^1P^2-Diadenosine-5′ pyrophosphate	4·6	0·06	—
Diphosphopyridine nucleotide	1·9	0·10	0·46
Triphosphopyridine nucleotide	6·7	0·06	0·27

We thank the Rockefeller Foundation for its support and the Department of Scientific and Industrial Research for a Maintenance Grant (to N. A. H.). We are also deeply indebted to Dr. M. Dixon, Biochemistry Department, Cambridge, and Dr. D. E. Hughes, Biochemistry Department, Oxford, for generously making independent enzymic tests.

UNIVERSITY CHEMICAL LABORATORY, CAMBRIDGE. [*Received, March 28th,* 1957.]

[25] Krebs and Hems, *Biochim. Biophys. Acta*, 1953, **12**, 172.