Enzymes in Organic Synthesis

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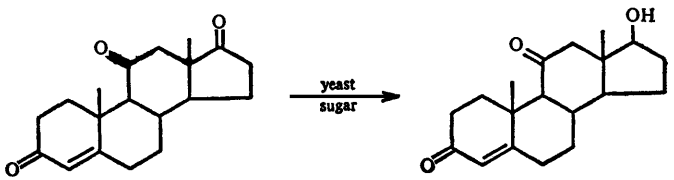
1 Introduction

Why should a chemist consider using an enzyme as a catalyst in synthesis when there exists already a vast number of chemical reagents? The answer is simply that there are several synthetic operations which enzymes can carry out better than conventional reagents. Enzymes are the proteins which catalyse reactions in living cells and they have evolved to cope with specific tasks in biosynthetic or metabolic reactions. There are enzymes known which will catalyse most of the common transformations of organic synthesis such as oxidation–reduction, hydrolysis, and condensation reactions. Not only are the enzymes specific with respect to the reaction that they catalyse, they are also specific in their position of attack on a molecule. Further, many enzymes show stereospecificity in their reactions in addition to regiospecificity and all operate under very mild conditions, usually dilute aqueous solution at less than 50 °C. In order to profit from these advantages however, certain difficulties must be overcome. The practical difficulties include stability and solubility problems to which we shall return shortly, but there is a conceptual difficulty in accepting enzymes into the usual body of synthetic reagents. One source of this latter difficulty is that to date most synthetic work using enzymes has been carried out by chemists or biochemists studying biosynthesis or the mechanism of enzyme catalysis and their work is disguised from the sight of synthetic chemists. Also there has been much effort spent in studying microbiologically mediated reactions particularly in industry, often with products such as antibiotics in mind. Now it is possible to perceive some generally useful techniques using enzymes or micro-organism cultures which can be applied to synthesis. The most obvious fields of application are in natural product chemistry since the natural products are themselves biosynthesized by enzymes. In this review, we attempt to show what advantages enzymes have in a wider synthetic context and to suggest some further avenues for exploration.

2 The Strategy of Synthesis using Enzymes

The major value of enzymes as catalysts in synthesis is their selectivity and accordingly the most successful strategies of synthesis will exploit selectivity to

the full, *e.g.*, non-enzymically it would not be possible to differentiate between the carbonyl groups in steroid (1) but yeast fermenting on sugar has been shown



(1) 62%

to reduce only the 17-keto function and the product alcohol was isolable in 62% yield.[1] It is in this context of selectively carrying out single steps in a synthesis that enzymes should be applied. There is no reason to expect them to be able to provide the final magical key step in a synthesis any more than a conventional reagent because enzymes are no more than macromolecular catalysts, much larger molecules than normal synthetic reagents.

Although enzymes catalyse particular reactions and often in nature will accept only specific substrates, it has been generally found that enzymes will transform many compounds provided that certain structural similarities with the natural substrate are conserved. Further research is widening the range of 'foreign' substrates and the mere fact that no enzyme-catalysed reactions of the molecule in question have been described should not discourage the chemist from attempting an enzymic reaction provided that the molecule is an analogue of a known substrate of the enzyme. Exactly how close the analogy should be will become clearer as we proceed with our discussion of examples.

In most synthetic applications one enzyme is used to catalyse a single chemical step. This contrasts with the biological situation where organized enzyme systems catalyse multiple transformations. The organization of biological multienzyme systems is difficult to imitate *in vitro* for two main reasons. Firstly the natural systems frequently have the enzymes ordered in space in relation to each other. This allows efficient transfer of substrate from one enzyme to another. Secondly, the rate at which each enzyme can operate and its affinity for substrate are matched so that the sequence can operate in a controlled manner.

Nevertheless there are several examples which show that these difficulties can be overcome in synthesis. Perhaps the most outstanding is Khorana's synthesis of a gene.[2] He used chemically synthesized polynucleotides as substrate and joined these together, using the nucleotide polymerase which had already been isolated by molecular biologists, to give a complete gene.

[1] H. L. Herzog, M. A. Jevnik, P. L. Perlman, A. Nobile, and L. B. Hershberg, *J. Amer. Chem. Soc.*, 1953, **75**, 266.

[2] K. L. Agarwal, H. Büchi, M. H. Caruthers, N. Gupta, H. G. Khorana, K. Kleppe, A. Kumar, E. Ohtsuka, V. L. Rahjbandary, J. H. van der Sande, V. Sgaramella, H. Weber, and T. Yamada, *Nature*, 1970, **227**, 27.

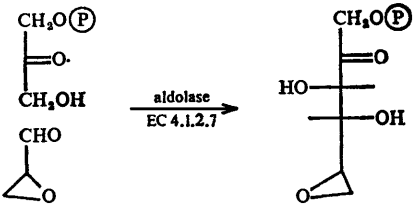
Smaller molecules too may be synthesized by an enzyme system. Cornforth and Eggerer have described a multi-stage one-pot synthesis using enzymes in their work on chiral acetic acid.[3] These 'enzyme cocktails', as Cornforth has called them, take advantage of the fact that it is unusual for isolated enzymes to inhibit each other's catalytic activity whereas the requisite chemical reagents may react with each other rather than with the substrate. The sequence of reactions was the activation of acetic acid *via* its phosphate to the coenzyme-A ester which was condensed with glyoxylate to yield *S*-malic acid. One enzyme catalysed each step of the sequence (Scheme 1).

$$MeCO_2H + ATP \xrightarrow{i} MeCO{\cdot}OPO_3H \xrightarrow{ii} MeCO{\cdot}SCoA$$

Reagents: i, Acetate kinase (EC 2.7.2.1); ii, Phosphotransacetylase, CoASH.

Scheme 1

It is not always necessary to use the natural substrate of an enzyme. Aldolase (EC 4.1.2.7)* specifically requires dihydroxyacetone phosphate as one substrate but is less discriminating with regard to the aldehyde component of the condensation. Rose[4] has taken advantage of this to prepare a number of hexose epoxides from dihydroxyacetone phosphate and a variety of aldehydes (Scheme 2). The products were used as inhibitors in a mechanistic study of sugar isomerases.



Scheme 2

The more common applications of enzymes fall into two groups. Firstly, the enzyme may be used to introduce an important structural element into an intermediate, such as a chiral centre. Many syntheses have followed this strategy

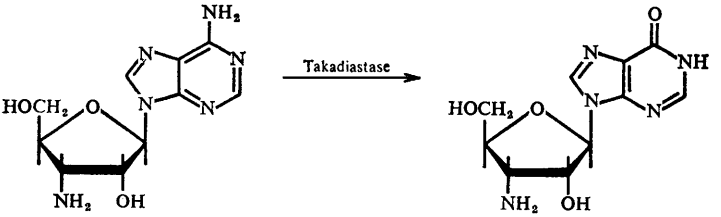
* The EC (Enzyme Commission) number is the official systematic classification of enzymes by the International Union of Biochemistry. Enzymes are grouped together according to the reactions that they catalyse. Thus for aldolase, 4 shows that it is a lyase, 1 that it cleaves C—C bonds, 2 that aldehydes are formed and 7 that it is the seventh of this class listed. The numbers are important because they provide a reliable correlation between the many trivial names and trade names that have been given to enzymes. We shall quote EC numbers wherever they are known and shall distinguish applications of purified enzymes from those of crude preparations.

[3] J. W. Cornforth, J. W. Redmond, H. Eggerer, W. Buckel, and C. Gutschow, *Nature*, 1969, **221**, 1212.

[4] I. A. Rose, E. L. O'Connell, and K. J. Schray, *J. Biol. Chem.*, 1973, **248**, 2214.

especially if the chiral centre is to contain a stereospecifically inserted isotopic label. Thus in his studies of hydroxylation reactions in alkaloid biosynthesis, Battersby enzymically prepared chiral labelled benzyl alcohols which were then converted through a sequence of chemical steps into the required precursor (Scheme 3).[5] The stereospecificity of enzymes may be applied in the synthesis of building blocks of defined chirality: it is here that the application of enzymes to synthesis has greatest potential.

The second typical use of enzymes is to make small selective modifications to a substrate, such as the cleavage of a protecting group, or the selective reduction of a carbonyl group, or the selective oxidation of an amino function. Thus a crude commercial enzyme preparation, 'Takadiastase', catalyses the oxidation of only the aromatic amino group in 3′-amino-3′-deoxy-adenosine (2) through



(2) 75%

an adenosine deaminase enzyme (EC 3.5.4.2).[6] There are many examples of unusual regioselectivity in enzyme-catalysed reactions of non-natural substrates, and from the point of view of synthesis some systematic investigation of specificity would be desirable. However, the regioselectivity of enzymes does offer the opportunity to work without time-consuming protection and deprotection of reactive functional groups.

Since enzymes are catalysts, all enzyme-mediated reactions are in principle reversible. Thus many oxidizing enzymes will also catalyse reduction, many hydrolytic enzymes will catalyse ester formation, and aldolase, formally classified as a lyase, can as we have seen be used to couple substrates. It is therefore important to choose substrates and conditions such that the equilibrium is shifted in the direction required. The usual chemical methods of displacing the position of equilibrium are applicable provided that the enzyme is stable to these conditions.

At present, enzymes are not cheap and should not be used wastefully. However it is often inevitable that during the work-up of a reaction, the enzyme will be destroyed. Recently methods have been developed for the preparation of water-insoluble enzyme derivatives which can be reisolated after reaction and used as

[5] A. R. Battersby, J. E. Kelsey, J. Staunton, and K. E. Suckling, *J.C.S. Perkin I*, 1973, 1609.
[6] N. N. Gerber, *J. Med. Chem.*, 1964, 7, 204.

many times as required. Another expense which may arise is the requirement of many enzymes for a coenzyme. Coenzymes are relatively small molecules which combine with the enzyme or substrate to form the catalytically active species. For example, coenzyme-A is a complex thiol whose esters are active in enzyme-catalysed condensation reactions (Scheme 1). The free carboxylic acids are inactive. It would be an advantage if these relatively expensive coenzymes could be replaced by simpler analogues. Lynen[7] has shown that the relatively simple thiol *N*-acetylcysteamine can act enzymically as a substitute for coenzyme-A, but such tolerance is rare and enzymes usually show greater specificity towards their coenzymes than towards their substrate. It is often possible, however, to use only catalytic quantities of both enzyme and coenzyme and so lessen the cost.

3 Practical Aspects of Enzymes as Synthetic Catalysts

A. The Chemical Nature of Enzymes.—In order for an enzyme to be active, the polypeptide chain must be coiled and folded in such a way that certain reactive amino-acid sidechains are brought together to form what is known as the catalytically active site. This site usually takes the form of a groove or a cleft in the essentially globular overall structure of the protein and near it or associated with it is a similar site to which the substrate binds by electrostatic or hydrophobic forces. It is the detailed geometric arrangement of the side-chains of amino-acids composing the peptide chain at these sites that is responsible not only for the catalysis but also, through the binding sites, for the selectivity of the enzyme-catalysed reactions. The three-dimensional structure, of course, is the origin of the stereospecificity. As would be expected with molecules of molecular weights of up to a million, such structures as the reactive arrangement or conformation, known as tertiary structures, have limited stability. With most enzymes, the range of stability reflects the fact that they have evolved to function in a predominantly aqueous environment at around 37 °C. Accordingly, it is necessary to handle enzymes under similarly mild or milder conditions so that activity may be maintained. In general, extremes of heat, high concentrations of some salts or urea, and high concentrations of organic solvents must be avoided. More detailed discussion of these aspects of enzyme and protein chemistry can be found in a number of textbooks.[8] Fortunately as we shall see, it is usually possible to get round the problems which the solubility and stability of enzymes pose.

B. Sources and Availability of Enzymes.—Common criteria for choosing a reagent are its availability, its stability to storage, and, of course, its suitability for the reaction in question. It is a tedious and difficult job for chemists to isolate enzymes in a pure state but fortunately the range of commercially purified

[7] L. Jaenicke and F. Lynen, in 'The Enzymes', ed. P. D. Boyer, H. Lardy, and K. Myrback, Academic Press, London and New York 2nd Edn., 1960, vol. 3, p. 3.
[8] M. Dixon and E. C. Webb, 'The Enzymes', 2nd Edn., Longmans, London, 1964.

enzymes is rapidly widening. Many of these preparations require only mild refrigeration to guarantee months of service. Sometimes, however, it is not necessary to work with a highly purified enzyme: crude preparations obtained simply by breaking open the cells of the source organism often possess the required activity and the chemist should not feel afraid to prepare and apply such systems as he requires. Common sources of crude preparations are yeasts and other micro-organisms. Oxidations carried out by micro-organisms have been studied very widely and this field has been thoroughly reviewed in both strategic and practical aspects elsewhere.[9] The practical advice offered by Fonken and Johnson[9] applies to all reactions using micro-organisms, and their book, written with chemists in mind, has value even outside its immediate remit of oxidations. Accordingly, we shall make only passing reference to such reactions and concern ourselves chiefly with laboratory synthesis using purified enzymes or readily available crude preparations from plant, animal, or micro-organism sources.

C. Solvents.—In order to catalyse a reaction, an enzyme must be present in its reactive conformation which, for many reasons is usually adopted automatically in dilute aqueous solution. Such a medium is a poor solvent for many organic compounds and it is fortunate that many enzymes will tolerate significant quantities of organic solvents mixed with water; *e.g.*, the stereospecific reduction of benzaldehydes to benzyl alcohols used by Battersby was carried out in a 5% organic solvent–phosphate buffer medium with high dilution (0.25 g substrate l^{-1}) and excellent yields were obtained given sufficient time.[5] Similarly, acetone has been used to solubilize cholesterol in a crude rat liver preparation.[10] In general, the use of small quantities of organic solvents presents few problems.

Special cases are known in which reactions are run in predominantly organic media or in two-phase systems.[11] Usually these involve lipases (EC 3.1.1.3) which have long-chain fatty acid glycerides as their natural substrates. Not only do these enzymes hydrolyse esters, but they also demonstrate the reversibility of enzyme-catalysed reactions by synthesizing esters, provided that water is present at a concentration of less than 5%.[12] Interestingly, it is easier to synthesize tertiary esters of carboxylic acids than primary. Solvents as apolar as hexane have been used in such work.

D. Insolubilized and Immobilized Enzyme Preparations.—It would be an economy if the catalytic enzyme could be reused in many runs of a reaction and it has been found that enzymes can very readily be attached to polymer matrices by chemical or physical means, or be enclosed within a membrane whose pores are

[9] G. S. Fonken and R. A. Johnson, 'Chemical Oxidations with Microorganisms', Marcel Dekker, New York, 1972.
[10] M. G. Kienle, R. K. Varma, L. J. Mulheirn, B. Yagen, and E. Caspi, *J. Amer. Chem. Soc.*, 1973, **95**, 1996.
[11] G. V. Nevgi and C. Ramakrishnan, *J. Indian Chem. Soc.*, 1950, **27**, 260; 1951, **28**, 271, 333, 471.
[12] N. N. Li, R. R. Mohan, and D. R. Brusca, G.P. 2 222 067 (*Chem. Abs.*, 1973, **78**, 70 225m).

too large to allow the enzyme to pass through. In either case, the enzyme is trapped within a water-insoluble material and such preparations are referred to as insolubilized enzymes. If the enzyme is attached to a polymer in some way, the enzyme can also be said to be immobilized. However it is possible to enclose enzymes within microcapsules of polymer beads yielding a mobile, insoluble preparation. There is much interest in the preparation of immobilized enzymes for use as 'enzyme reactors' in industrial processes.[13] A comprehensive compilation of immobilized enzymes has recently been published,[14] and this book also details many of the available techniques that are outlined below.

Covalent attachment of enzymes to synthetic polymers has been achieved for a number of enzymes using methods based upon fibre reactive dye technology involving substitution products of cyanuric chloride.[15] The majority of enzymes studied catalyse hydrolyses, *e.g.*, chymotrypsin (EC 3.4.4.5), β-galactosidase (EC 3.2.1.23) and penicillin amidase (EC 3.5.1.11), but the oxido-reductase lactate dehydrogenase (EC 1.1.1.27) was also successfully immobilized. A conjugate of β-galactosidase and cellulose has been shown to retain its catalytic activity even after 3 years' storage.[16] Other techniques which have been applied on a large scale include cyanogen bromide-activated sepharose, the workhorse of affinity chromatography,[17] or ethylene–maleic anhydride copolymers.[18] It is usual to employ polar polymers for immobilization (cellulose, sepharose, polyacrylamides) rather than hydrophobic polymers (polystyrenes) since the latter would surround the enzyme with an unfavourable environment akin to an organic solvent.

Physical methods of immobilization include the adsorption of enzymes upon porous glass beads, but the stability of such materials to desorption is limited.[19] More successful has been microencapsulation of enzymes within semi-permeable polymeric membranes to yield insolubilized preparations. Conditions suitable for carrying out polymerizations in the presence of enzyme have been described;[20] the enzyme is trapped within the capsule during polymerization. Alternatively, enzymes can be entrained within a cross-linked polyacrylamide gel lattice. Unfortunately acrylamide, which is required for the polymerization, denatures some enzymes and so limits the applicability of this method.[21] Recently, a novel variation of dialysis has been used to prepare 'enzyme reactors'. Rony has adsorbed enzymes within hollow semi-permeable fibres themselves enclosed within a beaker to prevent damage. By placing a solution of substrate in the beaker, high conversions were obtained in both hydrolytic and oxidation reac-

[13] M. D. Lilly and P. Dunnil, *Process Biochem.*, 1971, **6**, 39.
[14] O. R. Zaborsky, 'Immobilised Enzymes', CRC Press, Cleveland, 1973.
[15] M. D. Lilly, G. Kay, R. J. H. Wilson, and J. H. Sharp, B.P. 1 183 260 (*Chem. Abs.*, 1970, **72**, 118 062m); G. Kay and M. D. Lilly, *Biochim. Biophys. Acta*, 1970, **198**, 276.
[16] M. D. Lilly, *Biotechnol. and Bioeng.*, 1971, **13**, 589.
[17] H. Guildford, *Chem. Soc. Rev.*, 1973, **2**, 249.
[18] Ref. 14, p. 14.
[19] Ref. 14, p. 76.
[20] T. M. S. Chang, F. C. McIntosh, and S. G. Mason, *Canad. J. Physiol. Pharmacol.*, 1966, **44**, 115; Ref. 14, p. 93.
[21] Ref. 14, p. 84; Y. Degani and T. Miron, *Biochim. Biophys. Acta*, 1970, **212**, 362.

tions.[22] The beakers used are commercially available and the system is exceptionally convenient to operate. The enzyme can be removed from the fibres by flushing with a concentrated buffer solution.

Most of the enzymes that have been insolubilized are hydrolytic enzymes but the techniques developed seem nonetheless general and applicable to all classes of enzyme. Insolubilized enzyme preparations have the values of reusability and convenience, both important in synthesis, but there are two new difficulties which the physical nature of the enzymes in their new environment creates. Firstly, enzymes enclosed within semi-permeable pockets must be accessible to their substrates. Thus the porosity of the membrane controls what reactions are possible, like the mitochondrial membrane in a cell. This physical selectivity could be turned to advantage as an addition to the chemical selectivity of the enzymes themselves. Secondly, many enzymes, especially redox enzymes, require the presence of coenzymes to function. The coenzyme is often a much larger molecule than the substrate and may find difficulty in reaching the active site through the membrane, and so it becomes the expensive item. There is a need, therefore, to find methods of conserving this component or indeed of replacing it by a cheaper, simpler analogue. As a step in this direction, the coenzyme NAD has been immobilized on sepharose and this preparation had a reusable activity of 0.2% of the free natural coenzyme.[23]

E. Substrate Modification.—A third possible technique for bringing enzyme and substrate together into the same phase is to modify the substrate to make it water-soluble. This could be achieved by coupling a hydrophilic side-chain such as an amino-acid to the substrate. After reaction, the side-chain could be cleaved chemically or enzymically, just like a protecting group. In the latter case, isolation of the product before cleavage would be unnecessary because the hydrolytic enzyme could be added directly to the reaction mixture itself and would selectively remove the solubilizing group. Such applications of 'enzyme cocktails' do not seem to have been studied.

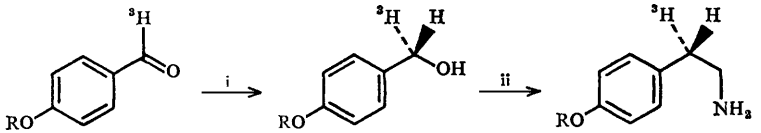
4 Examples of the Synthetic Applications of Enzymes

Having discussed in general terms the properties of enzymes when considered as chemical reagents, some reactions may now be examined in which the advantages that enzymes have to offer have been exploited. The reactions are classified following the order chosen by the Enzyme Commission but as we hinted earlier, exploitation has been uneven. Redox (EC1) and hydrolytic enzymes (EC3) have been most extensively studied whereas transferases (EC2), lyases (EC4), isomerases (EC5), and ligases (EC6) have received relatively little attention as synthetic catalysts. Accordingly it is mainly examples of the first

[22] P. R. Rony, *J. Amer. Chem. Soc.*, 1972, **94**, 8247.
[23] P. O. Larsson and K. Mosbach, *Biotechnol. and Bioeng.*, 1971, **13**, 393; M. K. Weibel, H. H. Weetall, and H. J. Bright, *Biochem. Biophys., Res. Comm.*, 1971, **44**, 347.

group that are cited; but suggestions are made of ways in which enzymes of the second group may prove of value.*

A. Enzymes Catalysing Redox Reactions.—*Redox at Carbonyl Functions.* The chief value of dehydrogenase enzymes in synthesis is their stereospecificity. Of the wide range of conventional reagents available for oxidation and reduction of carbonyl groups, only one, a complex borane, is the equal of the alcohol dehydrogenases in stereospecificity and then only in cyclic systems.[25] There are many alcohol dehydrogenases and often they are very selective (steroid dehydrogenases,[26] malate dehydrogenase and many others) but both yeast and liver alcohol dehydrogenases (EC 1.1.1.1) have wide tolerance for non-natural substrates including aliphatic, alicyclic, and aromatic aldehydes and ketones, and heterocyclic aldehydes.[27] Many of these substrates also contain other functional groups which would react with a borane unless they were protected, and protection is not necessary when using an 'enzymic reagent'. Thus an aldehyde bearing a tritium or deuterium label can be stereospecifically reduced to the corresponding alcohol (Scheme 3),[5] and the body of evidence, at least for the



R = CH_2Ph

Reagents: i, Liver alcohol dehydrogenase (EC 1.1.1.1), NADH; ii, two steps.

Scheme 3

liver enzyme, suggests that the configuration of the product will usually be *S*. (However the enzyme should not be relied upon to produce the same stereospecific result with widely different substrates and the configuration, if it is of importance, should be independently checked.) In these reactions, the expensive coenzyme NADH, the biological equivalent of sodium borohydride, is required. However it need only be used in catalytic amounts; either the enzyme itself can regenerate the coenzyme by oxidizing ethanol present in the reaction mixture (a typical example of the coupling of reactions using enzymes),[28] or flavins can

* General references describing the known specificity of enzymes can be found in Dixon and Webb's comprehensive book[8] and further more detailed discussions of properties of individual enzymes can be found in the appropriate volume of '*Methods in Enzymology*'[24] or 'The Enzymes'.[24]

[24] *Methods in Enzymology*, Vols. 1—28, Academic Press, London and New York; 'The Enzymes', ed. P. D. Boyer, 3rd Edn., Academic Press, London and New York, 1967—74, vols. 1—8.

[25] H. C. Brown and S. Krishnamurthy, *J. Amer. Chem. Soc.*, 1972, 94, 7159.

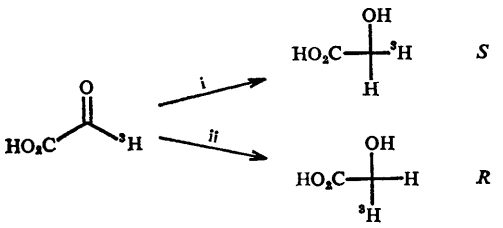
[26] H. U. Bergmeyer, 'Methods of Enzymatic Analysis', Verlag Chemie, Weinheim, 1965, p. 485.

[27] R. Bentley, 'Molecular Asymmetry in Biology', Academic Press, New York, 1970, Vol. 2, p. 164.

[28] H. R. Levy, F. H. Loewus, and B. Vennesland, *J. Amer. Chem. Soc.*, 1957, 79, 2949.

be added which will reduce the oxidized coenzyme NAD, a pyridinium salt, to NADH, a 1,4-dihydropyridine.[29] In cases where a large quantity of material is to be reduced, it may be possible to use actively fermenting bakers' yeast as the reducing agent.[30] Deuteriated 1-formyl-adamantane is a large molecule that has been reduced stereospecifically by this method.[31]

Chiral labelled molecules such as these were prepared with stereochemical mechanistic studies in mind and for rigour in such work it is necessary to prepare both enantiomers. An enzyme is specific for one enantiomer so the optical antipode must be obtained by another method, *e.g.* the product of enzyme reduction could be inverted chemically,[5] the coenzyme could be labelled instead of the substrate, or an enzyme of opposite stereospecificity could be used. Arigoni has used the third approach in his synthesis of chiral acetic acid which began with the enzyme-catalysed reduction of tritiated glyoxylic acid (Scheme 4).[32] Lactate



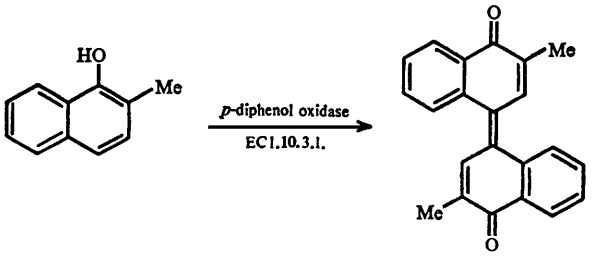
Reagents: i, Lactate dehydrogenase (EC 1.1.1.27); ii, Glyoxylate reductase (EC 1.10.3.1).

Scheme 4

dehydrogenase (EC 1.1.1.27) and glyoxylate reductase (EC 1.1.1.26) led respectively to the *S* and *R* enantiomers of glycollic acid and these were subsequently converted into acetic acid chemically. Perhaps the most important stereospecific reduction of this kind has been used to prepare chiral isotopically labelled mevalonic acids for biosynthetic studies of terpenes and steroids.[33]

Not only can enzymes reduce aliphatic aldehydes and ketones to alcohols but the same alcohol dehydrogenases can also oxidize alcohols to carbonyl compounds. There are also enzymes, the phenol oxidases (EC 1.10.3.1 and 2), that will oxidize phenols or aminophenols to the corresponding *ortho-* or *para*-quinones.[34] Monophenols too are substrates and in this case unusual transformations have been observed. 2-Methyl-1-naphthol on oxidation with *p*-diphenol oxidase yielded the bis-naphthoquinone (3).[35] Presumably this product is

[29] J. B. Jones and K. E. Taylor, *J.C.S. Chem. Comm.*, 1973, 205.
[30] P. Levene and A. Walti, 'Organic Syntheses, Collected Volumes', Vol. 2, 545, 1943.
[31] P. R. von Schleyer, E. Funke, and S. H. Liggero, *J. Amer. Chem. Soc.*, 1969, **91**, 3965.
[32] D. Arigoni, J. Luthy, and J. Retey, *Nature*, 1969, **221**, 1213.
[33] J. W. Cornforth, *Quart. Rev.*, 1969, **23**, 125; C. Donninger and G. Popjak, *Proc. Roy. Soc.*, 1966, **B163**, 465; P. Blattmann and J. Retey, *J.C.S. Chem. Comm.*, 1970, 1394.
[34] A. C. Maehly, *Methods Enzymol.*, 1955, **2**, 801.
[35] B. R. Brown and A. H. Todd, *J. Chem. Soc.*, 1963, 5564.



(3)

63%

the result of dimerization of an intermediate *p*-phenolate radical generated by the enzyme as it attempts to oxidize the foreign substrate to a quinone. This result suggests a novel approach to the use of enzymes in synthesis. If the mechanism of catalysis by the enzyme in question is known or if its action upon its natural substrate can reasonably be inferred on chemical grounds, as in this case, then it may be possible to use the enzyme to generate a suitable reactive intermediate which may then be trapped to yield the desired product. The behaviour of many hydrolytic enzymes as transferring reagents for esters and glycosides can be considered in the same way (see section 4C).

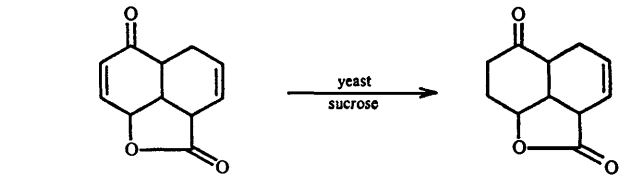
Hydrogenations and Dehydrogenations of C–C Bonds. Hydrogenations of C–C double bonds have been effected most commonly with crude enzyme preparations rather than with purified materials and most published work refers to steroids. It is difficult to generalize where attack in the steroid molecule will occur but there seems to be a preference for attack at ring A when an $\alpha\beta$-unsaturated system can be generated.[9] The preference for reaction at $\alpha\beta$-unsaturated sites extends to non-steroidal substrates too[36] and both results lead one to suggest that a carbonyl group coplanar with the $\alpha\beta$-plane is required for binding to the enzyme. If reduction is to be accomplished, a terminal reducing agent must be added to the reaction mixture; a reducing sugar such as sucrose is usually employed (*cf.* ref. 30). Thus in twenty-four hours in aqueous ethanol, a 67% yield of the saturated ketone (4) can be obtained from the $\alpha\beta$-unsaturated precursor.[36] For oxidation, on the other hand, a terminal electron acceptor must be present and for most aerobic organisms, oxygen itself is most convenient [*e.g.*, the formation of (5)].[37] An unusual case shows the use of artificial co-enzymes in synthesis; 2-methylnaphthoquinone was used as an oxidizing agent

[36] M. Protiva, A. Capek, J. O. Tilek, B. Kakac, and M. Tadra, *Coll. Czech. Chem. Comm.*, 1961, **26**, 1537.

[37] R. W. Thoma, J. Fried, S. Bonanno, and P. Grabowich, *J. Amer. Chem. Soc.*, 1957, **79**, 4818.

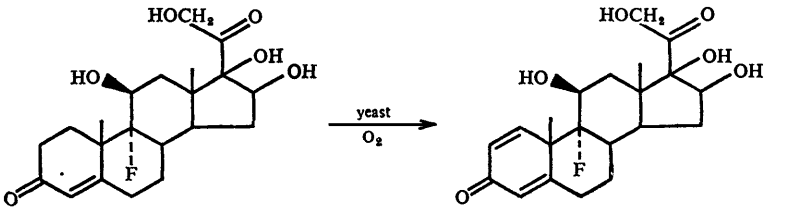
with a crude cell-free preparation to obtain a high yield of the ring A $\alpha\beta$-unsaturated ketone (6).[38]

For non-cyclic molecules, a range of carboxylic acid dehydrogenases is known and several have been shown to act stereospecifically.[39] They offer further routes to the synthesis of chiral labelled molecules.



(4)

67%



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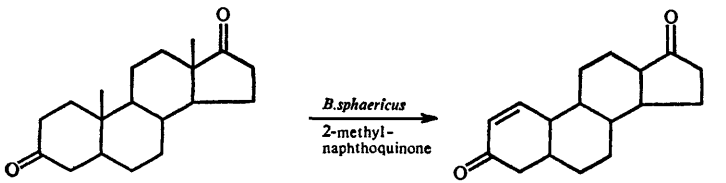


(6)

80%

Hydroxylation and Related Oxidations. Of particular interest to the synthetic chemist are oxidation reactions at chemically non-activated positions in molecules. A large number of such reactions catalysed by enzymes are known, but it is hard to find useful correlations of reactivity although the oxidizing species

[38] H. J. Ringold, M. Gut, H. Hayano, and A. Turner, *Tetrahedron Letters*, 1962, 835.
[39] J. Retey, J. Seibl, D. Arigoni, J. W. Cornforth, G. Ryback, W. P. Zeylemaker, and C. Veeger, *European J. Biochem.*, 1970, **14**, 232.

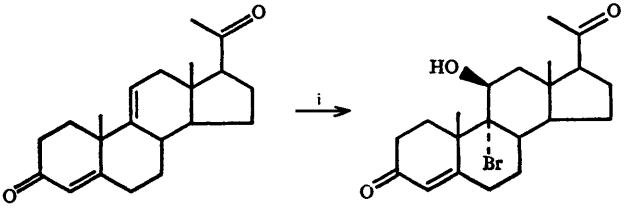
has electrophilic character. Fonken and Johnson have given exhaustive discussions of the state of current knowledge.[9,40]

It is usually difficult to oxidize electron-deficient heterocyclic compounds but enzymes have developed useful reactivities. Xanthine oxidase (EC 1.2.3.2) catalyses the oxidation of many heterocyclic compounds not closely related to xanthine, its natural substrate, and it has been described as omnivorous.[41] It is available in crystalline form and has also been immobilized. Aldehyde oxidase (EC 1.2.3.1) is a closely related enzyme and the two have similar characteristics, chief amongst which is the great substrate range which they will accept. Purines and pteridines are oxidized to the corresponding hydroxy-compounds normally at the electron-deficient positions.[42,43] Similarly, *N*-methyl nicotinamide can be oxidized to the 6-oxo derivative.[44] A further interesting reaction of xanthine oxidase is its ability to reduce the *N*-oxides of pyridines and purines, and presumably other tertiary amines, to the corresponding amines.[45] This reaction requires anaerobic conditions and the presence of an electron donor such as sodium dithionite. Xanthine oxidase is not restricted to aqueous solution; it has been shown to oxidize crotonaldehyde in a variety of organic solvents at rates less than a tenth of the rate in homogenous aqueous reaction.[46] The success of this reaction opens the way to a more extensive application of xanthine oxidase to synthesis. It is able to overcome the physical problems of reacting with organic compounds in non-aqueous media and chemically has a wide spectrum of reactivity. In general the most electron-deficient centres in a molecule are attacked most rapidly by the nucleophilic oxidizing agent generated by the enzyme.

Neidlemann[47] has discovered some unusual halogenations catalysed by the enzyme chloroperoxidase (Scheme 5). Electron-rich molecules are halogenated by the enzyme in the presence of hydrogen peroxide and an alkali metal halide. The enzyme seems to protect functionalities in ring A of the steroid series, but, with these exceptions, the electrophilic halogenating agent generated by the enzyme attacks the most electron-rich positions either by substitution [in the thiazole (7)] or by addition [in the steroid, (8)]. The isolated yields of product are reported to be over 50%. A systematic study of the regiospecificity of such reactions and extensions to the range of substrates halogenated would be valuable but the essential reactivity of the enzyme is already clear.

[40] G. S. Fonken and R. A. Johnson, *loc. cit.* ref. 9, p. 58, 130.
[41] H. R. Mahler and E. H. Cordes, 'Biological Chemistry', Harper and Row, New York, 1971, p. 653.
[42] B. L. Horecker and L. A. Heppel, *Methods Enzymol.*, 1955, **2**, 483.
[43] D. M. Valerino and J. J. McCormack, *Biochim. Biophys. Acta*, 1969, **184**, 154.
[44] G. Greenlee and P. Handler, *J. Biol. Chem.*, 1964, **239**, 1090.
[45] K. N. Murray and S. Chaykin, *J. Biol. Chem.*, 1966, **241**, 3468; G. Stoehrer and G. B. Brown, *J. Biol. Chem.*, 1969, **244**, 2498.
[46] F. R. Dastoli and S. Price, *Arch. Biochem. Biophys.*, 1967, **118**, 163.
[47] S. L. Neidlemann, P. A. Diaszi, B. Junta, R. M. Palmere, and S. C. Pan, *Tetrahedron Letters*, 1966, 5337; S. L. Neidlemann and M. A. Oberc, *J. Bacteriol.*, 1968, **95**, 2424; S. L. Neidlemann, A. I. Cohen, and L. Dean, *Biotechnol. and Bioeng.*, 1969, **11**, 1227.

(7)



(8)

Reagent: i, chloroperoxidase, KBr, H_2O_2.

Scheme 5

Oxidation of Amino Functions. In contrast to the heterocyclic compounds described above, the presence of acyclic heteroatoms in a molecule often has the effect of increasing that molecule's susceptibility to oxidation. Controlled selective methods of oxidation are therefore in demand in synthesis and enzymes offer some new approaches with amino-compounds as substrates. The amine and amino-acid oxidases oxidize the amino-group to a carbonyl group. Amines thus become latent aldehydes or ketones. For example, the amino-acid oxidases are specific for either the D or the L enantiomer and oxidize their substrates to keto-acids. L-Leucine is oxidized to the corresponding keto-acid by L-amino-acid oxidase (EC 1.4.3.3), and catalase (EC 1.11.1.6) can be used to prevent further oxidation by 'mopping up' the hydrogen peroxide produced as a by-product.[48]

Like xanthine oxidase, the amine oxidases, both monoamine oxidase (EC 1.4.3.4)[49] and diamine oxidase (EC 1.4.3.6),[50] have broad specificities. Studies of substrate specificity[51] suggest the presence of both a polar and an apolar binding site on the enzyme which are reflected by high rates of oxidation of polymethylene alkylamines and *p*-substituted phenyl methylamines. Enzymic oxidation of amines is thought to occur *via* a nucleophilic addition of the amine to a carbonyl-like group of coenzyme (either a flavin or pyridoxal phosphate)

[48] A. Meister, *Biochemical Preparations*, 1953, 3, 66; D. Wellner, *Methods Enzymol.*, 1971, **17b**, 587; M. Nakano and T. S. Danovsky, *ibid.*, p. 601; K. Yagi, *ibid.*, p. 608.

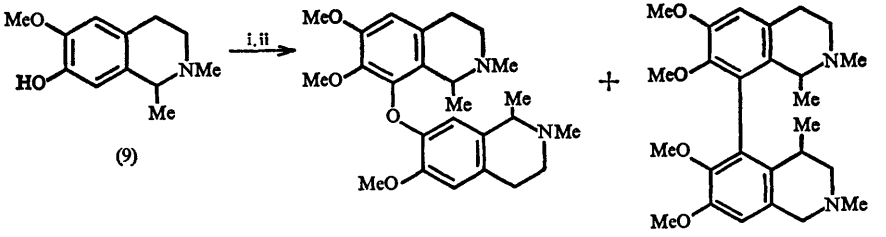
[49] E. Buffoni and H. Blaschko, *Methods Enzymol.*, 1971, **17b**, 682; C. M. McEwen, *ibid.*, pp. 686, 692; H. Yamada and O. Adachi, *ibid.*, p. 705; K. T. Yasunobu and B. Gomes, *ibid.*, p. 709; K. F. Tipton, *ibid.*, p. 717.

[50] J. M. Hill, *Methods Enzymol.*, 1971, **17b**, 730; B. Mondori, G. Rotilio, M. T. Costa, and A. F. Agro, *ibid.*, p. 735.

[51] V. Z. Gorkin, N. A. Kitrosskii, L. B. Klyashtonin, N. V. Komissarova, G. A. Leont'eva, and V. A. Puchov, *Biokhimiya*, 1964, **29**, 88; J. M. Hill and P. J. G. Mann, *Biochem. J.*, 1964, **91**, 171; C. M. McEwen, jun., and A. J. Sober, *J. Biol. Chem.*, 1967, **242**, 3068.

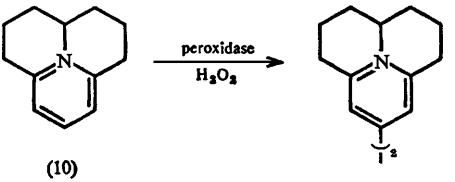
and this is consistent with the observations that *o*-hydroxyphenylmethylamines are not oxidized by monoamine oxidase because intramolecular hydrogen bonding protonates the amine.

Oxidative Coupling Using Enzymes. It might have been hoped that biogenetic-type syntheses of alkaloids based upon oxidative coupling would be more successful using enzymes as one-electron oxidizing agents than they are with simple transition-metal salts. Unfortunately, it seems that neither the yield of coupled products nor the selectivity which can be obtained is improved by using radical-generating enzymes such as peroxidase (EC 1.11.1.7). Inubushi, for example, has found that the simple *N*-methyltetrahydroisoquinoline (9) (Scheme 6) gives a mixture of dimers in low yield upon oxidation with peroxidase and



Reagents: i, peroxidase, H_2O_2; ii, CH_2N_2.
Scheme 6

hydrogen peroxide.[52] It would be interesting to attempt reactions of this type using one of the phenol oxidases which act directly upon the substrate without requiring the intermediacy of hydroxyl radicals. However peroxidase is capable of generating radicals in many molecules and, if reactivity is limited by the structure of the substrate as it is in (10), then preparative yields can be obtained.[53]



[52] Y. Inubushi, Y. Aoyagi, and M. Matsuo, *Tetrahedron Letters*, 1969, 2363; T. Kametani, S. Takano, and T. Kobari, *J. Chem. Soc.* (*C*), 1969, 9.
[53] V. R. Holland and B. C. Saunders, *Tetrahedron*, 1971, **27**, 2851.

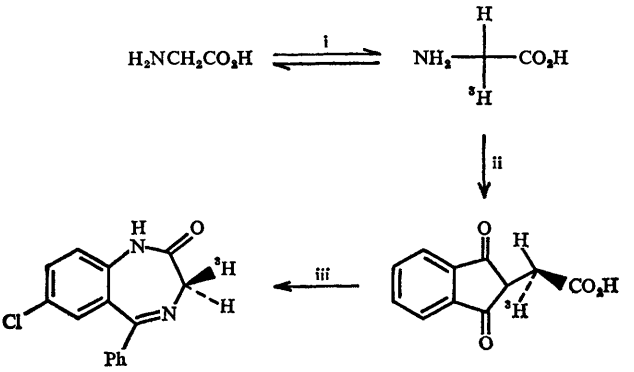
B. Enzymes Catalysing Group Transfer Reactions.—Synthetically, not only the enzymes classed as transferases (EC2) but also a number of hydrolytic enzymes (EC3) can usefully catalyse group transfer reactions. The classification refers to the biological function. With the exception of the nucleotidyl transferases,[2] few transferases have been applied to synthesis. This reflects the narrow range of substrates that are known for most transferases. However studies of the nicotinamide coenzymes and their reactivity have been greatly aided by the ability of a ribosyl transferase (EC 2.4.2.12) to catalyse the interchange of a substantial variety of pyridines as aglycones. In this way, a series of coenzyme analogues has been prepared and the structural requirements for biological activity of pyridine nucleotides has been established.[54]

One of the few transferases which shows a wide specificity is arylamine transferase (EC 2.3.1.5). In nature this enzyme utilizes the coenzyme-A esters of carboxylic acids and condenses them with aromatic amines to form amides. Fortunately the coenzyme-A esters, which are expensive to prepare, do not appear to be essential and amides have been prepared from a wide variety of aromatic amines including aniline, *p*-aminobenzoic acid, *p*-nitroaniline, and *o*-phenylenediamine.[55] However, so wide is the range of chemical methods available for the preparation of amides that it seems unlikely that this enzyme has much to offer to synthesis. The same can be said of catechol *O*-methyl transferase (EC 2.1.1.6) which methylates a wide range of phenols.[56] Once again, an expensive coenzyme, in this case *S*-adenosyl methionine, is required as source of the methyl groups.

The range of chiral isotopically labelled compounds that can be prepared has been valuably extended by alanine aminotransferase (EC 2.6.1.2). Arigoni[57] has shown that only the *pro-R* hydrogen of glycine is exchanged by this enzyme in the presence of the coenzyme pyridoxal phosphate. The coenzyme is a small and inexpensive molecule and it is required only in catalytic amounts to enable substantial quantities of chiral labelled glycine to be obtained. The building block thus produced has been transformed into the tranquillizer diazepam (11) (Scheme 7) and the fate of the label has been examined after the drug had been metabolized by the host. The enzymic hydroxylation that occurs during metabolism was thereby shown to be stereospecific.[58]

C. Enzymes Catalysing Hydrolysis Reactions.—As we pointed out above, these enzymes not only catalyse the hydrolysis of glycosides, esters, and amides but can also be made to catalyse transesterification and transglycosidation. Chemically, all that is required to differentiate between hydrolysis and synthesis is to balance correctly the leaving group and the nucleophile. Thus a good leaving group such as *p*-nitrophenol present in the parent ester or glycoside increases the

[54] B. M. Anderson, C. J. Ciotti, and N. O. Kaplan, *J. Biol. Chem.*, 1959, **234**, 1219.
[55] H. Tabor, A. H. Mehler, and E. R. Stadtman, *J. Biol. Chem.*, 1953, **204**, 127.
[56] J. Axelrod, *Methods Enzymol.*, 1962, **5**, 748.
[57] P. Besmer, Dissertation No. 4435, ETH Zurich, 1970.
[58] A. Corbella, P. Cariboldi, J. Jommi, A. Forgione, F. Marucci, P. Martelli, E. Mussini, and F. Mauri, *J.C.S., Chem. Comm.*, 1973, 721.



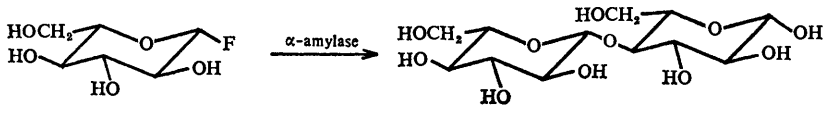
(11)

Reagents: i, L-alanine aminotransferase, pyridoxal phosphate; ii, *N*-ethoxycarbonylphthalimide; iii, three chemical steps.

Scheme 7

probability that a transfer reaction will occur. Many hydrolases are known to form covalent intermediates with their substrates, *e.g.*, chymotrypsin is acylated on a serine hydroxy-group. This intermediate can be attacked by water, and so hydrolysed, or by another nucleophile in which case transfer of the acyl group occurs. Glycoside-hydrolysing enzymes are thought to react *via* a stabilized oxonium ion derived from the sugar which also can react with a nucleophile or with water. There are many examples of such transfer reactions.

Acid phosphatase (EC 3.1.3.2) will transfer phosphate from aryl esters to aliphatic alcohols in 58—80% yield even in the presence of a seventy-fold molar excess of water. Presumably the binding of the alcohol to the active site of the enzyme more than compensates for the excess of water in the bulk solvent.[59] Similarly fluoroglucosides, *e.g.* (12), can serve as donors of the sugar moiety



(12)

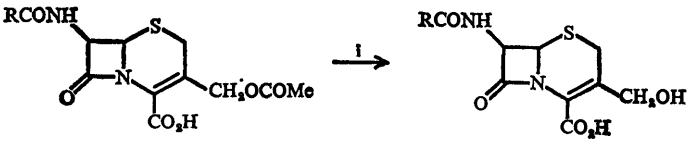
using one of a number of enzymes for example α-amylase[60] (EC 3.2.1.1). Here another glucose molecule is the acceptor and a maltose is formed. β-Galactosides have been particularly well studied and ^{14}C labelled glycosides can be prepared

[59] B. Axelrod, *Adv. Enzymol.*, 1956, **17**, 159; V. N. Nigam and W. H. Fishman, *J. Biol. Chem.*, 1959, **234**, 2394.

[60] E. J. Hehre, D. S. Genghof, and G. Okada, *Arch. Biochem. Biophys.*, 1971, **142**, 382.

from free unactivated galactose by use of β-galactosidase (EC 3.2.1.23).[61] Acetyl cholinesterase (EC 3.1.1.7) which in nature catalyses the hydrolysis of acetyl choline, a molecule with a positively charged leaving group, can be used to carry out transacetylations. No other transesterifications have been found using this enzyme.[62] We have already referred to the ability of lipases to synthesize esters of carboxylic acids.[11,12]

Hydrolytic enzymes in their usual function have three possible applications to synthesis. Firstly, they can provide extremely mild methods of removing protecting groups, both amides and esters, *e.g.*, an esterase from orange peel will selectively remove acetate esters from a range of substrates including the sensitive β-lactam antibiotics (Scheme 8).[63] Acetyl cholinesterase may be used



Reagents: i, orange peel enzyme.

Scheme 8

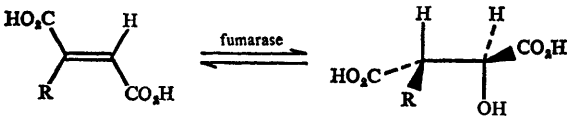
similarly and is selective for acetates. Thus the possibility of distinguishing between an acetate and a butyrate, say, in the same molecule is opened up. This is the second contribution of hydrolases to synthesis: the broadening of the methods available for selective protection when enzymes are used for deprotection. This may be illustrated by the peptidases trypsin (EC 3.4.4.1) and chymotrypsin (EC 3.4.4.5). These enzymes and their relatives compose the most thoroughly studied group of enzymes and their specificities, as reflected by the rates of hydrolysis of a range of substrates, have been widely investigated.[64] Both trypsin and chymotrypsin will hydrolyse esters but have maximal efficiencies for amides formed from the carboxy-group of an L-amino-acid, respectively an amino-acid with a basic side-chain and a hydrophobic side-chain. The possibilities of selective protection are obvious. In this particular case there is a useful check to see if the proposed synthesis has a good chance of success. The *X*-ray structures of both trypsin and chymotrypsin have been determined[65] and hence the active sites of the enzyme have been located. The geometry of the active sites is also known as is the relative position of the adjacent binding sites which control specificity. One can therefore check, preferably using molecular models, whether

[61] K. Wallenfels and R. Weil, in 'The Enzymes', ed. P. D. Boyer, 3rd Edn., Academic Press, London and New York, Vol. 7, 1973, p. 658; K. Wallenfels and O. P. Malhotra, *Adv. Carbohydrate Chem.*, 1961, **16**, 239.
[62] W. Leutzinger, *Methods Enzymol.*, 1971, **17b**, 782.
[63] J. d'A. Jeffery, E. P. Abraham, and G. G. F. Newton, *Biochem. J.*, 1961, **81**, 591.
[64] A. Williams, *Quart. Rev.*, 1969, **23**, 1.
[65] D. M. Blow, J. J. Birktoft, and B. S. Hartley, *Nature*, 1969, **221**, 337.

the proposed substrate will fit with the binding and activity constraints imposed by the enzyme's structure.

The third application of enzyme-catalysed hydrolysis to synthesis arises from the three-dimensional and chiral tertiary structures of enzymes. Peptidases and glycosidases show strict stereochemical selectivity and it is thus practicable to use them as resolving agents for α-amino-acids[66] and for glycosides.[67] The commercial availability of both the crystalline enzymes and some insolubilized derivatives adds to the attraction of these applications.

D. Condensation and Addition Reactions Catalysed by Enzymes.—Enzymes classified as lyases (EC4) are chiefly of interest here since isomerases (EC5) have apparently little synthetic potential and ligases (EC6) have narrow or unexplored specificities. Once again, the synthesis of chiral building blocks is easily achieved by lyases. For example, fumarase (EC 4.2.1.2) will accept a variety of fumaric acid derivatives as substrates and will catalyse their stereospecific hydration to malic acids (see below).[68] This is an interesting reaction because fumarase was at one time thought to be very specific with regard to its substrates. Further research has shown that a large number of αβ-unsaturated dicarboxylic acids including halofumaric acids and acetylenedicarboxylic acid can be hydrated. In the case of the olefins, the *trans*-addition of water to the double bond always results in the formation of the *S* configuration at the carbon atom bearing the hydroxy-group. Aconitase (EC 4.2.1.3) is a similar hydrating enzyme which will accept a number of foreign substrates.[69] There are probably many useful reactions hidden behind the unexplored specificities of enzymes which would be useful in synthesis.



R = Cl, Br, Me, H

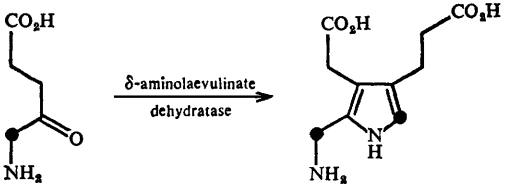
threo S

An unusual stereospecific addition reaction that is catalysed by an enzyme is the addition of hydrogen cyanide to aldehydes by an un-numbered enzyme, D-hydroxy-nitrilolyase.[70] This enzyme, which can tolerate 50% concentrations of ethanol in aqueous buffer solutions, will react with aliphatic, aromatic, and heterocyclic aldehydes and the resulting nitriles have been hydrolysed to optically

[66] J. P. Greenstein, *Methods Enzymol.*, 1957, **3**, 554.
[67] Ref. 27, p. 110.
[68] R. L. Hill and J. W. Teipel, in 'The Enzymes', ed. P. D. Boyer, 3rd Edn., Academic Press, London and New York, 1971, Vol. 5, p. 556.
[69] O. Gawron and K. P. Mahajan, *Biochemistry*, 1966, **5**, 2343.
[70] W. Becker, H. Freund, and E. Pfeil, *Angew. Chem.*, 1965, **77**, 1139; W. Becker and E. Pfeil, *Biochem. Z.*, 1966, **346**. 301.

active α-hydroxy-acids. In the case of benzaldehyde a 90% optically pure product was obtained in 96% yield.

The lyases are an exceptional group of enzymes in that it has proved possible to use some of them to catalyse the synthesis of whole target molecules rather than building blocks. Rose's use of aldolase to prepare sugar epoxides was described in the introduction.[4] Recently Battersby has described another example. He used the enzyme δ-aminolaevulinate dehydratase (EC 4.2.1.24)[71] to prepare ^{13}C labelled samples of porphobilinogen (13) in high yield. This highly reactive



(13)

● = ^{13}C

pyrrole is notoriously difficult to synthesize chemically and the expense involved in ^{13}C labelling made an alternative route essential. The product was converted enzymically into porphyrins and the incorporation of the label allowed a distinction to be made between the many hypothetical mechanisms that had been proposed for porphyrin biosynthesis.

5 Conclusion

From the discussion of examples it is clear that the use of enzymes in organic synthesis is in its infancy. However there are many reasons for feeling that the child is healthy and likely to develop well. Undoubtedly further biochemical research will uncover new possibilities and meanwhile the ever widening range of enzyme preparations which can be bought extends the scope for large scale use. Most important for chemists is the fact that the same chemical reasoning that is used to consider the reactivities of conventional reagents is also applicable to 'enzymic reagents' as we have tried to show. Indeed were this not the case, then the extant range of examples from which we have drawn but a few would have been much smaller. It is to be hoped that potentially useful and generally applicable results from the use of enzymes in synthesis will not remain tucked away in the inaccessible corners of the chemical literature but will be discussed openly for the good of enzymology and the good of synthesis.

[71] A. R. Battersby, E. Hunt, and E. McDonald, *J.C.S. Chem. Comm.*, 1973, 442.