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Asymmetric Synthesis Using Cofactor-Requiring Enzymes

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The use of cofactor-requiring enzymes as catalysts for large-scale requires efficient and economical procedures for *in situ* regeneration of these cofactors. This manuscript summarizes the procedures which are now available for cofactor preparation and regeneration. ATP can be effectively regenerated from ADP (and AMP) using acetyl phosphate and acetate kinase (and adenylate kinase), and it can be prepared inexpensively from RNA. Use of ATP-requiring enzymes is now routine (at least as far as the ATP regeneration is concerned). The use of the nicotinamide cofactors is more difficult, because these materials decompose in solution. The best procedure for regenerating NAD(P)H from $NAD(P)^+$ are those based on formate/formate dehydrogenase, glucose 6-phosphate/glucose-6-phosphate dehydrogenase, and ethanol/alcohol dehydrogenase/aldehyde dehydrogenase. The best procedures for regenerating $NAD(P)^+$ from NAD(P)H use dioxygen/methyl viologen or ketoglutarate/glutamic dehydrogenase.

Although enzymes can be effective catalysts for enantioselective reactions, they have been relatively little used for this purpose in practical organic synthesis. The relative indifference of synthetic chemists to the potential of this group of catalysts is a consequence of a number of circumstances. First, enzymes are unfamiliar: they require aqueous environments; they are prepared, characterized, and manipulated using specialized techniques having little in common with techniques used in other areas of synthetic organic chemistry; and they appear to be unstable. Second, certain generally interesting classes of enzymatic reactions (including many reactions which *form* bonds between organic molecules and most reactions which involve oxidation or reduction) involve cofactors; these reactions are expensive. Third, the substrate selectivity

of enzyme-catalyzed reactions often limits the generality of their application. Nonetheless, in these reactions in which they are applicable, they can be very efficient catalysts, and their ability to catalyze reactions of naturally-occurring substances (which are, of course, products of and reactants in the reactions which take place in life) makes them of particular interest in pharmaceutical, food, and agricultural chemistry.

The research summarized in this manuscript was directed toward one particular problem in enzymology: that is, the development of techniques which would make possible the use of cofactor-requiring enzymes in organic synthesis. The central problem in this area has been one of expense. ATP costs approximately $800/mole when purchased in mole quantities; the costs of the nicotinamide cofactors range from $1500/mole (for NAD^+) to $250,000/mole (for NADPH). There are few organic reactions which can tolerate costs of this magnitude for stoichiometric reagents. The solution to this problem of cost is, in principle, straightforward, and has been the subject of extensive previous work. The most efficient way of lowering the effective cost of the cofactors is to develop procedures which make possible their regeneration from inexpensive reagents *in situ* (Figure 1)

Among the considerations which determine the usefulness of a synthetic sequence which involves a cofactor-requiring enzymatic step are:

1) The character of the reaction used for regeneration of the cofactor. The reagent A should be readily available, inexpensive, and stable; the product B should not complicate workup; the equilibrium constant for the reaction $A + X \rightleftharpoons B + Y$ should lie far to the right; the enzymes used (if any) should have low cost, high stability, and high specific activity.

2) The intrinsic stabilities of the cofactors X and Y under the conditions of the reaction.

3) The original cost of the cofactor.

4) The operational simplicity of the regeneration scheme.

Here we divide the discussion of approaches to cofactor regeneration into three sections: one each for ATP, oxidized nicotinamide cofactors (NAD^+ and NADP), and reduced nicotinamide cofactors (NADH and NADPH). Most of the other cofactors which appear in biochemistry are either easily regenerated or of little importance, and we shall not discuss their regeneration here.

Although either purely chemical or enzymatic procedures might be used to effect the regeneration reactions, in general enzymatic procedures are superior. To be able to recycle the cofactors a large number of times it is necessary to have high yields for the reactions which regenerate them. Thus, to have 50% of the cofactor remaining after 100 cycles of reaction and regeneration, the yield for each cycle must be 99.3% (100 log 0.993 = log 0.50), and for 1000 cycles, the corresponding yield must be 99.9%. This type of selectivity is most easily obtained by enzymatic catalysis, and we have therefore used only enzymatic methods in our work.

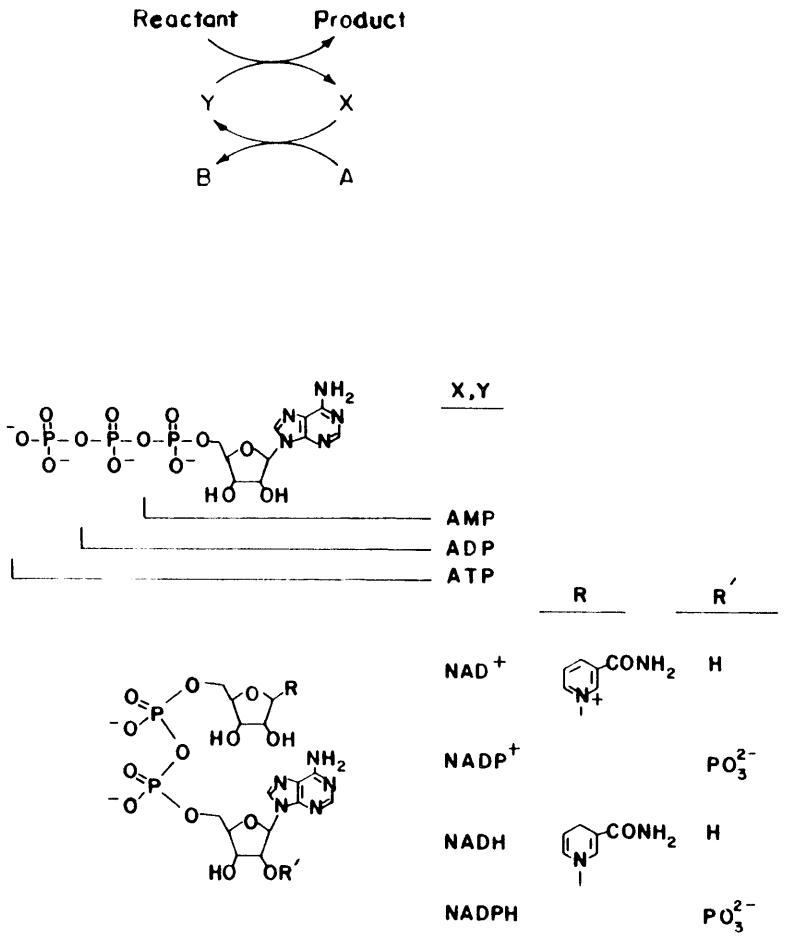


Figure 1. General scheme for cofactor regeneration (top) and structures of adenosine and nicotinamide cofactors (bottom). Key: X, Y, cofactors; A, regenerating agent; and B, product from this reagent.

ATP

Regeneration. Most biochemical reactions which involve ATP as a cofactor convert it to ADP or AMP; adenosine itself is important only as a product of the small group of reactions which proceed through S-adenosyl methionine. Thus, it is necessary to have regeneration procedures which will convert both AMP and ADP to ATP. Chemical methods for these phosphorylation reactions can be rejected out of hand: they are incompatible with the enzymes which would be present in the system as catalysts for reactions which *use* the ATP, and lack the specificity required to give high yields and high total turnover numbers (TTN) for the ATP (TTN = moles of product produced in the reaction per mole of cofactor or enzyme present). The stability of ATP is good: the hydrolysis of ATP at pH 6-8 is slow compared with any synthetic reaction of practical interest.

The choice of phosphorylating agents which might, in principle, be used to convert AMP or ADP to ATP is limited. Table I summarizes values of $\Delta G^{\circ\prime}$ for the reaction XP + ADP $\rightleftharpoons$ X + ATP for those compounds XP which are (relatively) readily available and exergonic with respect to phosphorylation of ADP. Of these, PEP

Table I. Free energy of phosphorylation of ADP to ATP

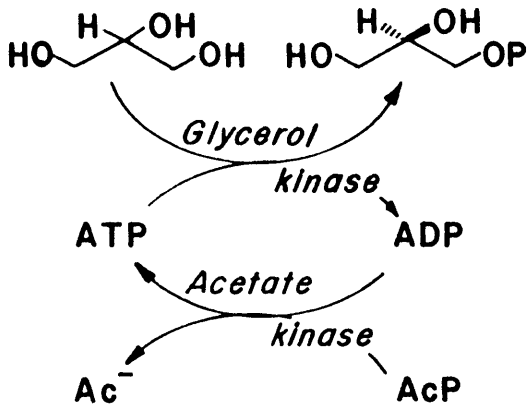
XP	$\Delta G^{\circ\prime}$ (kcal/mole)
Phosphoenolpyruvate (PEP)	-7.5
Carbamyl phosphate	-5.0
Acetyl phosphate (AcP)	-3.0
Pyrophosphate (PP_i)	-0.7

is relatively expensive (although regeneration systems based on PEP have many advantages in simplicity), carbamyl phosphate has very poor stability in solution, and pyrophosphate is only a weak phosphorylating agent and requires enzymes which are available only with difficulty. Acetyl phosphate (AcP) is a reagent which offers a practical combination in its characteristics: it is prepared easily from inexpensive reagents; the enzymes it requires for use in ATP regeneration are commercially available and acceptably stable; it is a good phosphorylating agent. In this manuscript we focus attention on procedures for ATP regeneration based on AcP. The only other procedure which seems useful for laboratory-scale is that based on PEP; details of this procedure will be published elsewhere.

AcP can be prepared easily by acylation of phosphoric acid with acetic anhydride or with ketene, and isolated as a fairly

stable ammonium salt (1,2). Acetate kinase (the enzyme which catalyzes the reaction of acetyl phosphate with ADP) and adenylate kinase (the enzyme which catalyzes phosphate transfer between ATP and AMP) are readily available and inexpensive. The ATP regeneration schemes based on these enzymes are shown in Figure 2 (3,4,5).

These schemes have now been used to prepare organic materials on scales of several moles. An example relevant to asymmetric synthesis is the glycerol kinase-catalyzed phosphorylation of glycerol (equation i) (6).



(i)

This reaction yields enantiomerically pure sn-glycerol-3-phosphate ((R)-glycerol-1-phosphate, a compound having the correct configuration to serve as the basis for the synthesis of phospholipids). The turnover numbers (TTN = moles product per mole cofactor) achieved in these syntheses (TTN ≃ 100) have been limited primarily by convenience: we normally use a relatively large quantity of ATP, to keep reaction rates high. The ATP is, however, essentially all still present at the conclusion of the reaction. For laboratory-scale synthesis of fine chemicals, the methods shown in Figure 2 represent an effective solution to the problem of ATP regeneration.

Synthesis. A final problem related to ATP utilization is that of obtaining the initial quantity of ATP to be used in the reaction. ATP as a pure biochemical is expensive. A material suitable for use in recycling can be obtained from RNA (approximately $80/kg) by the process outlined in equation ii (7).

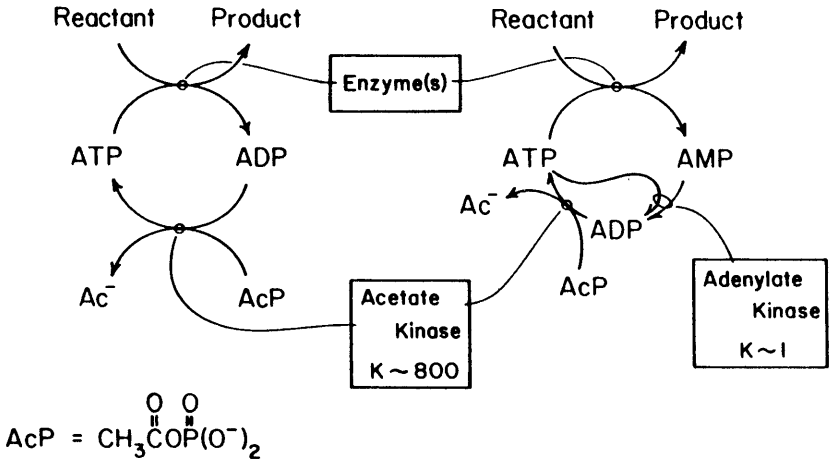
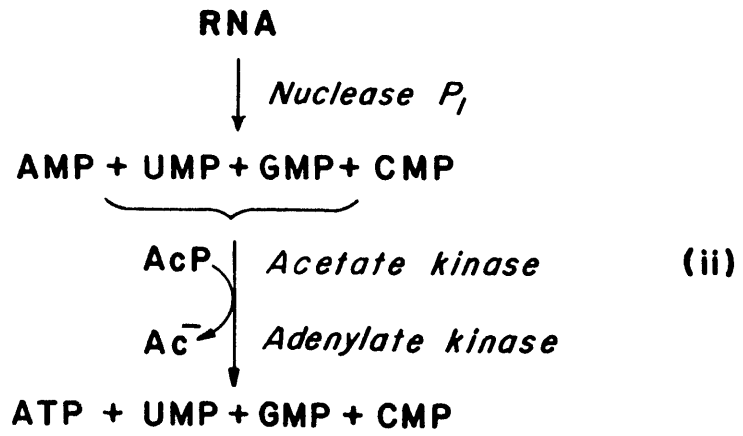


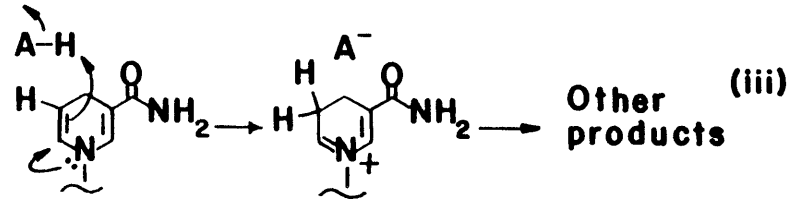
Figure 2. Schemes used for regenerating ATP from ADP (left) and AMP (right).



Cleavage of RNA using nuclease P_1 yields a mixture of nucleoside monophosphates, contaminated with oligonucleotides and other materials. The AMP present in this mixture can be converted selectively to ATP by treatment with acetyl phosphate and a mixture of adenylate kinase and acetate kinase. The resulting mixture can be used directly, *without purification*, to supply ATP for use in cofactor recycling.

$NAD(P)^+$

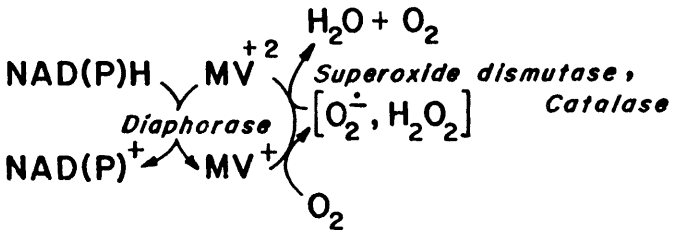
Regeneration. The oxidized nicotinamide cofactors ($NAD(P)^+$) are considerably more difficult to work with than ATP, but are more tractable than the reduced nicotinamide cofactors (NAD(P)H). The oxidized cofactors are sensitive to nucleophiles (8), but are relatively stable at pH 7; the reduced cofactors decompose by acid-catalyzed processes involving protonation at C-5 of the dihydropyridine ring as the rate-limiting step (equation iii) (9,10).



Phosphate, a common component of enzymatic systems and an integral part of $NADP^+$ and NADPH, is a particularly effective acid catalyst for this reaction.

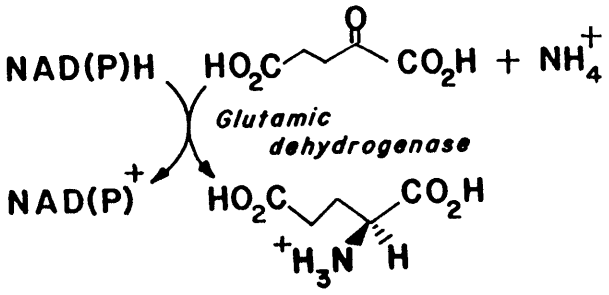
The efficient utilization of the nicotinamide cofactors requires not only a scheme for their recycling but also a method for dealing with their limited lifetime in solution. A number of approaches to recycling have been considered, and some appear to be quite satisfactory. The only obvious approach to the economic problem posed by limited lifetime is to lower the initial cost of the cofactor, and we can only offer suggestions concerning this problem.

The recycling of NAD(P)H to $NAD(P)^+$ can be accomplished by any of several methods. If dioxygen can be used in the system, the most straightforward recycling method involves methyl viologen (MV)-catalyzed oxidation (equation iv). The details of the several



(iv)

reactions which may be involved in this recycling scheme are not known, but in practice, it has proved to be a useful synthetic method. When *anaerobic* recycling is required, a procedure based on glutamic dehydrogenase works well (equation v) (11).



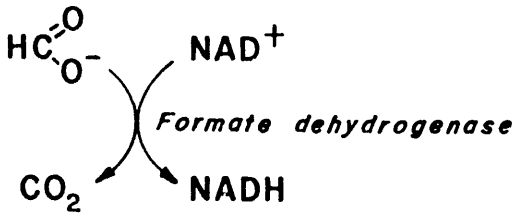
(v)

We defer discussion of the problem of *synthesizing* $NAD(P)^+$ to the next section.

NAD(P)H

Regeneration. A large number of systems have been tested for utility in regeneration of reduced nicotinamide cofactors. Of these, only three seem likely to be useful in the short term. Here we briefly describe those systems which, in our opinion, have the practicality required for use in mole-scale organic synthesis.

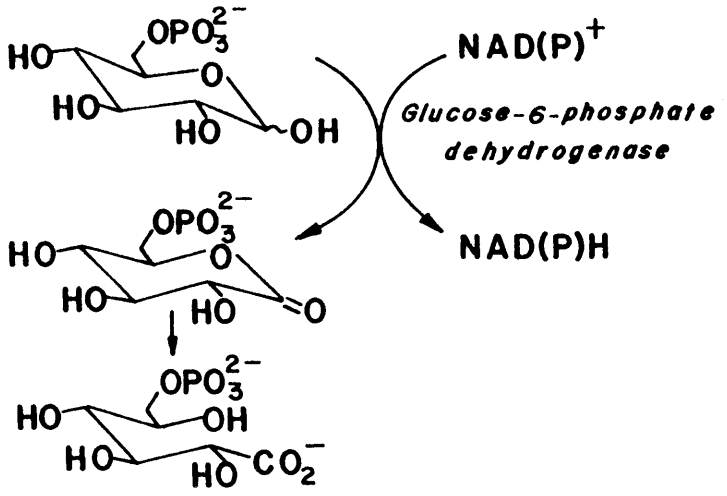
Formate/Formate Dehydrogenase. This system (equation vi) (12) has several advantages: it requires only one enzyme, and this enzyme is readily available in quantity (although it is still moderately expensive when purchased commercially); formate is inexpensive, and removal of CO_2 causes no problem during workup; formate is a strong reducing agent. The principal disadvantages



(vi)

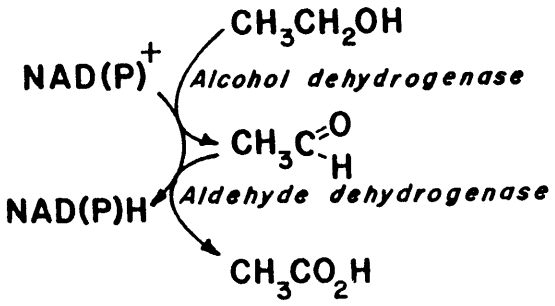
of the system are that it is specific for NAD^+ (and thus requires a transhydrogenase for use with $NADP^+$), that the specific activity of the enzyme (3 U/mg; 1 U = 1 μmole of NAD^+ consumed/min) is only modest (13) (thus large reactor volumes are required when immobilized enzymes are being used), and that the enzyme is sensitive to autoxidation.

Glucose-6-Phosphate/Glucose-6-Phosphate Dehydrogenase. The advantage of this system (equation vii) are that it requires a single enzyme (10), that this enzyme is stable and available with high specific activity (700 U/mg for NAD^+; 500 U/mg for $NADP^+$) (14), that the same enzyme catalyzes the reduction of both NAD and NADP , and that the reaction is irreversible because 6-phosphogluconolacetone hydrolyzes rapidly to 6-phosphogluconate. Its disadvantages are that glucose-6-phosphate is not commercially available (although it is relatively readily prepared), that 6-phosphogluconate may cause significant problems in workup, and that glucose-6-phosphate and 6-phosphogluconate both catalyze the decomposition of NAD(P)H (10). In practice, the convenience of having an easily manipulated, stable, active enzyme outweighs the disadvantages of having to prepare glucose-6-phosphate and of suffering a short lifetime for NAD(P)H. Overall, the method is a useful one in laboratory-scale preparations.



(vii)

Ethanol/Alcohol Dehydrogenase/Aldehyde Dehydrogenase. The combination of ethanol and alcohol dehydrogenase has been used extensively to reduce NAD^+ to NADH (15,16). This system is unsatisfactory for two reasons: it is only weakly reducing, and the acetaldehyde produced deactivates many enzymes. By adding an excess of aldehyde dehydrogenase (equationviii)the system becomes a very good one (17,18). Its advantages are that ethanol is

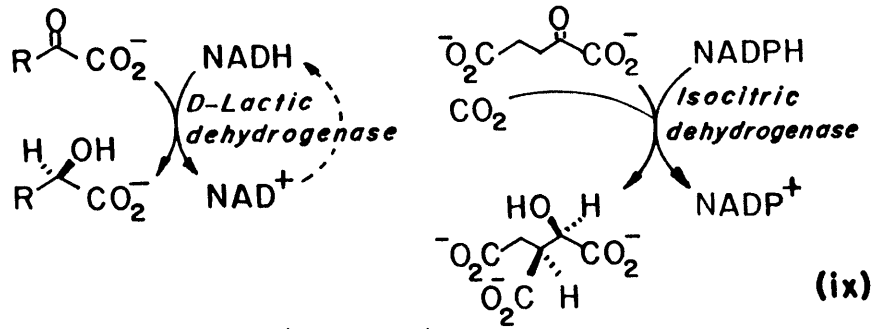


(viii)

inexpensive, the coupled system is strongly reducing, the enzymes have high specific activities (19) (alcohol dehydrogenase: 400 U/mg for ethanol; aldehyde dehydrogenase; 80 U/mg for acetaldehyde), and acetate seldom complicates workup. Its disadvantages are that the system requires two enzymes, that the enzyme which must be in excess (aldehyde dehydrogenase) is the more expensive and the more sensitive, and that it is more reactive toward NAD^+ than $NADP^+$. A similar system which works well for NADH regeneration is based on methanol as substrate and three combined enzymes, including alcohol dehydrogenase, aldehyde dehydrogenase and formate dehydrogenase, as catalysts (18). The side product (CO_2) in this system does not complicate the work-up and methanol is less expensive than ethanol, but the specific activities of the rate-limiting enzymes of this system are less than those based on ethanol as ultimate reducing source.

Others. In addition to these procedures, a number of others have been established to be effective in reducing $NAD(P)^+$. Many of these methods have been reviewed (15,16). Recently a promising procedure based on a secondary alcohol dehydrogenase has been described by Zeikus (20), and methods which use dihydrogen (21) and electrons from a cathode (22,23) have been described. With the exception of the procedure of Zeikus, these methods are not as convenient for laboratory-scale work as those described above.

There are many examples of the use of NAD(P)H to effect organic syntheses in systems in which the reduced nicotinamide cofactor is regenerated *in situ* (15,16,24). Recent examples include syntheses of D-lactic acid (10,12), isocitric acid (10,21) and other α-hydroxy acids (D or L) on scales of 0.1 to 0.5 mole (equation ix). The turnover numbers for NAD(P)(H) in these reactions are TTN ≃ 1000 - 2000.



(ix)

Synthesis of NAD^+ and $NADP^+$. The nicotinamide cofactors are now isolated from yeast (25,26). A major difficulty in this preparation is simply that of separation of the NAD(P)(H) from the other components in the cell. To reduce the cost of these materials, either the yield must be improved from the yeast preparation, the isolation must be simplified, or some type of synthesis must be developed. We have taken a step toward developing a new synthesis by the combined enzymatic/conventional synthetic procedure summarized in Figure 3(27). The overall conversion from

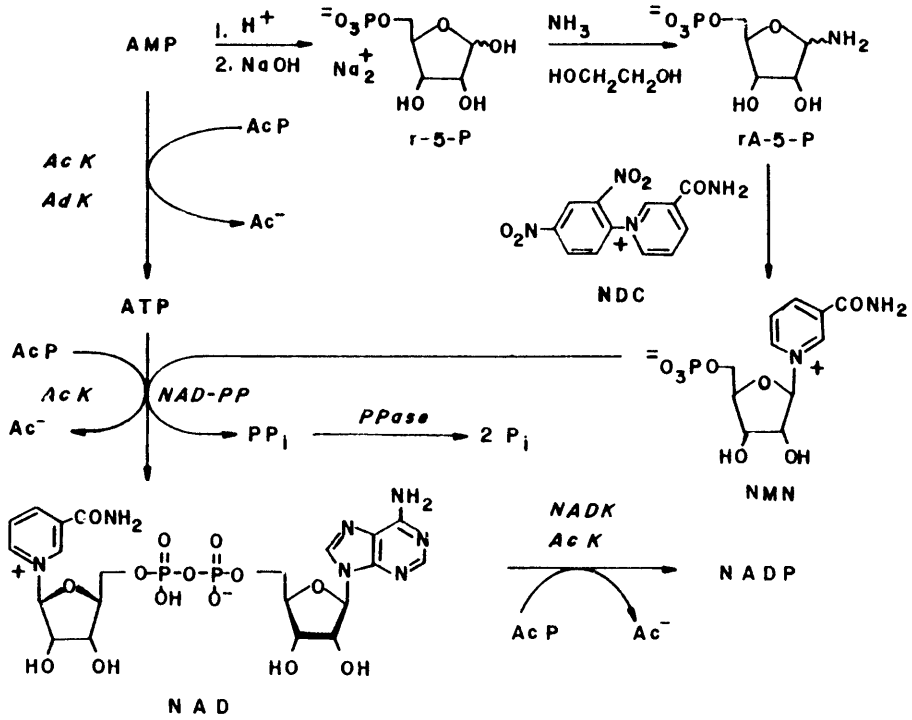


Figure 3. Combined chemical and enzymatic synthesis of NAD+ (27). Key: AcK, acetate kinase; AdK, adenylate kinase; NAD-PP, NAD pyrophosphorylase; PPase, pyrophosphorylase; NADK, NAD kinase; r-5-P, ribose-5-phosphate; rA-5-P, ribosylamine-5-phosphate; NMN, nicotinamide mononucleotide; AcP, acetyl phosphate; PP_i, pyrophosphate; and NDC, N_1-(2,4-dinitrophenyl)-3-carbamoylpyridinium chloride.

ribose-5-phosphate to NAD^+ in this procedure is approximately 60% on small scale; that from AMP to NAD^+ via ATP is essentially quantitative. The procedure involves only one isolation (that of ribose-5-phosphate). The solution containing the NAD^+ can be used directly for cofactor recycling: whatever components are present as impurities in this solution apparently do not inactivate or inhibit enzymes. This procedure (after development) or some related procedure may provide the best hope for reducing the cost of the nicotinamide cofactors.

Acknowlegements

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