formed from pyruvate, fumarate, lactate or acrylate had relatively low specific activity. This is taken as evidence that the metabolism of propionate involves carbon dioxide fixation to form succinate.

4. There was negligible labelling of a succinate pool when labelled propionate was metabolized.

5. Fumarate and malate were produced from succinate by rumen epithelium.

6. Propionate suppressed ketone-body formation from pyruvate and lowered the total amount of pyruvate metabolized.

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A Comparison of the Action of Penicillinase on Benzylpenicillin and Cephalosporin N and the Competitive Inhibition of Penicillinase by Cephalosporin C

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A species of *Cephalosporium* (I.M.I. 49137) has been found to produce two hydrophilic antibiotics, named cephalosporin N and cephalosporin C (Abraham, Newton & Hale, 1954; Newton & Abraham, 1955). Cephalosporin N, a monoaminodicarboxylic acid which is highly sensitive to penicillinase and yields penicillamine (β -thiolvaline) and D- α -aminoadipic acid on hydrolysis, is probably

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(D-4-amino-4-carboxy-n-butyl)penicillin (Newton & Abraham, 1954). It appears to be identical with synnematin B (Abraham *et al.* 1955). Cephalosporin C is a monoaminodicarboxylic acid which is similar to cephalosporin N in chemical composition and also yields D- α -aminoadipic acid on hydrolysis. It resembles the penicillins in some of its properties but differs from them strikingly in others. For example, it is not sensitive to penicillinase and does not give β -thiolvaline on hydrolysis, but it does

yield valine when hydrolysis is preceded by hydro-genolysis with Raney nickel (Abraham & Newton, 1956).

The discovery that cephalosporin C was a penicillin-like substance raised the question whether it would prove to be a competitive inhibitor of the enzyme penicillinase. Preliminary experiments, in which benzylpenicillin was estimated by anti-bacterial assay, showed that cephalosporin C could protect the penicillin from enzymic inactivation. The present paper gives the results of manometric experiments which were undertaken to obtain more precise information about this phenomenon and to compare the action of penicillinase on benzylpenicillin and cephalosporin N.

METHODS

Materials. The benzylpenicillin used was a crystalline sodium salt (Glaxo Laboratories Ltd.). Phenoxymethylpenicillin was obtained as the free acid from The Distillers Co. (Biochemicals) Ltd. and was converted into the sodium salt. The cephalosporin N was a sodium salt prepared by the method of Abraham *et al.* (1954), which was about 50% pure and did not contain more than 3% of cephalosporin C. The molar concentrations of solutions of cephalosporin N used in manometric experiments were estimated from the total CO_2 evolved in the presence of NaHCO_3 and penicillinase, assuming a molecular weight of 381 for the pure sodium salt (Newton & Abraham, 1954). Cephalosporin C was used as a crystalline sodium salt prepared by the method of Newton & Abraham (1956). The amorphous sodium salt of the benzoyl derivative of cephalosporin C was prepared as described by Abraham & Newton (1956). The preparation of synnematin B was a gift from Parke Davis and Co. The penicillinase, for which we are greatly indebted to Dr M. R. Pollock, was a highly purified preparation of the penicillin-induced enzyme from *Bacillus cereus* NRRL 569 (Kogut, Pollock & Tridgell, 1956). All dilutions of the stock enzyme solution were made in 0.3% (w/v) aqueous gelatin (Harrington Brothers Ltd.). When diluted in the absence of gelatin the enzyme lost a variable amount of activity (cf. Pollock, Torriani & Tridgell, 1956).

Reaction velocities. (a) *Enzymic hydrolysis.* The manometric method of Henry & Housewright (1947) and Pollock (1950) for measuring the activity of penicillinase was used for determining the velocities of enzymic reactions at pH 7.0. The main compartment of each Warburg vessel contained 1.6 ml. of enzyme solution and 0.5 ml. of 0.043 M- NaHCO_3 . The side bulb contained a solution of the substrate (1.63–8.7 μmoles), or a mixture of the substrate and inhibitor, in a mixture of 0.4 ml. of water and 0.1 ml. of 0.043 M- NaHCO_3 . The vessels were gassed with $\text{CO}_2 + \text{N}_2$ (5:95, v/v). The first readings were taken 3 min. after tipping the substrate into the enzyme, when a steady state appeared to have been reached. The majority of the measurements were made at 30°. In duplicate experiments with substrate alone the measured rates of evolution of CO_2 seldom differed by more than 1%. The concentration of enzyme was such that 8.7 μmoles of benzylpenicillin were almost completely hydrolysed in about 30 min. Under these conditions no hydrolysis of cephalosporin C could be detected.

(b) *Alkaline hydrolysis.* The non-enzymic inactivation of benzylpenicillin in alkaline solution was also studied. In this case the reaction was followed by measuring changes in optical rotation. Sodium benzylpenicillin has a strong positive rotation which is more than twice as great as that of the sodium salt of D- α -benzylpenicilloic acid (Mozingo & Folkers, 1949). A solution of sodium benzylpenicillin in water (7.1 mg./ml.; 0.02 M) was added to an equal volume of 0.032 N- NaOH , the temperature of both solutions having previously been brought to that at which the reaction was to be carried out. Optical rotations were measured in a 1 dm. jacketed polarimeter tube at temperatures from 8° to 25°. The initial rotation (α_0) was +1.05°. A difficulty was encountered in the determination of the precise value to be assigned to the final rotation (α_∞). A gradual downward drift in rotation continued after the primary bimolecular reaction should have virtually reached its end, presumably because D- α -benzylpenicilloic acid underwent slow mutarotation in alkaline solution. An arbitrary value was therefore assigned to α_∞ (0.48°) which represented the rotation when the reaction at 25° had proceeded for 90 min. and 1 equiv. of alkali (determined by titration) had been consumed. The subsequent rate of change of rotation was less than 0.02°/hr. It was therefore reasonable to assume that the secondary reaction did not exert a significant influence on the measurements of the primary hydrolysis until the latter approached the end of its course. The concentration of product at time t was assumed to be $b(\alpha_0 - \alpha_t)/(\alpha_0 - \alpha_\infty)$, where b was the initial concentration of penicillin.

In plotting results the best straight lines were fitted by the method of least squares.

RESULTS

Comparison of the hydrolysis of benzylpenicillin and cephalosporin N catalysed by penicillinase

The results of a typical experiment showing the relative rates of hydrolysis at 30° of benzylpenicillin, phenoxymethylpenicillin and cephalosporin N in the presence of penicillinase from *Bacillus cereus* NRRL 569 are plotted in Fig. 1. In each case the rate of reaction was constant over most of its course. The reaction was therefore of pseudo zero order and the enzyme was apparently saturated with its substrate. There was no significant change in the reaction rate following a two- or four-fold reduction in the concentration of benzylpenicillin or cephalosporin N. This confirmed the finding of Manson, Pollock & Tridgell (1954) that the Michaelis constant of the benzylpenicillin–penicillinase system was too small to measure by the manometric technique.

The values obtained for the relative rates of hydrolysis (V_{max}) of benzylpenicillin, phenoxymethylpenicillin, cephalosporin N and synnematin B are shown in Table 1. The value of V_{max} for phenoxymethylpenicillin was significantly greater than that for benzylpenicillin. The value of V_{max} for cephalosporin N was not distinguishable from the corresponding value for synnematin B. Cephalosporin C underwent no measurable hydrolysis under the conditions used.

The relative values of the Michaelis constants for the benzylpenicillin–penicillinase and cephalosporin N–penicillinase systems were estimated from the rates of hydrolysis of each substrate alone and of mixtures of the two. If each reaction is described by the conventional Michaelis equation, then

where E represents the enzyme, S_1 and S_2 the two substrates and P_1 and P_2 the two products. Suppose that the total amount of enzyme present is always E_0 and that each substrate is present in excess. The

Fig. 1. Rates of hydrolysis of different substrates, at 30°, in the presence of the same concentration of penicillinase. ●, Benzylpenicillin (3.35 mM); ○, phenoxymethylpenicillin (3.30 mM); □, cephalosporin N, (3.30 mM); ▲, cephalosporin C (2.40 mM).

Table 1. Relative values of V_{max} at 30° for the hydrolysis of different penicillins catalysed by penicillinase from *Bacillus cereus* NRRL 569.

All values are relative to that for benzylpenicillin. The value for cephalosporin N is the mean of five determinations. Values for phenoxymethylpenicillin and synnematin B are the means of duplicate determinations.

Substrate	V_{max}
Benzylpenicillin	1.00
Phenoxymethylpenicillin	1.25 ± 0.03
Cephalosporin N	0.54 ± 0.01
Synnematin B	0.54 ± 0.01

rates of reaction with substrates S_1 and S_2 alone will be

$$V_{1, max} = k_3[ES_1] = k_3[E_0], \quad (3)$$

$$V_{2, max} = k'_3[ES_2] = k'_3[E_0]. \quad (4)$$

When the two substrates are present together, the total rate of reaction at a given time will be represented by

$$V_t, max = k_3[ES_1] + k'_3[ES_2], \quad (5)$$

where $[ES_1]$ and $[ES_2]$ are the concentrations of the two enzyme–substrate complexes at the time concerned. Since both substrates are present in high concentration and are competing for the same enzyme

$$ES_1 + ES_2 = E_0 \quad (6)$$

$$\text{and } [ES_1]/[ES_2] = [S_1] K_{m,2}/[S_2] K_{m,1}, \quad (7)$$

where the Michaelis constants, $K_{m,1}$ and $K_{m,2}$, are equal to $(k_2 + k_3)/k_1$ and $(k'_2 + k'_3)/k'_1$ respectively.

Combination of equations (5), (6) and (7) gives

$$V_t, max = k'_3[E_0] + \frac{(k_3 - k'_3)[E_0]}{1 + (K_{m,1}[S_2]/K_{m,2}[S_1])}. \quad (8)$$

Combination of equations (3), (4) and (8) and rearrangement gives

$$\frac{1}{V_t, max - V_{2, max}} = \frac{1}{V_{1, max} - V_{2, max}} \left[1 + \frac{K_{m,1}[S_2]}{K_{m,2}[S_1]} \right]. \quad (9)$$

In the special case where $[S_1] = [S_2]$, equation (9) reduces to an equation proposed by Whittaker & Adams (1949).

A plot of $1/(V_t, max - V_{2, max})$ against $[S_2]/[S_1]$ should give a straight line with a slope

$$K_{m,1}/K_{m,2}(V_{1, max} - V_{2, max})$$

and an intercept

$$1/(V_{1, max} - V_{2, max}).$$

In Fig. 2 the results of an experiment with benzylpenicillin (S_1) and cephalosporin N (S_2) are plotted in this way. The rates of hydrolysis of benzylpenicillin alone (3.35 mM) and cephalosporin N alone (3.03 mM) and the initial rates of hydrolysis of mixtures of the two compounds in which the total concentration was between 3.03 and 3.35 mM were measured (in arbitrary units) in the presence of a standard solution of penicillinase. The slope of the line (Fig. 2) corresponded to a value of $1.0 + 0.05$ for the quotient $K_{m,2}/K_{m,1}$.

The energies of activation (E) of the enzyme-catalysed hydrolysis of benzylpenicillin and of cephalosporin N at pH 7.0 were calculated from the values obtained for V_{max} at various temperatures (25–35°) in the presence of a standard concentration of penicillinase. In Fig. 3, $\log_{10} V_{max}$ for each substrate has been plotted (in arbitrary units) against $1/T$. According to the Arrhenius equation the slopes of the resulting straight lines are $-E/2.303R$. The slopes obtained with benzylpenicillin

and cephalosporin N corresponded to energies of activation of 5.3 ± 0.3 and 5.7 ± 0.4 kcal.mole⁻¹ respectively.

Alkaline hydrolysis of benzylpenicillin

The hydrolysis of benzylpenicillin in dilute sodium hydroxide solution, like that catalysed by penicillinase, leads to the formation of D- α -benzylpenicilloic acid. The hydrolysis followed the course of a second-order reaction, since straight lines were

Fig. 2. Variation in the initial rate of enzymic hydrolysis (V_i) of mixtures of benzylpenicillin (S_1) and cephalosporin N (S_2) with changes in the initial molar ratio of the substrates. $V_{2, \max}$ represents the rate of hydrolysis of cephalosporin N alone.

Fig. 3. Plot of the logarithm of velocity constants against the reciprocal of the absolute temperature. $\circ$, Enzymic hydrolysis of benzylpenicillin; $\bullet$, enzymic hydrolysis of cephalosporin N; $\square$, alkaline hydrolysis of benzylpenicillin. The velocity constants of the reactions are in arbitrary units.

Fig. 4. Alkaline hydrolysis of benzylpenicillin, as measured by changes in optical rotation.

obtained when $\log_{10} a(b-x)/b(a-x)$ was plotted against time, a being the initial concentration of sodium hydroxide, b the initial concentration of benzylpenicillin and x the concentration of product at t minutes (Fig. 4). Values of k_{OH-} at temperatures from 8° to 25° were obtained from the slopes of the lines, by using the expression

$$k_{OH-} = \frac{2.303}{t(b-a)} \log_{10} \left[\frac{a(b-x)}{b(a-x)} \right].$$

At 25°, k_{OH-} was 5.0 l. mole⁻¹ min.⁻¹. The plot of $\log_{10} k_{OH-}$ against $1/T$ is shown in Fig. 3, k_{OH-} being measured in arbitrary units. The slope of the line corresponds to an energy of activation of

$$14.5 \pm 0.5 \text{ kcal. mole}^{-1}.$$

Competitive inhibition of penicillinase by cephalosporin C

In the presence of cephalosporin C the rate of the penicillinase-catalysed hydrolysis of benzylpenicillin was reduced, and the reaction in the presence of a high concentration of substrate was no longer of zero order over most of its course. Fig. 5 shows the rates of hydrolysis of different concentrations of benzylpenicillin (3.35–0.84 mM) in the presence of a single concentration of cephalosporin C (8.1 mM). Similar results were obtained when a different concentration of cephalosporin C (4.05 mM) was used, when benzylpenicillin was replaced by cephalosporin N, or when cephalosporin C was replaced by its benzoyl derivative.

The results of different experiments have been brought together in Fig. 6, the expression of Hunter & Downs (1945) being used for the relationship between the substrate concentration $[S]$, the inhibitor concentration $[I]$, and α (reaction velocity in the presence of inhibitor/reaction velocity in the

absence of inhibitor). The initial reaction velocities in the presence of inhibitor (Fig. 4) were used in calculating values of α . With benzylpenicillin and cephalosporin C the plot of $[I]\alpha/(1-\alpha)$ against $[S]$ gave a straight line whose slope corresponded to a value of 2.9 ± 0.05 for K_i/K_m , K_i being the dissociation constant of the enzyme-inhibitor complex and K_m the Michaelis constant of the enzyme-substrate system. The intercept of the line corresponded to a value for K_i which was too small to measure accurately, but which appeared to be less than 3×10^{-4} M. With cephalosporin N the corresponding

value for K_i/K_m was not significantly different from that obtained with benzylpenicillin.

In a single experiment with benzylpenicillin as substrate, the benzoyl derivative of cephalosporin C behaved as a more powerful inhibitor of penicillinase than cephalosporin C itself, the value of K_i/K_m being approximately 1.0. However, since the purity of the amorphous benzoyl derivative is uncertain, this value can only be regarded as provisional.

DISCUSSION

In the presence of penicillinase from *B. cereus* NRRL 569, benzylpenicillin, phenoxymethylpenicillin and cephalosporin N showed a similarity in behaviour which was consistent with the presence of the same β -lactam-thiazolidine ring system in all three compounds. The finding that the value of V_{max} for cephalosporin N was indistinguishable from that for synnematin B gave support to earlier evidence that these two compounds are identical (Abraham *et al.* 1955). Phenoxymethylpenicillin is considerably more stable than other penicillins in dilute acid (Brandl & Margreiter, 1954), and in this property it shows some resemblance to cephalosporin C. In the presence of penicillinase, however, phenoxymethylpenicillin and cephalosporin C behaved quite differently, the former being inactivated 1.25 times as fast as benzylpenicillin under conditions which brought about no measurable hydrolysis of the latter. Behrens & Kingcade (1948) had previously reported that phenoxymethylpenicillin was destroyed 1.4 times as rapidly as benzylpenicillin by a preparation of penicillinase whose origin was unspecified.

In spite of the striking difference between benzylpenicillin and cephalosporin N in physical and antibacterial properties, the values of V_{max} for the penicillinase-catalysed hydrolysis of these compounds differed by less than a factor of two, and the corresponding values for the energies of activation and the Michaelis constants were very similar. The energies of activation (about 5.5 kcal. mole⁻¹) were lower than those for many enzymic reactions (see Stearn, 1949; Huennekens, 1953), a fact which was not surprising since the turnover number of the penicillinase is one of the highest among known enzymes (Kogut *et al.* 1956).

The rate of the non-enzymic inactivation of benzylpenicillin was measured by Brodersen (1947) at different temperatures in glycine-sodium hydroxide buffer (pH 9.57 at 20°). With the use of data for the change in the dissociation constant of glycine with temperature the velocity constants for the first-order reaction were calculated at a constant hydrogen-ion concentration. Under these conditions a value of 22.7 kcal. was obtained for the energy of activation. The corresponding value at a

Fig. 5. Rates of hydrolysis in the presence of the same concentration of penicillinase of different mixtures of benzylpenicillin and cephalosporin C. O, Benzylpenicillin (3.35 mM); x, benzylpenicillin (1.67 mM); ●, benzylpenicillin (3.35 mM) plus cephalosporin C (8.1 mM); □, benzylpenicillin (1.67 mM) plus cephalosporin C (8.1 mM); ▲, benzylpenicillin (0.84 mM) plus cephalosporin C (8.1 mM).

Fig. 6. Effect of cephalosporin C on the rate of hydrolysis of benzylpenicillin and cephalosporin N in the presence of penicillinase. The results are plotted in the manner suggested by Hunter & Downs (1945). O, Benzylpenicillin as substrate; ●, cephalosporin N as substrate.

constant hydroxyl-ion concentration, obtained by subtraction of the heat of ionization of water from 22.7 kcal., was about 9.8 kcal. The latter value is smaller than that obtained in the present work for the energy of activation (14.5 kcal.) of the reaction in dilute sodium hydroxide. The reason for the difference is uncertain. However, it seems clear that the energy of activation of the hydrolysis catalysed by hydroxyl ions is considerably greater than that of the hydrolysis catalysed by penicillinase. A difference of this kind is often found when enzymic reactions are compared with the corresponding chemical ones.

In the transition-state theory (Eyring, 1935) the concept of energy of activation (E) is replaced by one of free energy of activation ($\Delta F^\ddagger$) which may be divided into terms containing the heat of activation ($\Delta H^\ddagger = E - RT$) and entropy of activation ($\Delta S^\ddagger$). An enzymic reaction may be assumed to involve a transition state of the enzyme-substrate complex (ES^*). At high concentrations of substrate the velocity constant of the reaction (k_3) is given by the equation (Laidler & Hoare, 1950)

$$k_3 = (kT/h) \exp - \Delta H^\ddagger/RT \exp \Delta S^\ddagger/R,$$

in which k is the Boltzmann constant, h is Planck's constant, and $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are the heat and entropy of activation respectively for the process $ES \rightleftharpoons ES^* \rightarrow P$. At 30° the value of kT/h is 6.3×10^{12} sec.⁻¹. Substitution in this equation of the value found by Kogut *et al.* (1956) for the molecular activity of the penicillinase at pH 7 and 30° with benzylpenicillin as substrate (2.47×10^8 molecules of substrate hydrolysed/sec./mol. of enzyme), and of 4.7 kcal. mole⁻¹ for $\Delta H^\ddagger$, gives a value of -27.3 cal. mole⁻¹ degree⁻¹ for the entropy of activation. For cephalosporin N the corresponding data (molecular activity, 1.44×10^8 ; $\Delta H^\ddagger$, 5.1) yield a value of -27.4 cal. mole⁻¹ degree⁻¹ for the entropy of activation. Hence the difference in $V_{\max}$ for the two reactions would be largely accounted for by the difference in the estimated values of $\Delta H^\ddagger$, with very little change in the values of $\Delta S^\ddagger$. However, since the differences in the values obtained for $\Delta H^\ddagger$ and $\Delta S^\ddagger$ are too small for the experimental error to be negligible, the data do not allow an accurate estimation of the relative contributions of changes in $\Delta H^\ddagger$ and $\Delta S^\ddagger$ to the change in $V_{\max}$.

A negative entropy of activation has frequently been found for enzymic reactions in the presence of high concentrations of substrate. In contrast, with low concentrations of substrate $\Delta S^\ddagger$ tends to be positive, a fact which has been attributed to an unfolding of the enzyme molecule during its initial combination with the substrate (Casey & Laidler, 1950).

Cephalosporin C behaved as a competitive inhibitor of the hydrolysis of benzylpenicillin and

cephalosporin N catalysed by penicillinase. On the assumption that $k_2 \gg k_3$ with each substrate (equation 1), the values found for $K_i/K_{m,1}$ and $K_i/K_{m,2}$ (about 2.9 in each case) represent the ratios of the dissociation constant of the enzyme-inhibitor complex to the dissociation constants of the complexes formed by the enzyme with benzylpenicillin and cephalosporin N respectively. The quotient $(K_i/K_{m,1})/(K_i/K_{m,2})$ gave a value for $K_{m,2}/K_{m,1}$ (about 1.0) which was in good agreement with the value calculated from the rates of hydrolysis of different mixtures of the two substrates in the absence of the inhibitor. This result indicates that the side chain of the penicillin molecule plays little part in the formation of the initial enzyme-substrate complex. Since the absolute value of K_i appeared to be less than 3×10^{-4} M, $K_{m,1}$ should be less than 10^{-4} M. This is consistent with the finding of Manson *et al.* (1954) that the Michaelis constant of the benzylpenicillin-penicillinase system is approximately 0.6×10^{-4} M with the induced enzyme from *B. cereus* 569.

The finding that cephalosporin C is a competitive inhibitor of penicillinase lends support to the earlier chemical evidence that it is a penicillin-like compound, though not a true penicillin. Whether the antibacterial properties of cephalosporin C, cephalosporin N and benzylpenicillin depend on a reaction involving the same cell receptors is uncertain, but the specific activities of these three compounds against a number of Gram-negative organisms, like their affinities for penicillinase, are of the same order, whereas the activity of benzylpenicillin against many Gram-positive organisms is very much greater than that of cephalosporin N or cephalosporin C (Newton & Abraham, 1954, 1955). It is possible that permeability or active transport plays a part in determining the relative toxicity of these compounds to bacterial cells.

SUMMARY

1. The relative rates of hydrolysis of high concentrations of different penicillins in the presence of penicillinase from *B. cereus* NRRL 569 have been measured manometrically at pH 7 and 30°. Phenoxymethylpenicillin was hydrolysed 1.25 times as fast, and cephalosporin N 0.54 times as fast, as benzylpenicillin. Cephalosporin C underwent no hydrolysis under the conditions used.

2. The energies of activation of the enzyme-catalysed hydrolysis of benzylpenicillin and cephalosporin N in high concentration were found to be 5.3 and 5.7 kcal. mole⁻¹ respectively, and the calculated entropies of activation were negative. A value of 14.5 kcal. mole⁻¹ was obtained for the non-enzymic hydrolysis in dilute sodium hydroxide.

3. Cephalosporin C behaved as a competitive inhibitor of the activity of penicillinase. With both

benzylpenicillin and cephalosporin N, K_i/K_m was about 2.9. The quotient (Michaelis constant for cephalosporin N/Michaelis constant for benzylpenicillin) was therefore close to 1.0. A similar value for this quotient was deduced from the total rate of hydrolysis of mixtures of the two substrates.

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The Enzymic Cleavage of Aromatic Ethers

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A major route of the metabolism of many aromatic ethers involves cleavage to yield phenolic metabolites. For example, *p*-nitrophenyl ethers (Huggins, Jensen & Cleveland, 1948), phenacetin and *p*-phenetidine (Brodie & Axelrod, 1949; Smith & Williams, 1949), codeine (Adler & Latham, 1950) and a variety of nuclear-substituted anisoles (Bray, Craddock & Thorpe, 1955) are converted into their corresponding phenols in the body. Although much information is now available concerning the metabolism *in vivo* of aromatic ethers, little is known about the cellular mechanism involved in their metabolism.

A previous study has shown that codeine is converted into morphine by an enzyme system localized in the microsomes of the liver (Axelrod, 1955c). The present investigation is concerned with further studies on the intracellular processes involved in the enzymic cleavage of aromatic ethers. It will be shown that aromatic ethers are converted into phenols and aldehydes by enzyme systems, localized in microsomes of the liver, which require both

reduced triphosphopyridine nucleotide and oxygen. Evidence for the presence of more than one ether-cleaving enzyme will be presented. The relationship between the structures of ethers and their rate of enzymic cleavage will also be described.

MATERIALS AND METHODS

Materials. Triphosphopyridine nucleotide (TPN⁺) of 80% purity, diphosphopyridine nucleotide (DPN⁺) and glucose 6-phosphate dehydrogenase were obtained from the Sigma Chemical Co. Reduced triphosphopyridine nucleotide (TPNH) was prepared by the procedure of Kaplan, Colowick & Neufeld (1952). Methyl and ethyl ethers were obtained from the Eastman Chemical Co.

Analytical methods. *p*-Acetamidophenol was hydrolysed to *p*-aminophenol by heating the biological material in *N*-HCl. *p*-Aminophenol was extracted into peroxide-free diethyl ether at pH 7, and then returned to 0.1 *N*-HCl. The compound was then determined by coupling with phenol in the presence of sodium hypobromite to form an indophenol dye (Brodie & Axelrod, 1948).

p-Ethoxy- and *p*-methoxy-acetanilide were hydrolysed to the corresponding amino compounds by heating the