6
Cofactor Regeneration for Enzyme-Catalysed Synthesis

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Introduction

Cofactors are low-molecular-weight non-protein species that are essential participants in enzyme-catalysed reactions. Certain cofactors (e.g. pyridoxal phosphate and thiamine pyrophosphate) are essentially self-regenerating (Baricos, Chambers and Cohen, 1976). Others act as functional group transfer agents and are essentially cosubstrates: they donate or accept a functional group (e.g. hydride or phosphate) to or from the other substrate of the enzymatic reaction. These cofactors (e.g. nicotinamide cofactors or nucleoside triphosphates) emerge from the enzymatic reaction in an altered form. In enzyme-catalysed synthesis, they therefore must either be used in stoichiomet-

Abbreviations: L-AcCarn, *O*-acetyl-L-carnitine; AcCoA, acetyl coenzyme A; AcOP, acetyl phosphate; ACS, acetyl coenzyme A synthetase; ADH, alcohol dehydrogenase; ADP, adenosine 5′-diphosphate; AK, acetate kinase; APS, adenosine 5′-phosphosulphate; ATP, adenosine 5′-triphosphate; CAT, carnitine acetyltransferase; CDP, cytidine 5′-diphosphate; CMP, cytidine 5′-monophosphate; CoA, coenzyme A; CP, carbamyl phosphate; CTP, cytidine 5′-triphosphate; L-Cys, L-cysteine; FDH, formate dehydrogenase; FMN, flavin mononucleotide; G6P, glucose-6-phosphate; G6PDH, glucose-6-phosphate dehydrogenase; GDH, glucose dehydrogenase; GluDH, glutamate dehydrogenase; HLADH, horse liver alcohol dehydrogenase; αKG, α-ketoglutarate; LDH, lactate dehydrogenase; MCP, methoxycarbonyl phosphate; N, nucleoside; NAD, nicotinamide adenine dinucleotide; NADH, 1,4-dihydronicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; NADPH, 1,4-dihydronicotinamide adenine dinucleotide phosphate; NeuAc, N-acetylneuraminic acid; NDP, nucleoside diphosphate; NMP, nucleoside monophosphate; NTP, nucleoside triphosphate; P_i, inorganic phosphate; PAN, polyacrylamide N-acryloxysuccinimide; PAP, 3′-phosphoadenosine 5′-phosphate; PAPS, 3′-phosphoadenosine 5′-phosphosulphate; PEP, phosphoenolpyruvate; PK, pyruvate kinase; PMS, phenazine methosulphate; PP_i, inorganic pyrophosphate; PTA, phosphotransacetylase; SAH, *S*-adenosyl-L-homocysteine; SAM, *S*-adenosyl-L-methionine; SMM, *S*-methyl-L-methionine; TN, turnover number; TTN, total turnover number; UDP, uridine 5′-diphosphate; UTP, uridine 5′-triphosphate; YADH, yeast alcohol dehydrogenase.

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Table 1. Cost of cofactors requiring regeneration

Cofactor	$/g mol
Acetyl CoA	1 300 000†
CoA	249 000†
ATP	220*
CTP	43 000†
GTP	64 000†
UTP	52 000†
NAD	710*
NADH	3 100*
NADP	25 800†
NADPH	216 000‡
PAPS	7 500 000‡
SAM	58 700†

* Kyowa Hakko Kogyo Co., Ltd; † United States Biochemical Corp; ‡ Sigma Chemical Co.

ric amounts or be regenerated *in situ* by a separate reaction. The high cost of cofactors (*Table 1*) precludes their large-scale use in stoichiometric amounts and so necessitates that they be regenerated.

Cofactor-requiring enzymes catalyse many synthetically useful reactions and the value of enzymes as catalysts for organic synthesis is becoming generally appreciated (Chibata, 1978; Suckling and Wood, 1979; Whitesides and Wong, 1983, 1985; Porter and Clark, 1985; Jones, 1986). The need for practical methods of cofactor regeneration, however, has impeded the incorporation of cofactor-requiring enzymes as catalysts into organic synthesis, especially for large-scale reactions.

A regeneration method must be capable of recycling the cofactor 10^2–10^6 times to be economical. The exact number of turnovers required depends on the initial cost of the cofactor and the value of the product formed by the enzymatic process. Clearly, the economical use of acyl coenzyme A or PAPS requires more turnovers of cofactor than does that of ATP (*Table 1*). High total turnover numbers require high selectivity for the formation of enzymatically active cofactor. If 50% of the original cofactor activity is to remain after 100 turnovers, the regeneration reaction must be 99.3% efficient for the formation of the active cofactor species. If 10^6 turnovers are required, the recycling step must be 99·99993% efficient. Almost invariably, this requirement for highly selective recycling necessitates enzymatic catalysis.

To be practical, a regeneration method must fulfil other requirements as well. All materials and equipment must be readily available, inexpensive, easily manipulated, and stable under the reaction conditions. The regeneration step must be favourable both kinetically and thermodynamically, and it must be compatible with the rest of the reaction system. Furthermore, a good regeneration system must permit convenient monitoring of the progress of the reaction and simple isolation of product.

Many strategies for cofactor regeneration have been examined. Chemical methods are perhaps most straightforward but often lack specificity for the formation of active cofactor and are incompatable with enzymatic systems.

Electrochemical or photochemical methods harness electrical or light energy to drive the regeneration step but suffer from the same drawbacks as chemical methods. Enzymatic methods use cell-free enzymes to catalyse the regeneration step while biological methods use whole cells or cell components as crude sources of the required enzymes. In this review, we focus on enzymatic methods because they are the most specific for the regeneration of enzymatically active cofactor and are generally most compatible with the other components of enzymatic reactions. Furthermore, cell-free enzymatic systems are cleaner than biological systems.

Most work on cofactor regeneration has focused on ATP and nicotinamide cofactors and this review emphasizes these cofactors. A few systems for regenerating acyl coenzyme A have been reported. Although the problems of regenerating *S*-adenosylmethionine (SAM) and 3′-phosphoadenosine 5′-phosphosulphate (PAPS) are unsolved, a discussion of the problems posed by these two systems is included because we believe that they will be foci of future investigations. Each section of the review briefly describes the structure and function of a cofactor and the methods for its preparation. It then discusses the various methods for regeneration, emphasizing methods used to form isolated product. Our evaluation of methods for cofactor regeneration is based on cost and convenience. Our calculations of cost reflect the prices of enzymes and cofactors as research biochemicals. These costs are therefore upper limits.

Two definitions concerning the turnover numbers for cofactors are important. The 'turnover number' (TN, eq.1) refers to the number of moles of product formed per mole of cofactor per unit time. It measures a rate and has units of s^{-1}. The 'total turnover number' (TTN, eq. 2) is the total number of moles of product formed per mole of cofactor during the course of a complete reaction.

$$\mathrm{TN} = \frac{\text{cofactor cycles}}{\text{time}} = \frac{\text{mol product}}{(\text{mol cofactor})\,(\text{time})} \tag{1}$$

$$\mathrm{TTN} = \frac{\text{mol product formed}}{\text{mol cofactor present in reaction}} \tag{2}$$

Whereas TN emphasizes the cost per unit rate of product formation, the TTN emphasizes the total cost per mole of product formed. The TTN is analogous to the 'productivity number' associated with heterogeneous catalysis (Simon *et al.*, 1985a, b; J.W. Nicoletti and G.M. Whitesides, unpublished work).

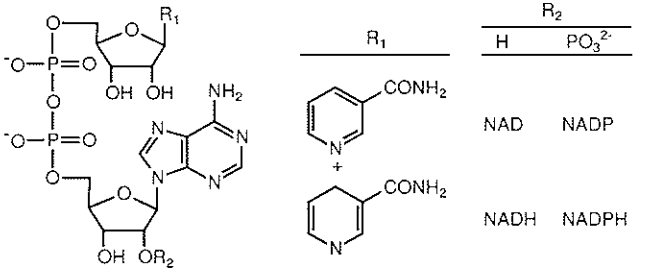
Nicotinamide cofactors

STRUCTURE AND FUNCTION

The nicotinamide cofactors NAD(P)(H)* are the general redox currency in living systems and mediate two-electron redox reactions. Unlike flavin

*We use parentheses to indicate generically the species within a class of the nicotinamide cofactors. For example, NAD(P) refers to both oxidized forms of cofactor, NAD and NADP; NAD(H) denotes both the oxidized and reduced forms of nicotinamide adenine dinucleotide; NAD(P)(H) denotes both redox forms of both cofactors.

cofactors ($K_D = 10^{-7} - 10^{-11}$M), the nicotinamide cofactors readily dissociate from their enzymes ($K_D = 10^{-3} - 10^{-5}$M), and NAD(P)H is stable against oxidation by O_2. Nicotinamide cofactors are therefore capable of shuttling reducing equivalents from oxidized metabolites to centres of energy production or to anabolic intermediates. Generally, NAD(H) functions in dehydrogenase-catalysed reactions involved in respiration (electrons transported ultimately to molecular oxygen), and NADP(H) serves in dehydrogenase-catalysed reactions that transfer electrons to intermediates of biosynthesis. Both cofactors participate in a broad range of synthetically useful, enantiofacially and enantiotopically selective redox reactions.



NAD(P)(H)

In both cofactors, electron transfer involves only the nicotinamide moiety. Enzymatic reduction of NAD(P) directs the hydride attack at C-4 of the pyridinium ring and forms exclusively the 1,4-dihydropyridine product, which is the only enzymatically active form of NAD(P)H. We shall see that the requirement for 1,4-reduction of the pyridinium ring severely limits the number of strategies practical for large-scale regeneration of NAD(P)H. The adenosine and pyrophosphate moieties act as handles which bind the cofactors to the active sites of enzymes. The 2′-phosphate on the adenosine of NADP(H) is the only difference between NADP(H) and NAD(H). It allows cells to separate the activities of the two cofactors since most nicotinamide-dependent dehydrogenases are specific for NAD(H) or NADP(H).

PREPARATION OF THE NICOTINAMIDE COFACTORS

NAD is isolated from yeast (Kornberg, 1957; Sakai, Uchida and Chibata, 1973; Watanabe *et al.*, 1975, 1979) or prepared by bacterial fermentation (Nakayama *et al.*, 1968). Enzymatic phosphorylation of NAD using microbial NAD kinase (EC 2.7.1.23) and ATP provides NADP (Uchida *et al.*, 1978; Hayashi, Tanaka and Kawashima, 1979; Murata, Kato and Chibata, 1979). Reduction of NAD(P) by chemical (Lehninger, 1957), enzymatic (Rafter and Colowick, 1957), or microbial (Eguchi, Nishio and Nagai, 1983) methods gives NAD(P)H. Synthesis of NAD remains inferior to isolation from biological sources (Kornberg, 1950; Hughes, Kenner and Todd, 1957; Traub, Kaufman and Teitz, 1969; Walt *et al.*, 1980, 1984; Whitesides and Walt, 1987).

STABILITY OF THE NICOTINAMIDE COFACTORS

The nicotinamide cofactors are somewhat unstable in aqueous solution, particularly at extremes of pH (Chenault and Whitesides, 1987). Acidic conditions catalyse the hydration and anomerization of the reduced cofactors (Lowry, Passoneau and Rock, 1961; Oppenheimer and Kaplan, 1974; Johnson and Tuazon, 1977; Wong and Whitesides, 1981). Basic conditions catalyse the hydrolysis of the nicotinamide–ribose bond of the oxidized cofactors (Schlenk *et al.*, 1937; Vistin, Schlenk and von Euler, 1937; Colowick, Kaplan and Ciotti, 1951; Kaplan, Colowick and Barnes, 1951; Guilbert and Johnson, 1977). Both reactions are catalysed by general as well as specific acids or bases. The reduced nicotinamide cofactors are particularly unstable in phosphate buffers, and NADPH, which bears an extra phosphate group, is less stable than NADH (Wong and Whitesides, 1981; Wu, Wu and Knight, 1986). Depending on the buffer or other ions present, the half-life of nicotinamide cofactors in solution, pH 7, 25°C, ranges from several hours to a month (Wu, Wu and Knight, 1986; Chenault and Whitesides, 1987).

NAD(P) is also sensitive to addition of nucleophiles at C-4 of the pyridinium ring (Ozols and Marinetti, 1969; Everse *et al.*, 1971b; Johnson and Smith, 1976, 1977; Biellman *et al.*, 1979). Several dehydrogenase enzymes catalyse the destructive addition of nucleophiles to NAD (Everse *et al.*, 1971b; Arnold and Kaplan, 1974; Parker, Lodola and Holbrook, 1978; Wilton, 1979; Burgner and Ray, 1984).

REGENERATION OF NICOTINAMIDE COFACTORS

Methods for regenerating nicotinamide cofactors have improved greatly in the past ten years, and at least one method (NADH regeneration by formate and formate dehydrogenase; EC 1.2.1.2) operates in commercial processes. A recent review describes in detail the problems involved in regenerating nicotinamide cofactors and the current solutions to these problems (Chenault and Whitesides, 1987). Immobilized nicotinamide cofactors are also well developed and facilitate the retention and reuse of NAD(P)(H) in hollow-fibre or membrane reactors (Lowe, 1978; Mansson, Larsson and Mosbach, 1982; Katayama, Urabe and Okada, 1983; Wandrey and Wichmann, 1985; Bückmann Morr and Kula, 1987; Goulos, 1987; Wahl and Chang, 1987).

The major obstacle to regenerating the reduced cofactors, NAD(P)H, is the demand for regioselective 1,4-reduction of the pyridinium ring. At present, only enzymatic methods provide the selectivity necessary to allow TTN $\geqslant$ 500 within a conveniently short time of reaction. Several good to excellent methods, each having its own particular advantages and disadvantages, regenerate NADH. Several good methods regenerate NADPH, although a really inexpensive and convenient method is still needed.

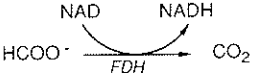
Enzymatic methods also remain the best methods of regenerating NAD(P). Because NAD(P) regeneration lacks the regiochemical demands of NAD(P)H

regeneration, electrochemical and chemical methods succeed better in oxidative regeneration than in reductive regeneration. Nevertheless, enzymatic methods provide higher TTN for cofactor and better compatibility with other components of enzymatic reactors than other methods. A major difficulty in regenerating NAD(P) is not so much the regeneration step but non-competitive or mixed inhibition by the product of the enzymatic oxidation. An analysis of product inhibition and strategies to minimize its effect on synthetic reactions has been described (Lee and Whitesides, 1985, 1986).

REGENERATION OF REDUCED NICOTINAMIDE COFACTORS NAD(P)H

Formate/formate dehydrogenase

The best and most broadly demonstrated method for recycling NADH uses formate dehydrogenase (FDH) to catalyse the NAD-dependent oxidation of formic acid to CO_2 (eq. 3) (Shaked and Whitesides, 1980; Tischer, Tiemeyer and Simon, 1980; Wichmann *et al.*, 1981).



(3)

This method has the advantage that formate is inexpensive, stable, innocuous to enzymes, and strongly reducing ($\triangle E_o' = -0{\cdot}42$ V, pH 7). The by-product, CO_2, is also innocuous to enzymes and is easily removed from the reaction. Progress of the reaction is easily monitored by assaying for formate. The enzyme FDH is commercially available, readily immobilized, and stable if protected from autoxidation.

A disadvantage of the system is the initial expense of FDH ($620/1000 U). For repeated use, however, the initial cost or effort of isolating FDH (Schütte *et al.*, 1976; Cordes and Kula, 1986) is warranted, since the effective cost of the enzyme is only the cost of the enzymatic activity lost per mole of product formed. Immobilized (Shaked and Whitesides, 1980) or soluble, membrane-retained (Wichmann *et al.*, 1981; Hummel *et al.*, 1987b) FDH loses only 0·5–3% of its activity per day of continuous operation. In these applications, formate/FDH is one of the least expensive methods for regenerating NADH.

The low specific activity of FDH (3 U/mg) is another disadvantage and may require large quantities of protein and immobilization support or long reaction times. In practice, however, this disadvantage may be relatively unimportant. One membrane-bound reactor for the continuous production of L-phenylalanine used formate/FDH to regenerate NADH and achieved a space–time yield of 2·76 mol $l^{-1}d^{-1}$ (Hummel *et al.*, 1987b).

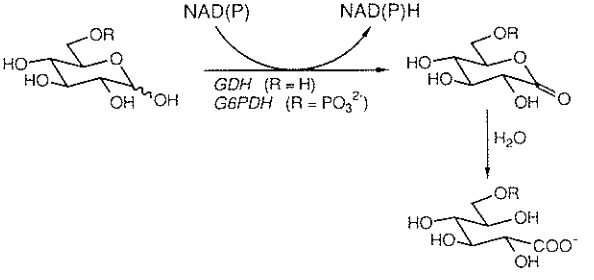
All things considered, formate/FDH is generally the most convenient and most economical method for regenerating NADH, especially for large-scale or repeated applications. It has been used for the continuous production of L-amino acids (Wichmann *et al.*, 1981; Hummel *et al.*, 1987a,b) and is employed for the commercial preparation of L-*tert*-leucine (Wandrey and Bossow, 1986; M.-R. Kula, private communication). Formate (or deuterioformate) and FDH

have also regenerated NADH (or NAD^{2}H) in preparations of D-lactate (Shaked and Whitesides, 1980), 12-ketochenodeoxycholic acid (Carrea *et al.*, 1984b), L-glutamic acid-α-d_1 and (*R*)-trifluoroethanol-1-d_1 (Wong and Whitesides, 1983a), and (*R*)-1,2-butanediol-2-d_1 (Lee and Whitesides, 1986). Total turnover numbers for cofactor range from 600 to 6×10^5.

Unfortunately, FDH is specific for NAD. NADPH regeneration with FDH would require a second enzyme, NAD(P) transhydrogenase (EC 1.6.1.1).

Glucose/glucose dehydrogenase

Glucose dehydrogenase (GDH, EC 1.1.1.47) catalyses the oxidation of glucose to gluconolactone, which spontaneously hydrolyses to gluconic acid (eq. 4, R = H).



(4)

The reaction is strongly exothermic ($\triangle E_o' = -0{\cdot}47$ V) and has been used to regenerate NADH in syntheses of (*S*)-ethanol-1-d_1 (Levy, Loewus and Vennesland, 1957) and L-carnitine (Vandecasteele and Lemal, 1980). Wong, Drueckhammer and Sweers (1985) used glucose and GDH from *Bacillus cereus* to regenerate NADH and NADPH in the enantioselective reductions of several ketones. The outstanding feature of GDH from *B. cereus* is its stability. It is stable in O_2. When immobilized on polyacrylamide *N*-acryloxysuccinimide (PAN) gel (Pollak *et al.*, 1980), the enzyme withstands heating at 55°C for 7 days in 0·5 M NaCl, pH 7·5, with no loss of activity. In a six-day synthesis of D-lactate, GDH regenerated NADH 36 000 times with no loss of activity (Wong, Drueckhammer and Sweers, 1985).

GDH from *B. cereus* accepts either NAD or NADP with high specific activity (250 U/mg). Glucose is readily available, inexpensive, stable, and strongly reducing. It is innocuous to NAD(P)(H) and actually enhances the stability of many enzymes. Like FDH, however, the initial cost of GDH ($280/1000 U) is a disadvantage. Also, the by-product, gluconate, may complicate the reaction work-up.

For processes in which gluconate does not complicate the isolation of products, however, glucose/GDH may be the best method of regeneration. The lower cost (compared with FDH) and high stability of GDH make the method attractive for one-time laboratory use. For large-scale or repeated uses in which the stability of enzymes is important, GDH may again be better than FDH. Glucose/GDH is the best method for regenerating NADPH.

Glucose-6-phosphate/glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase (G6PDH, EC 1.1.1.49) catalyses the oxidation of glucose-6-phosphate (G6P) to 6-phosphogluconolactone, which spontaneously hydrolyses to 6-phosphogluconate (eg. 4, $R = PO_3^{2-}$). The reaction is strongly exothermic ($\triangle E_o = -0{\cdot}43$ V) and has been used to regenerate NAD(P)H in syntheses of chiral α-hydroxy acids and alcohols (Wong and Whitesides, 1981; Hirschbein and Whitesides, 1982). G6PDH from *L. mesenteroides* is commercially available, inexpensive ($10–20/1000 U), high in specific activity (700 U/mg), and easily manipulated. It is stable against autoxidation and alkylation (Isaque, Milhausen and Levy, 1974) and accepts either NAD or NADP (yeast G6PDH accepts only NADP). G6P and 6-phosphogluconate are stable in solution and are innocuous to most enzymes. Reaction progress is monitored by assaying for G6P.

A major disadvantage of the system, however, is that G6P is expensive ($1300/mol) and therefore must be prepared from glucose, using hexokinase (EC 2.7.1.1) and ATP regeneration (Wong and Whitesides, 1981; Kazlauskas and Whitesides, 1985; Crans *et al.*, 1988), if it is to be used on large scale. The by-product of regeneration, 6-phosphogluconate, may complicate the isolation of products and, along with G6P and the inorganic phosphate impurity in G6P, may act as a general acid catalyst for the decomposition of NAD(P)H (Wong and Whitesides, 1981; Chenault and Whitesides, 1987). In syntheses using 0·06–0·17 M G6P and lasting four days, however, phosphate-catalysed degradation of NAD(P)H proved to be of no practical significance (Wong and Whitesides, 1981). Nicotinamide cofactors retained 50–85% of their original activity.

Alternatively, glucose-6-sulphate and G6PDH from *Saccharomyces cerevisiae* may be used to regenerate NADPH (Wong *et al.*, 1981b). Glucose-6-sulphate is not an acid catalyst of NAD(P)H hydration and is more easily prepared than G6P (Guiseley and Ruuoff, 1961).

The G6P/G6PDH system complements glucose/GDH as an excellent method for regenerating NADPH and a good method for regenerating NADH. Whereas glucose/GDH uses an expensive enzyme and an inexpensive reagent, G6P/G6PDH uses an inexpensive enzyme and an expensive (or difficult to prepare) reagent. Both G6PDH and GDH are stable and have high specific activities. Depending on the product formed, the presence or absence of a phosphate group on the by-product may facilitate its separation from the product.

Methods based on the oxidation of alcohols

Ethanol and alcohol dehydrogenase (ADH, EC 1.1.1.1) have been used extensively to regenerate NADH (Jones and Beck, 1976; Wang and King, 1979), especially in analytical procedures (Kato *et al.*, 1973; Bernofsky and Swan, 1973; Schulman *et al.*, 1974). The low cost of both ethanol and yeast alcohol dehydrogenase (YADH) and the volatility of both ethanol and

acetaldehyde make this system attractive. Because ethanol is only weakly reducing, however, only activated aldehydes or cyclic ketones are reduced in good yields (Zagalek *et al.*, 1966; Fink and Rodwell, 1975; Dodds and Jones, 1982). With other substrates, equilibria must be driven by excesses of ethanol or by removing acetaldehyde.

Methods for removing acetaldehyde include sweeping with nitrogen (Vandecasteele and Lemal, 1980; Mansson, Larsson and Mosbach, 1982), trapping with sodium bisulphite (Vandecasteele and Lemal, 1980), and oxidizing acetaldehyde further with aldehyde dehydrogenase (EC 1.2.1.5) (Vandecasteele and Lemal, 1980; Wong and Whitesides, 1982, 1983a). These methods give low TTN, however, or involve complex and unstable multienzyme systems. Low concentrations of acetaldehyde inhibit ADH non-competitively and uncompetitively (Wratten and Cleland, 1963), and both ethanol and acetaldehyde deactivate enzymes. Replacing NAD by an analogue of NAD as cofactor reduces product inhibition by acetaldehyde. The use of acetylpyridine adenine dinucleotide in oxidations of ethanol catalysed by horse liver ADH (HLADH) increases the K_i for acetaldehyde from 0·6 mM with NAD to 35 mM (Kazlauskas, 1987).

Regeneration methods based on the oxidation of methanol (Wong and Whitesides, 1982), cyclopentanol, and 2-cyclohexenol (Jones and Beck, 1976) have also been examined.

Methods based on the oxidation of dihydrogen

Several anaerobic bacteria produce hydrogenase (EC 1.12.1.2) enzymes that catalyse the direct reduction of NAD, MV (methyl viologen), or other redox dyes by H_2. Dihydrogen is an attractive chemical reductant because it is inexpensive, strongly reducing, and innocuous to enzymes and nicotinamide cofactors. Its consumption leaves no by-product and is easily monitored to provide a simple measure of the extent of reaction. Hydrogenase, in whole cell or partially purified form, has been immobilized and used to regenerate NADH (Wong *et al.*, 1981a; Danielsson *et al.*, 1982; Payen *et al.*, 1983). These methods are currently impractical, however. Hydrogenase is not commercially available and so requires preparative fermentation. It is also extremely sensitive to O_2 and other oxidants.

Other methods

Electrochemical methods for regenerating NAD(P)H are attractive because they require no secondary enzyme or reagent and produce no stoichiometric by-product. Electricity is inexpensive and allows convenient control of the reducing potential and monitoring of the progress of reaction. Unfortunately, direct cathodic reduction of NAD(P) suffers from poor regioselectivity, coupling of the 4-dihydropyridyl radical intermediate formed from one-electron reduction of NAD(P), and electrode fouling (Burnett and Underwood, 1968; Janik and Elving, 1968; Biellman and Lapinte, 1978; Jensen and Elving, 1984). Yields of enzymatically active NAD(P)H are low.

Several strategies improve the electrochemical behaviour of NAD(P). Immobilized NAD, which suppresses coupling of the radical intermediate (Aizawa, Coughlin and Charles, 1976), and coated electrodes (Aizawa, Suzuki and Kubo, 1976) both increase yields of active cofactor. Electron transport agents mediate the indirect reduction of NAD(P) (Day *et al.*, 1978; Wienkamp and Steckhan, 1982). The transfer of electrons from electron transport agents to NAD(P) may occur spontaneously or may be catalysed by enzymes (DiCosimo *et al.*, 1981; Shaked, Barber and Whitesides, 1981) or immobilized microbes (Simon *et al.*, 1985a, b). Biochemical catalysis increases the selectivity for the formation of active cofactor yet still uses an electrode as the ultimate source of electrons. All of these systems suffer from poor selectivity, instability, or complexity of operation.

Chemical (Jones *et al.*, 1972; Vandecasteele and Lemal, 1980) and photochemical (Wienkamp and Steckhan, 1983; Mandler and Willner, 1984, 1986a, b) methods of regenerating nicotinamide cofactors are known. These methods are inherently non-selective for the formation of enzymatically active cofactor and risk reductive inactivation of enzymes. TTN are low (10–100).

Whole cells or organelles can function as crude sources of enzymatic activity for cofactor regeneration (Benemann *et al.*, 1973; Rao, Rosa and Hall, 1976; Karube *et al.*, 1980; Miura *et al.*, 1981; Godbole, D'Souza and Nadkarni, 1983). The advantage of whole cells or organelles is the reduced cost of enzymatic activity. In certain cases, enzymes are more stable as whole-cell or organelle complexes than in purified form. The lifetimes of these systems are never very long, however, and the systems remain largely undemonstrated in preparative syntheses requiring NAD(P)H regeneration.

Conclusion

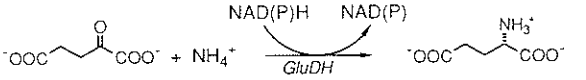
Only methods of regeneration in which the reduction of NAD(P) is enzyme catalysed provide the high selectivity and biochemical compatibility necessary for TTN of 10^3–10^4 and economical use of the cofactor. The best general method for regenerating NADH is formate/FDH. Glucose/GDH and G6P/G6PDH are also good methods, especially if enzyme stability is a concern. These latter two methods are useful for laboratory-scale preparations and are the best methods for NADPH regeneration. A really inexpensive and convenient method of regenerating NADPH, similar to formate/FDH for NADH, is still lacking.

Cofactor regeneration schemes for both NADH and NADPH are successful to the extent that cofactor is no longer the dominant cost of preparative reductions. The cost of enzymes or reagents is equal to, or (for systems using NAD(H)) much greater than the cost of cofactor. Further advances in cofactor regeneration will come through improved methods of enzyme isolation and stabilization or through the development of chemical or electrochemical methods having selectivities for enzymatically active NAD(P)H greater than 99·3%.

REGENERATION OF OXIDIZED NICOTINAMIDE COFACTORS NAD(P)

α-Ketoglutarate/glutamate dehydrogenase

The best and most broadly demonstrated method for regenerating NAD(P) uses glutamate dehydrogenase (GluDH, EC 1.4.1.3) to catalyse the oxidation of ammonium α-ketoglutarate (αKG) to glutamic acid (eq. 5).



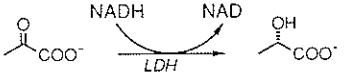
(5)

This method has been demonstrated in numerous syntheses of up to 0·1 mole (Wong, McCurry and Whitesides, 1980; Wong *et al.*, 1982; Carrea *et al.*, 1984a, 1985; Wong and Matos, 1985; Lee and Whitesides, 1985, 1986). GluDH also utilizes ammonium α-ketoadipate as the oxidant, allowing L-α-aminoadipate to be isolated as a useful by-product in addition to the oxidized product (Matos and Wong, 1986). αKG/GluDH oxidizes either NAD or NADP under anaerobic conditions and is thermodynamically sufficient to drive most biochemical oxidations ($\triangle E_o' = -0{\cdot}121$ V). Both αKG and GluDH ($3·1/1000 U) are inexpensive, and both αKG and glutamate are stable and innocuous to enzymes. The progress of the reaction is monitored by assaying for αKG.

A disadvantage of αKG/GluDH is that the specific activity of GluDH (40 U/mg) is only moderate. The stoichiometric by-product, glutamate, may complicate isolation of the product.

Pyruvate/L-lactate dehydrogenase

The use of pyruvate and L-lactate dehydrogenase (LDH, EC 1.1.1.27) to regenerate NAD (eq. 6) (Wong and Whitesides, 1982; Bednarski *et al.*, 1987) has the advantage that LDH is less expensive ($0·6/1000 U) and has a higher specific activity (1000 U/mg) than GluDH. Pyruvate/LDH is stable, and pyruvic acid is less expensive than α-ketoglutaric acid. Progress of the reaction is monitored by assaying for pyruvate.



(6)

A disadvantage of the pyruvate/LDH system is its reduction potential ($\triangle E_o' = -0{\cdot}185$ V), which is less favourable than that of αKG/GluDH. Also, LDH does not accept NADPH. Lactate formed as a by-product may complicate work-up.

Acetaldehyde/alcohol dehydrogenase

Acetaldehyde and YADH have been used to regenerate NAD from NADH with TTN = 1000–19 000 in preparations of fructose and cyclic ketones (Chambers *et al.*, 1978; Lemière, Lepoivre, and Alderweireldt, 1985). Advantages of acetaldehyde/YADH include the low cost of acetaldehyde and YADH ($0·4/1000 U) and the high specific activity of YADH (300 U/mg).

Both acetaldehyde and ethanol are volatile enough not to complicate product isolation. Disadvantages of the system are the low reduction potential of acetaldehyde ($\triangle E_o' = -0{\cdot}199$ V), possible deactivation of enzymes by acetaldehyde or ethanol, and the instability of acetaldehyde in solution (acetaldehyde is capable of both self-condensation and reaction with NAD (Everse *et al.*, 1971a, b)).

Other enzymatic methods

Regeneration methods based on NADH oxidase (EC 1.6.99.5; Chambers *et al.*, 1974; Baricos, Chambers and Cohen, 1976; Gwak *et al.*, 1982), flavin mononucleotide (FMN) reductase (EC 1.6.8.1; Drueckhammer, Riddle and Wong, 1985), and diaphorase (EC 1.8.1.4; Lee and Whitesides, 1985) enzymes have been examined. All of these systems, at least potentially, utilize dioxygen as the terminal oxidant. NADH oxidase transfers electrons directly to dioxygen. FMN reductase and diaphorase reduce FMN and a variety of electron-transfer dyes, respectively. FMN and some redox dyes can be reoxidized spontaneously by dioxygen. Although O_2 is inexpensive and convenient as an oxidant, many enzymes are unstable towards dioxygen, superoxide, and peroxide. The enzymes required by these systems are expensive or unavailable commercially, and some of the electron-transfer agents are expensive. TTN for NAD and the electron-transfer agents have been poor to moderate.

Electrochemical methods

The continuous electrochemical regeneration of NAD has been demonstrated in reactors oxidizing ethanol to acetaldehyde (Coughlin and Alexander, 1975; Coughlin *et al.*, 1975; Jaegfeldt, Torstensson and Johansson, 1978). In theory, electrochemical regeneration of NAD(P) is inexpensive and easily controlled and monitored. It forms no by-product and lacks the regiochemical demands of reductive regeneration. In practice, electrochemical oxidation of NAD(P)H remains difficult. The large overpotential required for oxidation and the fouling of electrodes by compounds adsorbed from solution are two major problems (Moiroux and Elving, 1978, 1979; Jaegfeldt, 1980). The electrochemical behaviour of NAD(P)H is irreproducible and highly dependent on the nature and history of the electrode surface.

Direct oxidation of NADH at carbon or platinum electrodes yields cofactor that is 90–99·3% enzymatically active (Aizawa, Coughlin and Charles, 1975; Kelly and Kirwain, 1977; Jaegfeldt, Torstensson and Johannsson, 1978). Both dye-mediated electron transfer (Tse and Kuwana, 1978; Kitani and Miller, 1981) and chemically modified electrodes (Jaegfeldt *et al.*, 1981; Laval, Bourdillon and Moiroux, 1984; Miyawaki and Wingard, 1985; Narasimhan and Wingard, 1985) reduce the required overpotential and improve the electrochemical activity of NAD(H). Gorton (1986) has recently reviewed the electrochemical oxidation of NADH with emphasis on chemically modified electrodes.

Chemical and photochemical methods

Jones and Taylor (1973, 1976) used FMN to mediate the spontaneous transfer of electrons from NADH to O_2. Their method is simple and has been used extensively by Jones and coworkers in preparative, HLADH-catalysed oxidations of alcohols and hemiacetals. A disadvantage of the system is the slow reduction of FMN by NADH (Lee and Whitesides, 1985), which necessitates high concentrations of the reactants. Typically, FMN ($250/mol) is present in almost stoichiometric concentrations, and the TTN for NAD seldom exceeds 25.

FMN covalently attached to a soluble support regenerated NAD (350 turnovers in 188 h) and NADP (1600 turnovers in 24 h) for the oxidation of ethanol and G6P, respectively (Montaine, Lenders and Crichton, 1987). The immobilized FMN can be used in high concentration but retained within a membrane reactor or recovered for reuse in subsequent reactions. FMN reductase, as mentioned above, catalyses the reduction of FMN by NADH and increases the turnover numbers of FMN and NAD (Drueckhammer, Riddle and Wong, 1985). In an HLADH-catalysed oxidation using FMN reductase, the TTN for FMN and NAD were 35 and 350, respectively.

Although other electron-transfer agents oxidize NAD(P)H and have been used in analytical applications, only phenazine methosulphate (PMS) has been used for preparative purposes. PMS is spontaneously reoxidized by O_2 and has been used to recycle NAD in small-scale oxidations of ethanol and androsterone (Legoy, Le Moullec and Thomas, 1978; Legoy *et al.*, 1979, 1980). Phenazine ethosulphate has been used in analytical applications (Bernofsky and Swan, 1973) to obviate difficulties due to the instability of PMS (McIlwain, 1937), but phenazine ethosulphate has not been investigated in preparative systems.

Photoexcitation by visible light accelerates the oxidation of NAD(P)H by several electron-transfer dyes (Chambers *et al.*, 1974) and facilitates the regeneration of NAD(P) by catalytic quantities of PMS and methylene blue (Julliard and Le Petit, 1982; Julliard, Le Petit and Ritz, 1986). TTN for NAD have reached 1125. Polymerized FMN (Chambers *et al.*, 1974) and immobilized acriflavin (Mansson *et al.*, 1976) have also been used to regenerate NAD photochemically.

Biological methods

Immobilized *Escherichia coli* (Burstein *et al.*, 1981; Chave, Adamowicz and Burstein, 1982) and *Leuconostoc mesenteroides* (Ergan, Thomas and Chang, 1984) have been used to regenerate NAD(P). Although these systems are potentially important for industrial use, their stability and general utility remain uncharacterized.

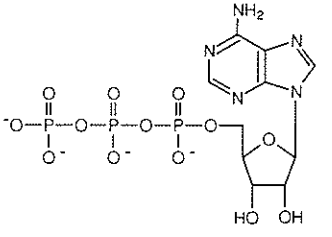
Conclusion

Methods for *in situ* NAD(P) regeneration are fewer and less developed than those for NAD(P)H regeneration. The αKG/GluDH system is the best general

method for NAD regeneration and virtually the only method for NADP regeneration. Pyruvate/LDH may have advantages of lower cost, greater stability, and ease of product isolation in certain applications. For industrial processes, no method is completely satisfactory. Methods based on the reduction of acetaldehyde, electrochemical oxidation of NAD(P)H, or whole cells are particularly attractive and deserve further development.

A frequent obstacle in enzymatic oxidations is not the cofactor regeneration itself but non-competitive or mixed product inhibition associated with the oxidation of substrate. Methods for identifying and minimizing the effects of product inhibition have been described (Lee and Whitesides, 1985, 1986).

Nucleoside phosphate cofactors



ATP

STRUCTURE AND FUNCTION

Nucleoside triphosphates serve as sources of nucleosides, nucleoside phosphates and phosphate in cells. Adenosine 5′-triphosphate (ATP) is the cofactor most often used as a phosphorylating agent. Other phosphate esters of nucleosides, such as uridine 5′-triphosphate (UTP) and cytidine 5′-triphosphate (CTP) usually donate the nucleoside phosphate moiety to form activated intermediates in biological pathways rather than donating only phosphate. UTP precedes the activated form of glucose, UDP-glucose, in the Leloir synthesis of polysaccharides, for example, and CTP precedes CDP-choline in the synthesis of phospholipids and CMP-NeuAc in the formation of glycosides of sialic acids.

In enzyme-catalysed organic synthesis, ATP has received the most attention of all the nucleoside phosphates because of its utility as a phosphorylating agent. The high cost of ATP initially limited the scale of its use in organic synthesis, but several satisfactory methods now allow regeneration of ATP from ADP on a mole scale. Other nucleoside phosphates have been used in organic synthesis mainly as sources of nucleoside phosphates rather than of phosphate. As a result, little effort has been spent on developing methods to regenerate nucleotides other than ATP.

PREPARATION OF NUCLEOSIDE PHOSPHATES

Most of the nucleoside mono- and triphosphates are commercially available and some, such as AMP and IMP (5′-inosine monophosphate) have been produced commercially on a 3000-ton scale (Hirose, Enei and Shibai, 1979; Samejima, Kimura and Ado, 1980). Fewer of the nucleoside diphosphates are commercially available. Enzymatic phosphorylation of RNA (extracted from yeast, US Biochemical Corp.) or fermentation produces nucleoside phosphates. Many reports describe the production of nucleoside phosphates by enzymatic methods (Langer *et al.*, 1976; Samejima *et al.*, 1978b; Yang and Colton, 1982; Crans and Whitesides, 1983; Kondo *et al.*, 1984; Nakajima *et al.*, 1984; Crans *et al.*, 1988) or by fermentation (reviews and leading references: Tanaka *et al.*, 1968; Hirose, Enei and Shibai, 1979; Samejima, Kimura and Ado, 1980; Asada *et al.*, 1981a, b; Kimura *et al.*, 1981; Fujio and Furuya, 1983, 1985; Linko and Linko, 1983; Kondo *et al.*, 1984; Yamaguchi *et al.*, 1984; Chibata, Tosa and Sato, 1985; Yonehara and Tani, 1987). Several simple methods, applicable on a laboratory scale, produce mixtures of nucleotides by the enzymatic hydrolysis of yeast RNA (Leuchs *et al.*, 1979; Wong, Haynie and Whitesides, 1983). These crude mixtures are acceptable for use in enzyme-catalysed synthesis (Wong, Haynie and Whitesides, 1983).

Chemical methods are inferior in their specificity and costs to enzymatic and biological methods for the large-scale production of nucleoside phosphates, but chemical methods are superior for the production of most analogues. Several good reports describe or review chemical routes to nucleotides (Scheit, 1980; Davisson *et al.*, 1987; Hobbs, 1987). Immobilized derivatives of ATP (Mosbach, Larsson and Lowe, 1976; Wang and King, 1979; Yamazaki and Maeda, 1981; Berke *et al.*, 1984; Smeds and Enfors, 1986) have proved less useful in enzyme-catalysed synthesis than similarly immobilized nicotinamide cofactors. ATP-utilizing enzymes tolerate the immobilized cofactors less well than NAD(P)(H)-utilizing enzymes, exhibiting greater reductions in catalytic activity.

STABILITY OF NUCLEOSIDE PHOSPHATES

The stability of nucleoside phosphates is, in general, not a problem because the cofactors are stable under the mild conditions usually used in enzyme-catalysed synthesis. They may be stored for months at temperatures below 0°C in the form of their neutral salts (Dawson *et al.*, 1982). The compounds are not sensitive to light or oxygen. Acidic conditions hydrolyse the phosphate esters.

REGENERATION OF NUCLEOSIDE PHOSPHATES

Most of the development of regeneration systems has focused on the regeneration of ATP. A regeneration system for ATP, and for other nucleoside phosphates, must meet several specifications in addition to those mentioned in

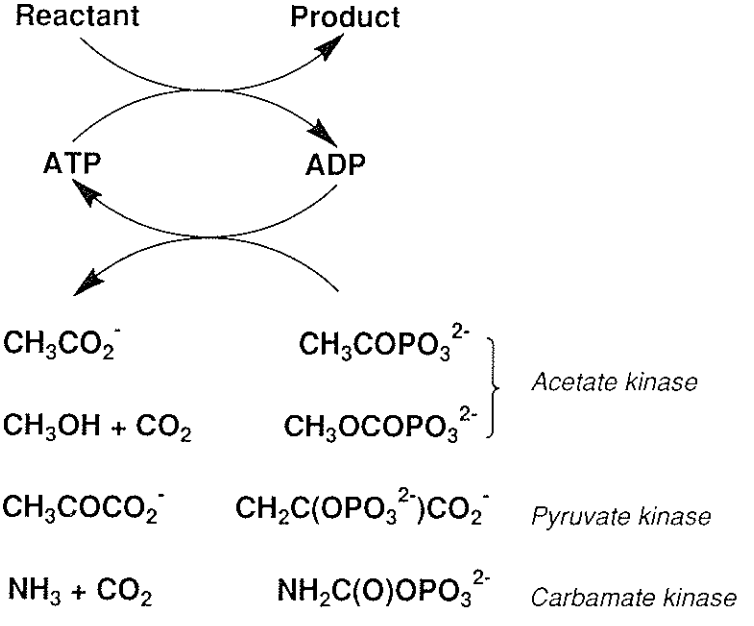
the introduction. First, the transfer of phosphate must be thermodynamically and kinetically favourable. In addition, a regioselective transfer of phosphate to form a high-energy bond must occur. Furthermore, the phosphate transfer agent must be accessible and stable under the reactions conditions.

Three basic strategies address these issues: enzyme-catalysed systems; biological systems, using whole cells, organelles, or methods of fermentation; and chemical synthesis. No report describes a practical method for the electrochemical regeneration of ATP, although proposals have been made (Wingard and Yoo, 1980). Enzymatic and biological systems have provided the main successes for the regeneration of ATP, and a discussion follows of the specific methods of these systems. Chemical methods often lack specificity and are not compatible with other simultaneous biological transformations.

Enzymatic methods require a greater initial effort or expense to obtain pure enzymes than do the biological methods. The biological methods, however, are less specific and often generate by-products. As a result, extraction of the product is often more difficult than with enzymatic methods. On a laboratory scale, enzymatic methods are more convenient.

REGENERATION OF NUCLEOSIDE COFACTORS USING ENZYMATIC SYSTEMS

Several procedures for the enzymatic regeneration of ATP in multigram-scale organic synthesis have been developed (*Scheme I*). These procedures use a cell-free enzyme to catalyse the transfer of phosphate from a high-energy phosphoryl donor to ADP. *Table 2* summarizes properties of the phosphorylating reagents used in ATP regeneration.



Scheme I. Enzymatic methods for the regeneration of ATP from ADP

Table 2. Properties of phosphorylating reagents used in ATP regeneration*

Property	PEP	AcOP	MCP	CP
Ease of preparation	+	+++	++	+++
$\Delta G°'$hyd (kcal/mole)†	−12·8	−10·1	−12·4	−12·3‡
Half-life for hydrolysis (h)				
pH 7, 25 °C	$\sim 10^3$	21	0·3	2·2
pH 7, 0 °C	$\sim 10^5$	960	15	16
Product inhibition (Ki, mM)	pyruvate 10, C¶	acetate 400, NC¶	§	HCO_3^- 500, NC¶

* PEP: phosphoenolpyruvate (Hirschbein, Mazenod and Whitesides, 1982); AcOP: acetyl phosphate (Crans and Whitesides, 1983); MCP: methoxycarbonyl phosphate (Kazlauskas and Whitesides, 1985); CP: carbamyl phosphate (Marshall, 1973a, b).

† W. P. Jencks, in *Handbook of Biochemistry*, 2nd ed. (H. A. Sober, ed.) pp. J–85. Chemical Rubber Company, Cleveland, 1970. Standard free energy of hydrolysis at pH 7, based on a standard state of 1 M total stoichiometric concentration of reactants and products, except hydrogen ion, and on an activity of pure water of 1·0.

‡ pH 9·5.

§ Kinetics for carbamate kinase are complex (Marshall, M. and Cohen, P. P., *J. Biol. Chem.* **241**, 4197–4208 (1966)).

¶ NC = non-competitive; C = competitive.

Phosphoenolpyruvate/pyruvate kinase

The best method for the enzymatic regeneration of ATP from ADP uses phosphoenolpyruvate (PEP) as the phosphate donor in a coupled reaction catalysed by pyruvate kinase (PK; EC 2.7.1.40). PEP is prepared from pyruvate on a mole-scale (Hirschbein, Mazenod and Whitesides, 1982; Whitesides, Hirschbein and Mazenod, 1986) and commercially available PK is inexpensive (~ $2/1000 U, rabbit muscle, Sigma Chemical Co.), has a high specific activity (~500 U/mg of protein), is relatively insensitive to oxygen, and is stable when immobilized (Campbell and Chang, 1975; Pollak *et al.*, 1980; Slegers *et al.*, 1986).

The primary advantages of PEP/PK are the excellent stability of PEP in solution and its strength as phosphoryl donor. This stability makes PEP particularly convenient for use with slowly reacting substrates.

The method has several minor disadvantages. The synthesis of PEP requires slightly more effort and expense than does the synthesis of acetyl phosphate or other phosphoryl donors; attempts to produce PEP by fermentation have not been successful (Moriguchi and Nagano, 1987). In addition, pyruvate inhibits PK (*Table 2*) and so the reaction must be carried out in dilute solution to keep the concentration of pyruvate low, pyruvate must be removed from the reaction mixture as it is formed, or high concentrations of PEP must be used to minimize the effects of this inhibition.

PEP/PK has been used in the synthesis of arabinose-5-phosphate (Bednarski *et al.*, 1988), arginine phosphate (Bolte and Whitesides, 1984), glucose-6-phosphate (Pollak, Baughn and Whitesides, 1977), and 5-phospho-D-ribosyl α-1-pyrophosphate (Gross *et al.*, 1983) on a 50–900 mmol scale. TTN typically are on the order of 100. Triphosphates have also been prepared: adenosine 5′-*O*-(3-thiotriphosphate) (Abril, Crans and Whitesides, 1984), (S_p)-adenosine 5-*O*-(1-thiotriphosphate) (Moran and Whitesides, 1983), and deoxyATP (Ladner and Whitesides, 1985).

Acetyl phosphate/acetate kinase

AcOP/AK is the most widely used method to regenerate ATP. AcOP is very easy to prepare on a mole scale (Crans and Whitesides, 1983), although it is only modestly stable in solution and its phosphoryl donor potential is lower than that of PEP (*Table 2*). Acetate kinase (AK; EC 2.7.2.1) is moderately expensive (~ $60/1000 U from *E. coli*, Sigma Chemical Co.), has useful specific activity (150–250 U/mg of protein), and can be stabilized by immobilization (Whitesides *et al.*, 1976, 1979). Oxygen inactivates the enzyme, but blocking the cysteine groups by treatment with *S*-methylmethanethiosulphonate reduces this sensitivity (Whitesides *et al.*, 1979). A thermostable enzyme from *B. stearothermophilus* that is not sensitive to oxygen has been immobilized on Sephadex (Nakajima *et al.*, 1984) and used to generate ATP from ADP using AcOP (Kondo *et al.*, 1984). Others have found the use of the *B. stearothermophilus* enzyme to be preferable to the enzyme from *E. coli* (Kim and Whitesides, 1988); the additional stability may compensate for the additional cost of the catalyst ($300/1000 U, 300–600 U/mg of protein, *B. stearothermophilus*, Sigma Chemical Co.)

AcOP/AK may be the most economical method for large-scale work because of the ease of preparing AcOP. In addition, inhibition is not a serious problem with AcOP/AK (*Table 2*). The relative instability of AcOP in solution compared with PEP is a major disadvantage of the method; the slightly higher cost of AK compared with PK is a minor disadvantage (the contribution of the enzymes to the total cost of the process is generally low when they are reused).

AcOP/AK has been used in the synthesis of creatine phosphate (Shih and Whitesides, 1977), dihydroxyacetone phosphate (Wong and Whitesides, 1983b), and glucose-6-phosphate (Pollak, Baughn and Whitesides, 1977).

An alternative phosphoryl donor, methoxycarbonyl phosphate (MCP), was designed to replace acetyl phosphate as a phosphoryl donor in regeneration schemes (Kazlauskas and Whitesides, 1985). The advantages of MCP are its ease of preparation and its strong phosphoryl donor potential. Furthermore, the product remaining after phosphoryl transfer from MCP to ADP, methyl carbonate, hydrolyses rapidly in solution to methanol and carbon dioxide ($t_{1/2}$ ~ 0·3 h, pH 7, 25°C). This decomposition simplifies work-up and also minimizes the effects of product inhibition. The rapid decomposition of MCP in solution is a serious drawback to its use (*Table 2*).

Other methods

The method based on carbamyl phosphate and carbamyl kinase (EC 2.7.2.2) has been demonstrated (Marshall, 1973a, b) but is not used (*Scheme I*). Preparation of carbamyl phosphate is facile, but it hydrolyses rapidly in solution and it is a slightly weaker phosphorylating agent than PEP (*Table 2*). The decomposition of carbamyl phosphate generates ammonium ion, and a complex ($MgNH_4PO_4$) may form during the reaction, removing magnesium (II) ions required for enzymatic activity from solution and filling the reaction vessel with a gelatinous precipitate.

Creatine phosphate and creatine kinase (EC 2.7.3.2) were used to regenerate ATP in the synthesis of fructose 1,6-diphosphate (Sakata, Kitano and Ise, 1981).

REGENERATION OF NUCLEOSIDE COFACTORS USING BIOLOGICAL SYSTEM

Fermentation

Fermentation provides an inexpensive source of enzyme activity and starting materials, such as glucose, are usually not costly. Fermentation is less convenient than enzymatic methods for laboratory-scale (< 1 mole) regeneration of cofactors. Fermentation systems do not provide specific activity and many by-products may form. Extraction of the product may be difficult, especially when reactions do not go to completion. Several reports demonstrate the true regeneration of ATP coupled to another synthetic reaction using fermentation: glutathione (Murata *et al.*, 1981; Murata, Tani and Chibata, 1981); NADP (Murata *et al.*, 1981); CoA (Shimizu, Tani and Ogata, 1979); CDP-choline (Ado *et al.*, 1979; Kimura *et al.*, 1981).

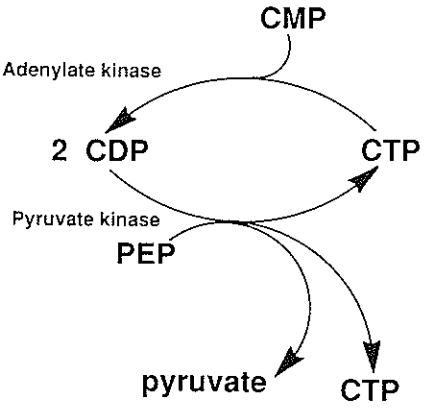
Fermentation mainly has been used to generate nucleoside triphosphates, particularly ATP (Ado *et al.*, 1979; Hirose, Enei and Shibai, 1979; Samejima *et al.*, 1978a; Samejima, Kimura and Ado, 1980; Linko and Linko, 1983; Chibata, Tosa and Sato, 1985). These methods convert glucose and adenosine to ATP using a combination of kinases and enzymes of glycolysis; methanol-utilizing yeasts have also been used (Tani and Yonehara, 1985; Yonehara and Tani, 1986, 1987). The types of cells generally used are yeast (Asada *et al.*, 1981b; Tachiki *et al.*, 1981; Murata, Tani and Chibata, 1981), *E. coli* (Yamaguchi *et al.*, 1984), and *Brevibacterium ammoniagenes* (Tanaka *et al.*, 1968; Fujio and Furuya, 1983, 1985). Genetic engineering has refined several of these systems (Shimosaka, Fukuda and Kimura, 1981; Fukuda *et al.*, 1984).

Organelles

Methods for regenerating ATP using organelles (i.e. chloroplasts, chromatophores or mitochondria) are not yet practical for synthetic applications. A review of efforts up to 1981–82 cites instability as a major problem (Ochiai, Tanaka and Fukui, 1983). More recent work has investigated this difficulty (Matsuoka, Suzuki and Aizawa, 1981; Smeds and Enfors, 1986) as well as the influence of external factors on the function of organelles (Laretta-Garde and Thomas, 1987). Although it is unlikely that regeneration systems based on chloroplasts will be applied in the near future, the concept of using photosynthetic energy to regenerate ATP is an attractive idea, deserving further research.

REGENERATION OF OTHER NUCLEOTIDES

Other nucleotiodes have not received as much attention as ATP; few, if any, methods exist for their regeneration. Many nucleoside triphosphates are



Scheme II. Generation of CTP from CMP using adenylate kinase

available from commercial sources, but these compounds are often more expensive than the corresponding monophosphates. Methods of generating nucleoside triphosphates by fermentation or by enzymatic phosphorylation of yeast RNA are mentioned above. Adenylate kinase (EC 2.7.4.3) has been used to generate CTP from CMP (*Scheme II*) and ribavirin triphosphate from ribavirin monophosphate using PEP/PK (Kim and Whitesides, 1988; Simon, Bednarski and Whitesides, 1988). Generation of nucleoside triphosphates (NTP) from nucleoside diphosphates (NDP) is generally not a problem because the enzymes used (PK and AK) have broad substrate specificity (Kim and Whitesides, 1988). The efficient generation of NDP from nucleoside monophosphates (NMP) is an unsolved problem and limits the generation of NTP from NMP. Nucleoside kinases that convert nucleosides (N) to NMP are known but are not available.

Nucleoside diphosphates are receiving increasing attention as pharmaceutical intermediates, but no practical biological or enzymatic routes have been demonstrated for their synthesis. A possible route is to transfer one phosphate unit in a enzyme-catalysed reaction from a triphosphate to an acceptor substance. A second is to generate the nucleoside diphosphates as an equilibrium mixture using RNA and nucleoside phosphorylase, and then to drive the (unfavourable) equilibrium by continuous separation using membranes or other procedures. The latter method has been demonstrated by *in situ* conversion of the nucleoside diphosphates into triphosphates (Wong, Haynie and Whitesides, 1983).

CONCLUSION

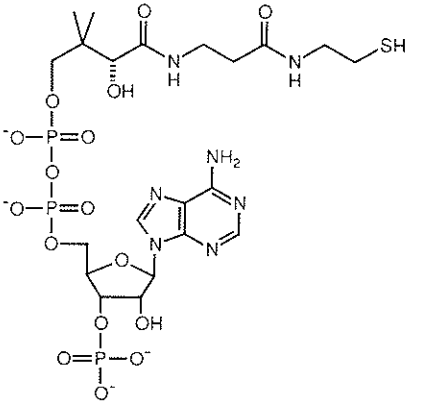
The regeneration of ATP from ADP is straightforward and most conveniently carried out on a laboratory scale using enzymatic methods. PEP/PK will probably be used most often because of the relative stability of PEP.

Several problems remain to be solved. More efficient methods for generating nucleoside triphosphates other than ATP from nucleoside monophosphates

must be developed. Perhaps the most straightforward route involves use of the commercially available nucleoside monophosphate kinases. These enzymes, however, are expensive ($10 800/1000 U; Sigma Chemical Co.), and more research must be done to improve their isolation and stability. The use of large quantities of adenylate kinase (either immobilized or confined within a dialysis membrane) has allowed the successful generation of CTP from CMP (Simon, Bednarski and Whitesides, 1988) and ribavarin triphosphate from ribavarin monophosphate (Kim and Whitesides, 1988). Adenylate kinases from rabbit muscle (Noda, 1973) and from baker's yeast (Ito, Tomasselli and Noda, 1980) accept a variety of nucleotides, suggesting that this catalyst will be useful for the practical preparation of other triphosphates. Useful methods for the regeneration of NDP from NMP and NMP from N do not exist.

Coenzyme A

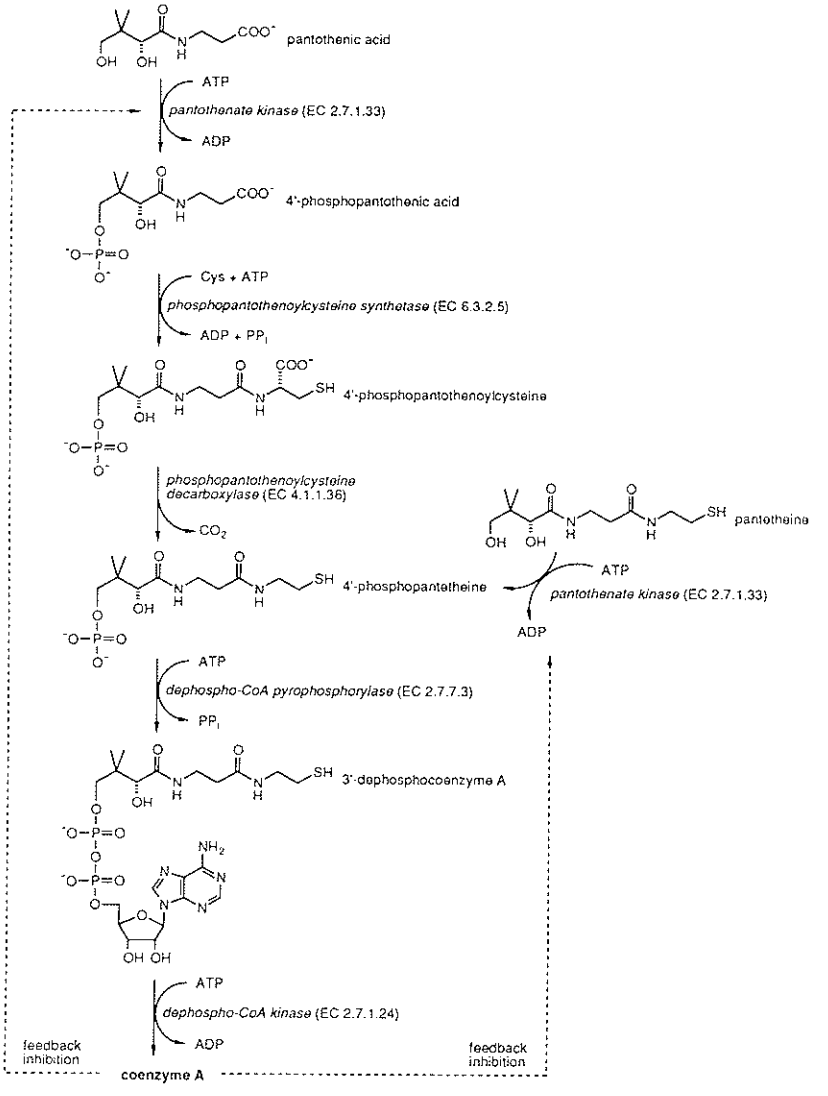
STRUCTURE AND FUNCTION



coenzyme A

Coenzyme A (CoA) functions in all organisms as an acyl carrier and activator. It participates in stereoselective carbon–carbon bond-forming reactions and in chemo- and regioselective acylations. Acetyl CoA (AcCoA) is a key intermediate in fatty acid metabolism, glucose metabolism through the citric acid cycle, malic acid formation, malonyl-CoA formation, and bacterial photosynthesis. Acyl-CoA derivatives participate in cholesterol and fatty acid biosynthesis.

CoA carries acyl groups as thioesters and thereby activates them both toward nucleophilic attack at the carbonyl carbon and toward Claisen or aldol condensation at the α-carbon (Bruice and Benkovic, 1966; Walsh, 1979a; Suckling and Suckling, 1980). Poor π overlap between sulphur and the carbonyl carbon makes nucleophilic addition to a thioester less energetically unfavourable than addition to an oxygen ester. Reduced resonance stabilization of the carbonyl group of a thioester also enables it to stabilize a negative charge on the α-carbon better than that of an oxygen ester. Thioesters are also thermodynamically less stable with respect to hydrolysis than oxygen esters and provide greater thermodynamic drive for acyl transfer. Free energies



Scheme III. Biosynthesis of coenzyme A

of hydrolysis for AcCoA and acetylcholine at pH 7 are −8 and −6 kcal/mol, respectively (Segel, 1976).

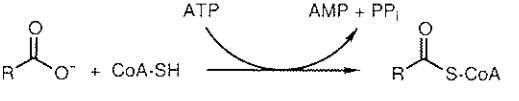
PREPARATION OF CoA

CoA is prepared (*Scheme III*) from pantothenic acid, AMP or ATP and L-cysteine (L-Cys) by fermentation (Wakamoto Pharmaceutical, 1985; Matsumoto *et al.*, 1986) or by synthesis with dried cells (Shimizu, Tani and Ogata, 1979; Asada *et al.*, 1982). Methods using dried cells require at least four

equivalents of ATP per equivalent of CoA formed. The major problem of both methods, however, is feedback inhibition of pantothenate kinase (EC 2.7.1.33) by CoA. Pantothenate kinase catalyses the phosphorylation of pantothenic acid or pantetheine, and its inhibition limits the total yield possible. Two strategies overcome this problem. Phosphopantothenate or phosphopantetheine prepared chemically reacts with ATP and L-Cys or ATP, respectively, in the presence of dried *Brevibacterium ammoniagenes* cells (Shimizu *et al.*, 1983). The enzymatic steps inhibited by CoA are thus avoided altogether. A mutant of *B. ammoniagenes* resistant to oxypantetheine shows reduced inhibition by CoA and produces higher concentrations of CoA in culture than wild-type cells (Shimizu *et al.*, 1984). CoA remains problematic for complete synthesis (Shimizu, 1970).

PREPARATION OF ACYL-CoA

Acyl-CoA derivatives are usually prepared by reacting CoA with acyl chlorides (Seubert, 1960; Lands *et al.*, 1966; Reitz *et al.*, 1968; Okuyama *et al.*, 1969; Bishop and Hajra, 1980), acid anhydrides (Simon and Shemin, 1953; Stadtman, 1957), mixed anhydrides of ethyl hydrogen carbonate (Stadtman, 1957; Goldman and Vagelos, 1961; Sánchez, Nicholls and Brindley, 1973), or *N*-hydroxysuccinimide esters (Al-Arif and Blecher, 1969) in buffered or basic solution. Wilson (1952) prepared AcCoA by reacting thioacetic acid with oxidized or reduced CoA. These methods, in general, suffer from non-specific acylation (Pullman, 1973) and low to moderate yields. Acylations with α, β-unsaturated acid derivatives suffer from Michael addition of CoA-SH to the acylating agent (Stadtman, 1957). Enzymatic acylations of CoA (eq. 7) using AcCoA synthetase (EC 6.2.1.1) or fatty acyl CoA synthetase (fatty acid: CoA ligase, EC 6.2.1.3) are highly selective for *S*-acylation but have been demonstrated in preparations of only 1–25 μmol (Kornberg and Pricer, 1953a; Galliard and Stumpfe, 1968; Branis *et al.*, 1976; Merrill, Gidwitz and Bell, 1982). The substrate specificity of both enzymes is broad (Tanaka *et al.*, 1979; Patel and Walt, 1987).



(7)

REGENERATION OF ACYL-CoA

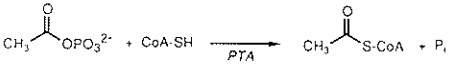
The expense of CoA (*Table 1*) places extreme demands on any method of regenerating acyl-CoA. Unless the product is of high value (> \$250/mol), economical use of CoA requires that acyl-CoA be recycled $\geq 10^4$ times. Such high turnovers demand not only extreme selectivity for the formation of enzymatically active cofactor but also high rates of reaction with low concentrations of CoA and high stability of all CoA species. Chemical methods almost certainly lack the selectivity necessary, and enzymatic methods operate slowly at low concentration of CoA.

Most attempts to recycle acyl-CoA *in situ* have focused on regenerating

AcCoA (Riecke, Barry and Mosbach, 1979; Patel, Conlon and Walt, 1986; Billhardt, Stein and Whitesides, 1988). Although a number of enzymatic methods are conceivable (Billhardt, Stein and Whitesides, 1988), only three have been demonstrated.

Acyl phosphate/phosphotransacetylase

Phosphotransacetylase (PTA, EC 2.3.1.8) catalyses the conversion of acetyl phosphate (AcOP) and CoA to AcCoA and inorganic phosphate (P_i) (eq. 8).



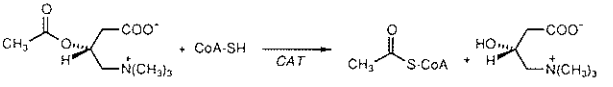
(8)

The reaction has regenerated AcCoA in 0·1–15 mmol-scale preparations of citric acid and *O*-acetyl-L-carnitine (L-AcCarn) (Patel, Conlon and Walt, 1986; Billhardt, Stein and Whitesides, 1988) and in μmol-scale acetylations of aminoglycosides (Breeze and Simpson, 1982). TTN for mmol-scale reactions range from 400 to 12 000. PTA is active with α-halo-acetyl phosphates, acryloyl phosphate, and a few short-chain alkanoyl phosphates (Satchell and Kyrtopoulos, 1973; Billhardt, Stein and Whitesides, 1988) and with CoA immobilized on Sepharose (Riecke, Barry and Mosbach, 1979).

An advantage of this system is the relatively low cost of PTA ($25/1000 U). Unfortunately, PTA is unstable in solution (Billhardt, Stein and Whitesides, 1988). Although synthesis of AcOP is straightforward (Crans and Whitesides, 1983; Kazlauskas and Whitesides, 1985), the preparation of acyl phosphates, in general, is difficult, and acyl phosphates hydrolyse readily in neutral solution.

Acyl carnitine/carnitine acetyltransferase

Carnitine acetyltransferase (CAT, EC 2.3.1.7) catalyses the transfer of acetate from L-AcCarn to CoA (eq. 9).



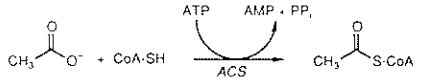
(9)

The reaction has recycled AcCoA 690 times in the synthesis of 1 mmol of citric acid (Billhardt, Stein and Whitesides, 1988). AcCarn/CAT has the advantage that acyl carnitines are far more stable in aqueous solution than acyl phosphates. CAT accepts short-chain alkanoyl and α,β-unsaturated acyl derivatives of L-carnitine and appears to be more stable than PTA (Billhardt, Stein and Whitesides, 1988). Unfortunately, CAT ($95/1000 U) is more expensive than PTA. Like acyl phosphates, acyl carnitines are difficult to prepare.

Acetate/ATP/acetyl-CoA synthetase

In theory, the use of acetyl-CoA synthetase or fatty acyl-CoA synthetase might be the simplest method for *in situ* acylation of CoA. Both enzymes couple a

broad range of underivatized carboxylic acids to CoA in the presence of ATP (eq. 10) (Tanaka *et al.*, 1979; Patel and Walt, 1987).



(10)

The difficulty with the system, of course, is that ATP must itself be regenerated (or used in stoichiometric quantities). Another disadvantage is that both synthetases are very expensive ($2–3/U). These systems have been used (without ATP regeneration) to regenerate AcCoA 1000 times in a small-scale synthesis of citrate (Patel, Conlon and Walt, 1986) and to regenerate acyl-CoA in nmol–μmol-scale syntheses of hydroxamic acids and diacyl-L-α-glycerophosphates (Kornberg and Pricer, 1953a, b).

Conclusion

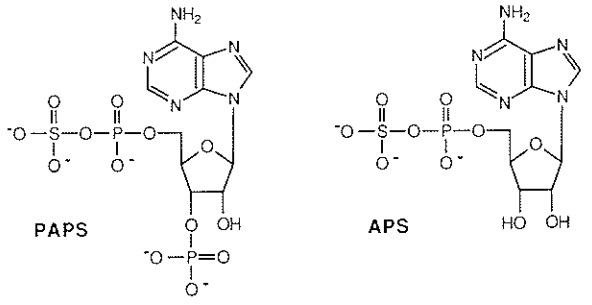
None of the methods of regenerating acyl-CoA have demonstrated sufficiently high TTN on a large scale to make the use of acyl-CoA economically feasible. Methods using PTA or CAT are most practical for laboratory-scale regeneration of short-chain acyl-CoA. The difficulty of preparing appropriate acyl derivatives and the narrow substrate range of the two enzymes detract from these methods, however. For large-scale synthesis, the use of ACS and fatty acyl-CoA synthetase deserve further development. Although ATP regeneration adds to the complication of these systems, methods of ATP regeneration are well developed. The ability to use free carboxylic acids as acylating derivatives is a disinct advantage.

Enzymatically active forms of immobilized CoA (Le Goffic, Sicsic and Vincent, 1978; Riecke, Barry and Mosbach, 1979) will be important for the economical use of CoA. They can be used in concentrations high enough for favourable reaction rates and regenerated enzymatically. Although the immobilized CoA has not been regenerated more than 1000 times in a single reaction, it can be recovered and reused in subsequent reactions. Another promising strategy to be pursued to lower the contribution of CoA to the cost of an enzymatic reaction is to use inexpensive analogues of CoA instead of CoA itself (Davis *et al.*, 1987).

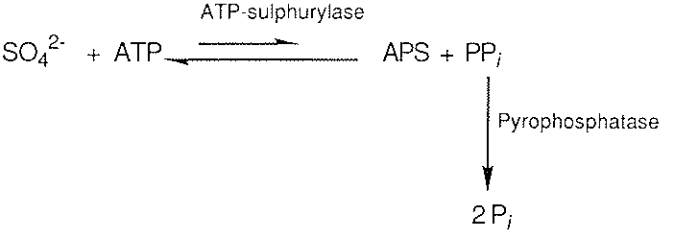
PAPS

STRUCTURE AND FUNCTION

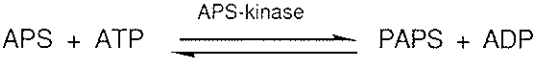
PAPS (3′-phosphoadenosine 5′-phosphosulphate) is the universal sulphate donor in biological systems. Several reviews describe the role of PAPS in sulphation (Schiff and Hodson, 1973; Peck, 1974; De Meio, 1975; Farooqui, 1978, 1981; Trudinger and Loughlin, 1981). Unlike ATP, the synthetic applications of PAPS have received scant attention.



Two enzymes generate PAPS from ATP and sulphate (DeMeio, 1975): ATP-sulphurylase (EC 2.7.7.4) condenses ATP and sulphate to produce adenosine 5′-phosphosulphate (APS, eq. 11) and APS-kinase (EC 2.7.1.25) uses an additional molecule of ATP to phosphorylate APS to PAPS (eq. 12).



(11)



(12)

Although the first reaction has an apparent equilibrium constant of 10^{-8} (Farooqui, 1981), the cleavage of pyrophosphate makes the formation of PAPS favourable (eq. 11). ATP-sulphurylase from a variety of sources has been isolated and studied (De Meio, 1975; Renosto, Seubert and Segel, 1984). Preparations of ATP-sulphurylase have a pH optimum near 8 and K_m for ATP is of the order of 1 mM and K_m for APS is of the order of 1 μM to 1 mM (De Meio, 1975). Recently, a relatively pure preparation of APS-kinase from *Penicillium chrysogenum* also has been isolated and studied (Renosto, Seubert and Segel, 1984). The enzyme exhibits maximal activity at pH 8·0, retaining 90% of its maximal activity between pH 7·5 and 8·5, and has K_m for APS = 1·4 μM and K_m for Mg-ATP = 1·5 mM. APS strongly inhibits the enzyme (K_i = 23 μM). Neither APS-kinase or ATP-sulphurylase are commercially available.

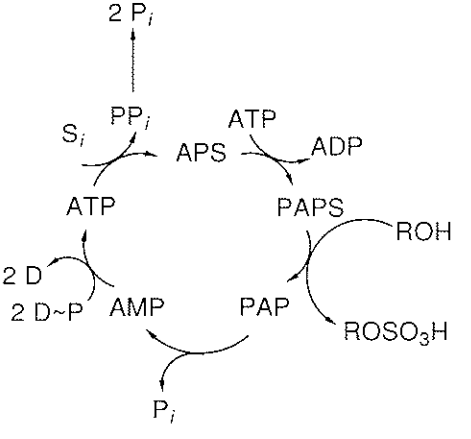
The sulphotransferases use PAPS as a source of sulphate during the synthesis of various compounds (Schiff and Hodson, 1973; De Meio, 1975). Many of the sulphated compounds are attractive synthetic targets: glycosaminoglycans (Conrad and Woo, 1980) such as heparin sulphate (Göhler, Niemann and Buddecke, 1984), keratin sulphate (Conrad and Woo, 1980), chondroitin sulphate (Renosto and Segel, 1977) and cholecystokinin (Vargas *et al.*, 1985); phenol or aryl sulphates, which are important in detoxification (Sekura, Duffel

and Jakoby, 1981); sulphamates; and sulpholipids, such as cerebroside sulphates or steroid sulphates (Farooqui, 1978; Lyon *et al.*, 1981).

PREPARATION AND STABILITY OF PAPS

A variety of chemical and enzymatic syntheses of PAPS have been reported. De Meio (1975) reviews the methods presented up to 1975, and other routes have been reported more recently (Tsang *et al.*, 1976; Horowitz *et al.*, 1977; Cooper and Trüper, 1979; Singer, 1979; Sekura, 1981). The route that seems capable of yielding the largest quantities of pure PAPS is based on a chemical synthesis of adenosine 2′, 3′-cyclic phosphate 5′-phosphosulphate from adenosine followed by enzymatic cleavage of the cyclic phosphate by T2-RNAase (EC 3.1.27.1; Horrowitz *et al.*, 1977). Adenosine 2′-phosphate 5′-phosphosulphate can also be prepared by treatment of the cyclic phosphate with spleen phosphodiesterase II. Sekura (1981) reports the synthesis of more than 400 mg of PAPS by a similar route in which adenosine 2′,3′-cyclic phosphate 5′-phosphate, prepared by treating a mixture of adenosine 2′,5′- and 3′,5′-diphosphates with dicyclohexylcarbodiimide, is treated with T2-RNAase.

PAPS is relatively stable to base, but the sulphate anhydride decomposes rapidly in acidic media; for example, when PAPS is placed in 0·1 N HCl at 37°C for 1·75 h, it decomposes to adenosine 3′,5′-diphosphate (Horowitz *et al.*, 1977). PAPS is reported to be stable to storage for months at temperatures below −20°C (Horowitz *et al.*, 1977; Sekura, 1981).



Scheme IV. Possible regeneration scheme for PAPS requires a multienzyme system. Sulphation of an alcohol (ROH) requires PAPS and generates PAP. The latter is hydrolysed to AMP, and AMP is then converted to ATP enzymatically using a phosphate donor (D~P) such as PEP. ATP is then converted to PAPS using ATP-sulphurylase, APS-kinase and pyrophosphatase

DIFFICULTIES OF REGENERATION OF PAPS

The critical role of PAPS in a wide variety of sulphation reactions, the small scale of the reported preparations of the cofactor, and its high cost, make PAPS an excellent candidate for regeneration. The possible need for multienzyme systems (*Scheme IV*; D~P = PEP or AcOP) is a major obstacle to the development of systems to regenerate PAPS. The unavailability of some of the enzymes required in this scheme (ATP-sulphurylase, APS-kinase and the sulphotransferases) represent another obstacle. These enzymes must be isolated, and many of the sulphotransferases have only begun to be studied.

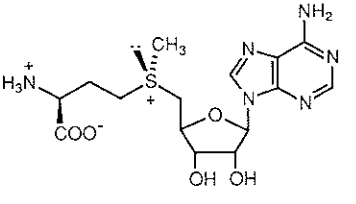
CONCLUSION

The availability of research quantities (~100 mg) of PAPS is not a significant problem, but a multi-gram synthesis of PAPS has not yet been demonstrated. Some of the enzymes needed for the synthesis, use and regeneration of PAPS have been isolated and studied, but only on a small scale. For the practical use of PAPS as a sulphate donor in organic synthesis to be possible, development of the current techniques (or invention of new ones) is necessary. The importance of sulphated compounds in biochemistry and medicine will direct effort to the solution of these problems in the next decade.

S-Adenosyl-L-methionine

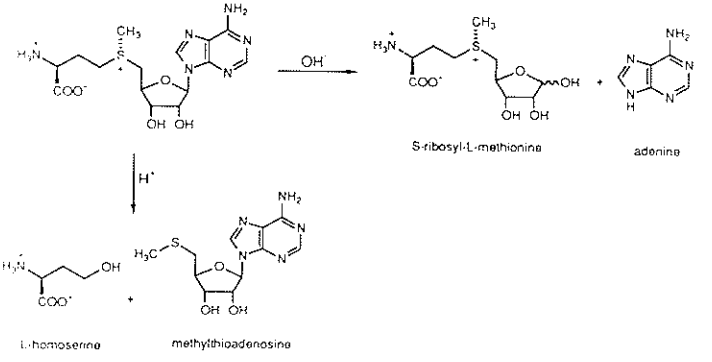
STRUCTURE AND FUNCTION

S-Adenosyl-L-methionine (SAM) is an activated alkylating agent that functions primarily in methylation of DNA, RNA, proteins, phospholipids, and a variety of small molecules (Salvatore *et al.*, 1977; Walsh, 1979b; Usdin, Borchardt and Creveling, 1982). SAM also operates in aminopropyl transfer for polyamine biosynthesis and in adenosyl transfer. It is the biosynthetic precursor to 1-aminocyclopropane-1-carboxylic acid. Enzymatically active SAM has the (*S*) configuration at both the amino acid and sulphonium centres (Cornforth *et al.*, 1977). The stereochemical requirement of the sulphonium centre, in particular, is a constraint on methods of synthesizing and regenerating SAM.



S-adenosyl-L-methionine

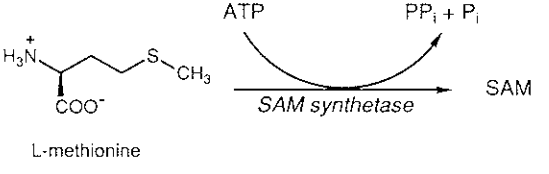
SAM is labile in aqueous solution (Parks and Schlenk, 1958; Schlenk, 1965; Borchardt, 1979). Alkaline conditions cleave the glycosidic bond to adenine, and acidic conditions hydrolyse the carbon–sulphur bond leading to the formation of homoserine and methylthioadenosine (*Scheme V*). The sulphonium centre racemizes at a rate competitive with alkaline hydrolysis at pH 7·5 (Wu *et al.*, 1983). Thus, any strategy for the economical use of SAM requires rapid turnover and stabilization of the cofactor.



Scheme V. Acid- and base-catalysed hydrolysis of SAM

PREPARATION OF *S*-ADENOSYLMETHIONINE

In vivo, SAM synthetase (ATP:L-methionine *S*-adenosyl transferase, EC 2.5.1.6) catalyses the formation of SAM from L-methionine and ATP (eq. 13).



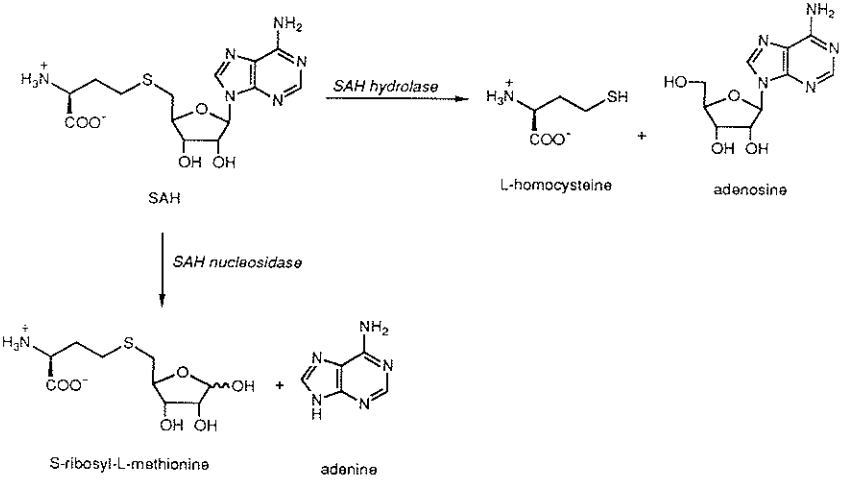
(13)

The adenylation of L-methionine is strongly inhibited by the product, SAM. Hence, enzymatic syntheses of SAM using SAM synthetase in whole cell or purified form suffer from product inhibition and require isolation of product from a dilute solution (Tanabe Seiyaku, 1980; Nippon Zeon, 1982; Gross, Geresh and Whitesides, 1983; Matos, Raushel and Wong, 1987). The best methods for preparing SAM require fermentation since SAM accumulates in high quantities in intracellular vacuoles of yeast grown in the presence of L-methionine (Shiozaki, Shimizu and Yamada, 1984, 1986). Chemically prepared *S*-adenosylhomocysteine (Ramalingam and Woodard, 1984) may be

methylated chemically (Borchardt and Wu, 1976), but this procedure forms both diastereomers of adenosyl-L-methionine.

REGENERATION OF *S*-ADENOSYLMETHIONINE

No attempt to regenerate SAM *in situ* has been reported. The product of transmethylation, *S*-adenosylhomocysteine (SAH), is not remethylated *in vivo* but is a powerful feedback inhibitor of most transmethylation reactions (Duerre and Walker, 1977). SAH is metabolized by SAH hydrolase (EC 3.3.1.1) to L-homocysteine and adenosine (*Scheme VI*). L-Homocysteine may then be utilized to re-form methionine and SAM. In bacteria, SAH nucleosidase (EC 3.2.2.9) metabolizes SAH to *S*-ribosyl-L-homocysteine and adenine.

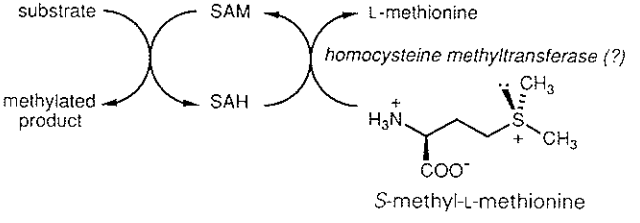


Scheme VI. *In vivo* metabolism of SAH

Two strategies, in principle, can regenerate SAM. Remethylation of SAH to form enzymatically active SAM is the most direct. This reaction might be accomplished by a diastereoselective chemical methylating agent or, more probably, by an enzymatic transmethylation. Alternatively, SAH could be broken down to the biosynthetic precursors of SAM and then built back up to SAM enzymatically.

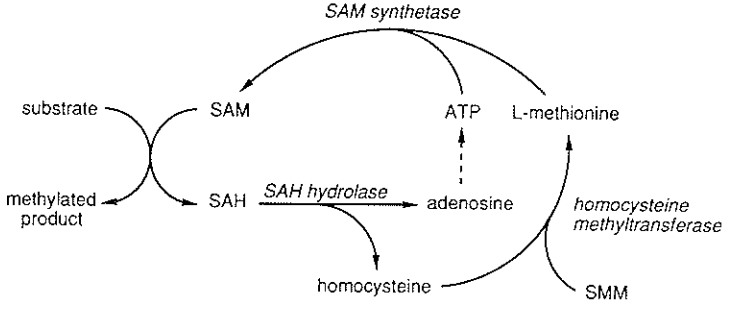
Regenerating SAM by an enzymatic transmethylation would use only a catalytic amount of SAH (which is far easier to prepare than SAM) and a high-energy methyl donor as cosubstrate. An obstacle to the transmethylation strategy is the fact that none of the SAM-dependent methylations have been demonstrated to be reversible. Another is that the relative transmethylation potentials of pertinent compounds are not known.

Even without data on transmethylation potentials, a probable methyl donor is *S*-methyl-L-methionine (SMM). SMM is a sulphonium compound like SAM and therefore should have a methyl-donor potential similar to that of SAM. It can be prepared conveniently by reacting L-methionine with methyl iodide (Toennies and Kollo, 1945). Transmethylation from SMM to SAH would afford SAM and L-methionine (*Scheme VII*). Extracts from jack bean seedlings and wheat germ are known to catalyse the reverse reaction (i.e. the methylation of methionine by SAM: Greene and Davis, 1960; Karr, Tweto and Albersheim, 1967), and reaction in the direction of SAM synthesis should be possible, especially if the SAM formed is consumed in a subsequent methylation step. Homocysteine methyltransferase from jack bean meal and other sources catalyses the methylation of homocysteine to form methionine and accepts either SAM or SMM as methyl donor (Shapiro, Yphantis and Almenas, 1964; Abrahamson and Shapiro, 1965; Dodd and Cossins, 1970). Perhaps this enzyme will also transfer a methyl group from SAM to methionine, and perhaps it is responsible for the activity observed with jack bean extracts. A disadvantage of SMM is that, like SAM, it is labile in solution.



Scheme VII. Possible regeneration scheme for SAM using *S*-methyl-L-methionine

Regenerating SAM along biosynthetic routes (*Scheme VIII*) would require several enzymes and considerably more complexity than a simple transmethylation. Particularly difficult is the regeneration of ATP from adenosine. While this strategy is probably impractical with purified enzymes, it may be possible with whole cells having enzymatic activities appropriately enhanced for the regeneration.



Scheme VIII. Possible multienzyme scheme for regenerating SAM

CONCLUSION

No attempt at regenerating SAM has been reported, but an attractive strategy is the enzymatic transmethylation of SAH, perhaps using SMM. Problems with this strategy that remain to be solved are questions of the reversibility of SAM-dependent methylations and the relative methylation potentials of enzyme substrates. The kinetic instability of SAM and other high-energy methyl donors is another problem.

Summary: accomplishments and prospects in cofactor regeneration

Much of the progress in regenerating cofactors for use in enzyme-catalysed organic synthesis has occurred since 1974, when the practical phosphorylation of AMP to form ATP was first demonstrated (Gardner *et al.*, 1974; Whitesides *et al.*, 1974; Whitesides, Siegel and Garrett, 1975). Progress has resulted as much from advances in chemistry as advances in enzymology. The demonstration that the enzymes formate dehydrogenase (Shaked and Whitesides, 1980; Tischer, Tiemeyer and Simon, 1980; Wichmann *et al.*, 1981) and glucose dehydrogenase (Wong, Drueckhammer and Sweers, 1985) were useful catalysts for regeneration was a critical advance for large-scale regeneration of the reduced nicotinamide cofactors. The practical chemical syntheses of acetyl phosphate (Crans and Whitesides, 1983; Crans *et al.*, 1988) and PEP (Hirschbein, Mazenod and Whitesides, 1982; Crans *et al.*, 1988), on the other hand, were the key developments making ATP regeneration possible.

Table 3. Utility of cofactor regeneration

Regeneration of A from B		Utility of current regeneration methods	Comments
NADH	NAD	+++	Formate/FDH is best method
NADPH	NADP	++	Glucose/GDH is best method
NAD(P)	NAD(P)H	++	Problems are product inhibition of enzymatic oxidations and rates and specificities of non-enzyme catalysed oxidations
ATP	ADP	+++	PEP and AcOP are the best P_i donors
ADP	AMP	++	Only an equilibrium mixture of ADP, ATP and AMP is easily available
(d)NTP	(d)NDP	+++	PK and AcK have wide specificities
(d)NDP	(d)NMP	+	AdK has low activity with some NMP; NMP kinases are expensive or unavailable
(d)NMP	(d)N	–	Enzymes are not available
AcCoA	CoA	+	AcCoA is expensive; demonstrated TTN are low
PAPS	PAP	–	No demonstrated methods
SAM	SAH	–	No demonstrated methods

Problems of regenerating NAD(P)H and ATP are largely solved (*Table 3*). Useful methods for regenerating NAD(P) exist, but have not been widely demonstrated. Strategies that obviate the mixed product inhibition often associated with biochemical oxidations need development. Regeneration of nucleoside diphosphates from the monophosphates is still a problem. Methods for regenerating acyl-CoA, PAPS, and SAM are in their infancy or are non-existent. Future research will address these unsolved problems as well as problems of enzyme availability and stability, reactor design, and application of known and newly discovered enzymatic reactions.

COFACTORS WITHOUT METHODS OF REGENERATION

In situ regeneration of acyl-CoA, PAPS, and SAM has suffered from several factors. Many of the systems for regenerating these cofactors require more than one enzyme and are inherently more complicated than the systems regenerating NAD(P)(H) and ATP. The enzymes required are expensive or not commercially available, relatively poorly characterized, and, in some cases, unstable. For regenerating PAPS and SAM, viable enzymatic routes have not even been determined. Impeding the regeneration of acyl-CoA is a lack of methods for preparing acyl phosphates and *O*-acylcarnitines. A problem in SAM regeneration is the general instability of SAM and of the high-energy methyl donors likely to be used for regeneration.

Another factor impeding progress in the regeneration of these cofactors is a perceived lack of utility for such regenerations. Future work will be stimulated by the need for these cofactors in complex synthesis and biochemical processing. Acyl-CoA will form acyl-and acylaminoglycosides and products of asymmetric aldol reactions. PAPS will form heparin sulphate and other sulphated polysaccharides. SAM will facilitate *in vitro* processing of DNA, RNA, and proteins.

ENZYME AVAILABILITY AND STABILITY

As mentioned previously, many enzymes for regeneration are expensive, not commercially available, poorly characterized, or unstable. For example, nucleoside and deoxynucleoside monophosphate kinases required to convert hydrolysates of RNA and DNA to nucleoside triphosphates are expensive or are not commercially available. Future work using classical screening techniques will identify useful sources for the large-scale isolation of enzymes, and protein engineering will provide enzymes having desired kinetic characteristics, substrate specificities, and stabilities.

REACTOR DESIGN

Key objectives in the area of reactor design are improvements of reactor productivity, reactor stability, and separation of products. Various immobilization techniques for the stabilization of enzymes exist, but the most practical methods have not yet been determined. An advance over stirred-tank and

column reactors (which use enzymes immobilized on solid supports) is membrane-bound and hollow-fibre reactors (which use soluble enzymes). Membrane-based reactors have the advantages of simplicity and high productivity. They allow the separation of products from reactions in progress and therefore can drive thermodynamically unfavourable reactions. Development and use of immobilized cofactors will be important for the practical application of membrane systems to cofactor-requiring reactions.

Advances in separation technology will include developments in filtration, chromatography, and differential precipitation. Other advances will avoid the separation process altogether. Intermediates, such as glycosyl nucleoside phosphates, will probably be generated *in situ* and used in further reactions rather than being isolated. The use of coupled reactions may also drive the overall equilibria of processes to favourable formation of product.

NEW APPLICATIONS OF ENZYMATIC REACTIONS

The development and use of methods for *in situ* cofactor regeneration will increase as new preparative processes utilize enzymatic reactions. Many non-cofactor-requiring enzymes (e.g. lipases, esterases, amidases, and aldolases) are already incorporated into the armamentarium of synthetic organic chemists (Whitesides and Wong, 1983, 1985; Jones, 1986). They offer particularly useful routes to chiral synthons and to amides, lipids, and sugars. The desire to accomplish more complex biochemical transformations (i.e. syntheses of oligosaccharides, lipids, polypeptides and nucleic acids) will necessitate cofactor-requiring enzymes and, with them, methods for cofactor regeneration. New uses for known enzymes will develop, and the desire for new types of reactions will stimulate the discovery of new enzymes.

Acknowledgements

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