E R N S T  B .  C H A I N

# The chemical structure of the penicillins

*Nobel Lecture, March 20, 1946*

Before beginning with the subject proper of this lecture let me give you a few details of the historical development of the chemical work on penicillin and its organization. Work on the purification and the structure of penicillin was started at Oxford immediately after the extraordinary chemotherapeutic value of the compound had been established conclusively by our group. The initial chemical work was done by my colleague Dr. E. P. Abraham and myself in the Department of Pathology. Towards the end of 1942 we joined forces with Dr. W. Baker (now Professor of Organic Chemistry at Bristol) and Sir Robert Robinson. This group of chemists - Dr. Abraham, Dr. Baker, Sir R. Robinson and myself - have formed the nucleus of research workers whose efforts have led to the elucidation of the chemical structure of the penicillins and the synthesis of some of their degradation products. The success of this work has been due to the combined efforts of all the members of our group, and I should like you to regard me tonight merely as its representative.

Shortly after the chemical work had been started at Oxford, a number of other British research centres, both academic and industrial, began similar studies. Of these I should like to mention in particular the Imperial College of Science whose group was under the leadership of Dr. A. H. Cook and Professor Sir Ian Heilbron, the chemical laboratories of Burroughs Wellcome Ltd. in which the work was directed by Dr. S. Smith, the laboratories of Imperial Chemical Industries Ltd., and the laboratories of the firm of Glaxo, under the direction of Dr. F. A. Robinson.

Simultaneously with the work in England, American chemists began an intensive study of the structure of penicillin with the aim of quickly achieving a synthesis. This work was carried out on a very large scale, with something like 200 academic and industrial research chemists taking part in the project. Until May 1944 this work was entirely independent of the British effort, and we in Britain had no information about the state of the American investigations, except for a few fragmentary rumours.

In 1943 the British and U.S. Governments imposed a ban on the publica-

tion of all chemical work on penicillin and simultaneously negotiations were begun between the two governments for the purpose of finding a suitable method for a complete exchange of information between the various groups of workers on both sides of the Atlantic. These negotiations were protracted, and while they were in progress we at Oxford got on with our studies and were able to propose the first complete structural formulae for penicillin in October, 1943. In February, 1944, agreement for exchange of information between the British and American workers was reached; in Britain the *Medical Research Council* (M.R.C.) formed the "Penicillin Synthesis Committee" to which were sent papers by British authors; in America the *Office of Scientific Research and Development* (O.S.R.D.) delegated Dr. Hans T. Clarke of Columbia University to co-ordinate the chemical research work on penicillin in the U.S.A. and to receive monthly reports from its contractors. These two bodies, the M.R.C. and O.S.R.D., agreed to exchange their reports at monthly intervals, and in April 1944 we received the first American reports on penicillin. As I have already mentioned, the Americans have put a tremendous effort into the investigations on the chemistry of penicillin, and the following groups of chemists in the U.S.A. have participated in the project: *Academic* - Dr. Du Vigneaud and his collaborators, of Cornell University, New York, Dr. W. Bachmann of Michigan University; Dr. Woodward of Harvard University. *Industrial* - the Merck group, who have made the most extensive and valuable contributions in the degradation work as well as in the synthetic studies; the Squibb group; the Pfizer group; the N.R.R.L. group of the U.S. Department of Agriculture at Peoria, Illinois; the Abbott group; the Eli Lilly group; the Upjohn group; the Shell group; and others.

We at Oxford have been greatly handicapped in our work by lack of material. Altogether we had about 2 g of penicillin at our disposal; of this 1.5 g were about 50% pure and only about 500 mg were about 90% pure. The American workers were in a more fortunate position; the Merck group alone has used up many hundred grams of pure crystalline penicillin.

The Anglo-American collaboration continued until October 1945, and altogether about 700 reports were sent to the coordinating government committees. These reports contain partly work directly concerned with the degradation of the penicillins, and partly synthetic work, concerned with the synthesis of degradation products, intermediates and model compounds. It is obviously impossible to give you a complete account of all the work embodied in the 700 reports, in which a good many new compounds have

been added to Beilstein. I shall limit myself to work bearing directly on the purification and structure of the penicillins and shall quote only as much of the synthetic work as is relevant to the arguments about the structure. For the sake of presenting a coherent and clear picture it will not be always convenient to follow strictly the historical course of events, but I shall try to do so whenever possible. A comprehensive account of all the chemical work on penicillin is being published in form of an Anglo-American monograph under the auspices of the National Academy of Sciences, Washington, U.S.A.

During the purification studies it became clear that there existed several penicillins which had very similar biological and chemical properties, but which differed in their chemical composition. Later work showed that all penicillins contain a common nucleus, but differ in the structure of their side chains. So far four different penicillins have been obtained in the form of their crystalline sodium salts. They are designated in England as penicillins I-IV, according to the sequence of their historical discovery; in America they are termed, F, G, X and K.

Let me briefly bring back to your memory the most important physical and chemical properties of penicillin. The penicillins are organic acids, readily soluble in different organic solvents, such as esters, chloroform or ether, but insoluble or only sparingly soluble in hydrocarbons. They are stable in water only in the form of their salts, in a pH ranging between 5 and 8, and rapidly lose their biological activity in aqueous solutions of higher acidity or alkalinity. In addition to acid and alkali, the penicillins are also inactivated by many other reagents, for example by most heavy-metal ions, including those of Zn and Cd, by primary alcohols and amines, thiols, aldehydic or ketonic reagents, oxidizing reagents and a specific enzyme, penicillinase, which occurs in some penicillin-resistant strains of bacteria.

There is not time to describe in detail the methods of purifying the penicillins and a few general remarks about them must suffice. In view of the high sensitivity of the penicillins to many reagents commonly used in purification processes we were limited almost exclusively to distribution of penicillin between different solvents and to various forms of chromatography. In particular, extensive use has been made of modifications of the method of partition chromatography, a method invented in England by Martin and Synge, which is capable of wide applicability.

The success of the purification process depends entirely on the nature of the starting material, in other words on the composition of the culture me-

dium, on the conditions of fermentation, and on *the* strain of *Penicillium notatum* used. With the starting material as it is now available, the purification of penicillins II and III presents no difficulty, and crystalline sodium penicillin II has become a readily available substance.

The first penicillin to be obtained pure was penicillin II, which was crystallized in the form of its sodium salt. This was achieved about July 1943 by Wintersteiner and MacPhillamy, working at the Squibb Institute in New Jersey. About one week later, we at Oxford obtained the sodium salt of penicillin I in the crystalline state. Only the alkali salts of the penicillins and their salts with a few simple organic cations have so far been obtained crystalline. Despite many attempts it has not yet been possible to obtain crystalline their salts with any divalent metals. The sodium salts of penicillins I, II, and III can be crystallized from a mixture of water and butanol (1:20). Crystalline sodium salt of penicillin II is now produced on an industrial scale.

The crystalline sodium salts of the penicillins are colourless needles. The pure substances are strongly dextro-rotatory, $[\alpha]_D$ of penicillin I and II being $+305°$. Elementary analysis of the crystalline sodium salts has shown that the penicillins have the following composition:

| | |
|---|---|
| Penicillin I | $C_{14}H_{20}O_4N_2S$ |
| Penicillin II | $C_{16}H_{18}O_4N_2S$ |
| Penicillin III | $C_{16}H_{18}O_5N_2S$ |
| Penicillin IV | $C_{16}H_{26}O_4N_2S$ |

On catalytic hydrogenation with Pt or Pd, penicillin-I takes up one mol of $H_2$. The other penicillins do not react with catalytically activated hydrogen.

Analysis of the salts and electrometric titration curves have shown that the penicillins are strong monobasic acids having p$K$'s about 2.9 (Fig.1). There is no indication of the presence of any basic group in the electrotitration curve. This fact has played an important role in structural considerations.

The acid group in the penicillins is a carboxyl group that can be esterified by the action of $CH_2N_2$. The methyl ester has been obtained in the crystalline state. Its activity *in vitro*, about 70 u./mg, is much less than that of penicillin salts, but *in vivo* it possesses about the same activity as the salts. This is due to the fact that it is hydrolyzed easily by enzymes occurring in the body tissues. The methyl ester of penicillin cannot by hydrolyzed chemically even under mild conditions (pyridine and one equivalent of alkali at 0°C) without appreciable loss of antibacterial activity.
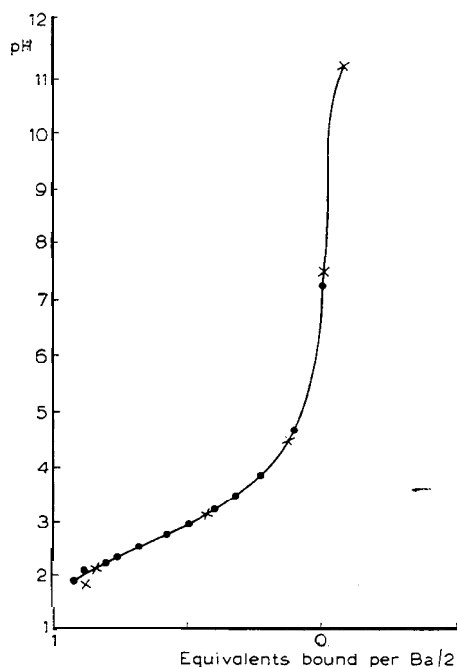
Fig. 1. Electrometric titration curve of 2-pentenylpenicillin (0°C).

Molecular weight determinations of the penicillins by several methods have shown that their molecular weights correspond to the simple formulae, shown above. Penicillin I and IV have no characteristic u.v. absorption, but penicillin II and III show clearly the fine structure of a benzene ring. When penicillin is inactivated by keeping at acid pH (Fig.2), the electrometric titration shows that a new very strong acidic group, about p$K$ 1.5, and a basic group p$K$ 7.6, is formed. The reaction product is insoluble in organic solvents, in accordance with its zwitterionic structure.

When penicillin is inactivated by alkali at pH 10, it is also converted into a zwitterion with the formation of new acidic and basic groups, but this compound differs from the product of acid inactivation, the newly formed acidic group having a p$K$ of 1.8, the new basic group a p$K$ of 5. Both products, that of acid as well as that of alkaline inactivation, have been obtained in the crystalline state. The product of acid inactivation is isomeric with penicillin and is termed penillic acid. The product of alkaline inactivation contains an additional molecule of $H_2O$; it is thus a hydrolysis product and is termed penicilloic acid. We shall discuss the structure of these important degradation products later on.
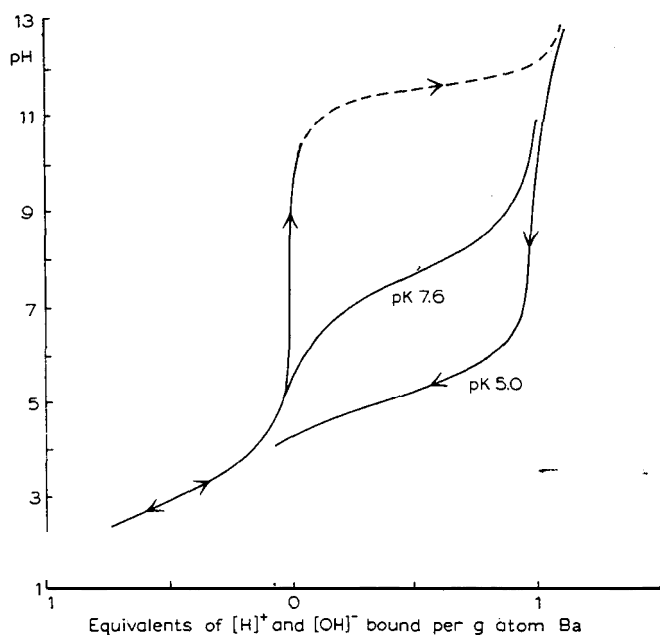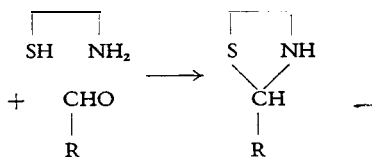
Fig. 2. Electrometric titration of 2-pentenylpenicillin (0°C) before and after inactivation with acid and alkali.

As a starting-point in the elucidation of the structure of the penicillin molecule we decided to investigate the nature of the two nitrogen atoms shown to be present by analysis. Some indication of the nature of these nitrogen atoms was obtained by hydrolysis of penicillin at 100° with normal acid. After short hydrolysis (30 min-1h) one of the two nitrogen atoms appeared in the form of $NH_2$-nitrogen, estimable by the Van Slyke procedure for the determination of a-amino groups; during prolongedhydrolysis (24 h) the other N was gradually liberated as ammonia. The acid hydrolysate of penicillin gave a strong ninhydrin reaction, confirming the presence of the a-amino acid suggested by the Van Slyke determination. This amino acid was the first degradation product of penicillin to be isolated in crystalline form. It is precipitated by $HgCl_2$ and obtained crystalline after decomposition of the mercury complex with $H_2S$. Elementary analysis shows that it is a hydrochloride, having the formula $C_5H_{11}O_2NSHCl$, and it is thus the moiety of the penicillin molecule which contains the sulphur atom. The S-containing amino acid was termed penicillamine; it gives strong nitroprusside and ferric. chloride reactions for SH. On oxidation with bromine it yields a crystalline compound which was termed penicillaminic acid. This

substance afforded better analyses than penicillamine hydrochloride. It contained three additional oxygen atoms indicating that it was the sulphonic acid corresponding to penicillamine. The titration curve showed that it contained two acid groups (the sulphonic acid and carboxylic groups) and one basic group (the $\alpha$-NH$_2$ group), but no SH group. Like penicillamine, it gives a strong ninhydrin reaction, and all its nitrogen appears as a-amino acid nitrogen in the Van Slyke determination. That the amino and thiol groups in penicillamine are in juxtaposition, is shown by the easy formation of thiazolidines when the substance is warmed with ketones and aldehydes :

$$\begin{array}{ccc} \overline{\text{SH} \quad \text{NH}_2} & & \overline{\text{S} \quad \text{NH}} \\ & \longrightarrow & \diagdown\diagup \\ + \quad \text{CHO} & & \text{CH} \\ \quad | & & | \\ \quad \text{R} & & \text{R} \end{array}$$

The titration of penicillamine shows clearly three proton binding centres that correspond to the carboxyl group (pK 1.8), the $\alpha$-amino group (p$K$ 7.9) and the SH group (p$K$ 10.5) (Fig.3.)
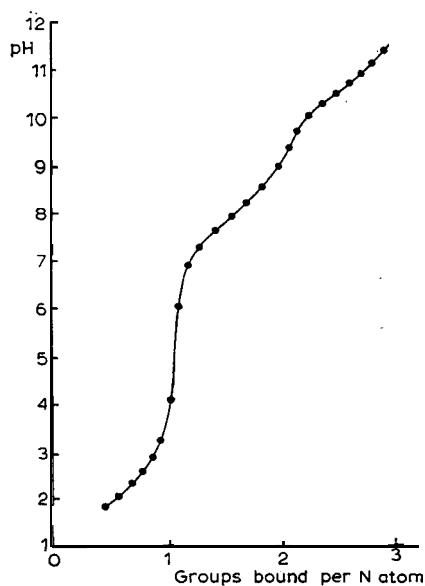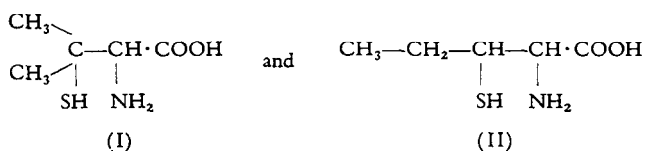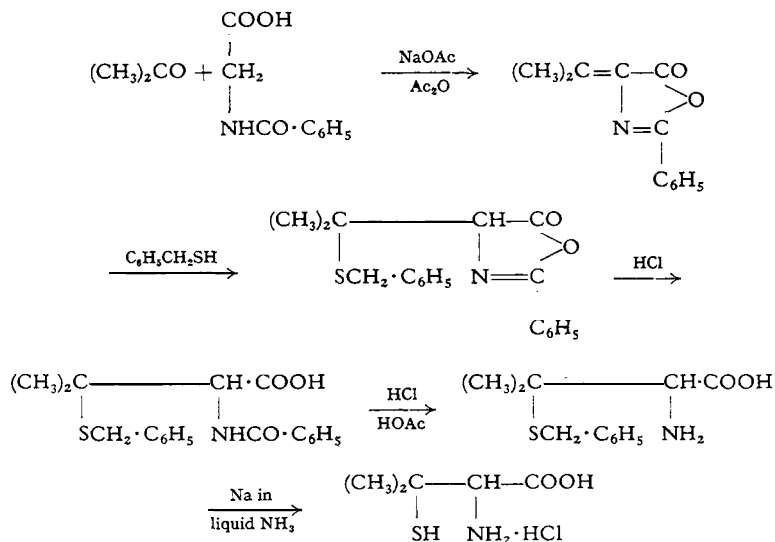


Fig. 3. Electrometric titration curve of penicillamine hydrochloride (25°C).

Of the two possible isomeric structures for penicillamine, (II) appeared

$$CH_3 \diagdown$$
$$C-CH \cdot COOH \quad and \quad CH_3-CH_2-CH-CH \cdot COOH$$
$$CH_3 \diagup | \quad | \qquad\qquad\qquad\qquad\qquad | \quad |$$
$$SH \quad NH_2 \qquad\qquad\qquad\qquad\qquad SH \quad NH_2$$
$$(I) \qquad\qquad\qquad\qquad\qquad\qquad\qquad (II)$$

improbable because C–CH$_3$ determinations gave very low values; on the other hand this finding was in accordance with structure (I), as it is known from the work of Kuhn and Roth that *gem*-dimethyl groups such as are present in structure (I) are not oxidized to acetic acid by chromic acid under the conditions of the C-CH$_3$ determination method. We concluded therefore that penicillamine had structure (I). This was conclusively proved by synthesis. This synthesis is based on a method evolved by Carter, Stevens and Ney (*J. Biol. Chem.*, 139 (1941) 247) for the synthesis of methyl cysteine; it involves the addition of benzyl mercaptan to the double bond of the azlactone obtained by condensation of hippuric acid with acetone. The steps in this synthesis are indicated in the following scheme:

$$COOH$$
$$|$$
$$(CH_3)_2CO + CH_2 \xrightarrow[Ac_2O]{NaOAc} (CH_3)_2C=C-CO$$
$$| \qquad\qquad\qquad\qquad\qquad\qquad | \quad \diagdown O$$
$$NHCO \cdot C_6H_5 \qquad\qquad\qquad\qquad N=C \diagup$$
$$|$$
$$C_6H_5$$

$$\xrightarrow{C_6H_5CH_2SH} (CH_3)_2C \underline{\qquad\qquad} CH-CO$$
$$| \qquad\qquad\qquad | \quad \diagdown O \quad \xrightarrow{HCl}$$
$$SCH_2 \cdot C_6H_5 \quad N==C \diagup$$
$$|$$
$$C_6H_5$$

$$(CH_3)_2C \underline{\qquad\qquad} CH \cdot COOH \quad \xrightarrow[HOAc]{HCl} \quad (CH_3)_2C \underline{\qquad\qquad} CH \cdot COOH$$
$$| \qquad\qquad\qquad | \qquad\qquad\qquad\qquad\qquad\qquad | \qquad\qquad\qquad |$$
$$SCH_2 \cdot C_6H_5 \quad NHCO \cdot C_6H_5 \qquad\qquad\qquad SCH_2 \cdot C_6H_5 \quad NH_2$$

$$\xrightarrow[\text{liquid } NH_3]{\text{Na in}} \quad (CH_3)_2C \underline{\quad\quad} CH-COOH$$
$$| \qquad\qquad |$$
$$SH \quad NH_2 \cdot HCl$$

*Several other methods of synthesising penicillamine have now been developed.*

The resolution of this amino acid is best achieved through fractionation of the brucine salt of the N-formyl compound. Natural penicillamine belongs
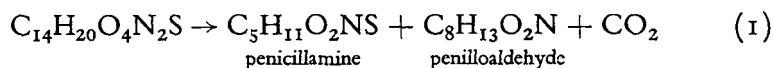
to the "unnatural" *d* -configuration. This was anticipated from the optical behaviour of penicillamine and its acetone derivative which was analogous to that of *d*-cysteine. The *d*-configuration of penicillamine was finally proved by treatment of the phenylureido derivative with Raney nickel, which led to the phenylureido derivative of *d*-valine. Penicillamine is a new amino acid which so far has not been found in any other biological material and it is yet another example of an amino acid of "unnatural" configuration produced by micro-organisms. These occur, for example, in the antibiotics gramicidin and tyrocidine and in the antigen from *Bacillus mesentericus.*

Penicillamine is similar to cysteine in many respects but it is much more soluble in water. The same applies to the disulphide. The disulphide, however, differs from cysteine in its far greater stability towards reducing agents; thus, unlike cysteine, it cannot be reduced by KCN. Neither *d*- nor *l*-penicillamine, nor their disulphides, are attacked by enzymes occurring in animal tissues.

Penicillamine, which is a constituent common to all penicillins, accounts for five of the 14 C atoms present in penicillin I. Another carbon atom is accounted for in the form of $CO_2$, one molecule of which is liberated when the free penicillin is heated to about 60⁰. The remaining eight carbon atoms are found in an aldehyde $C_8H_{13}O_2N$ which is isolated in small amounts from the acid hydrolysates of penicillin I, after removal of the penicillamine by $HgCl_2$. This aldehyde was obtained in the form of the 2,4-dinitrophenylhydrazone and as the dimedone derivative. It is obtained in larger amounts after treatment of penicillin with alkali ("alkali inactivation") and subsequent treatment of the solution with $HgCl_2$. This precipitates penicillamine in the form of its mercuric chloride complex and simultaneously one molecule of $CO_2$ is liberated; the supernatant solution now gives in good yield a precipitate with 2,4-dinitrophenylhydrazine of the hydrazone of the aldehyde $C_8H_{13}O_2N$. This aldehyde was termed penillo-aldehyde. Thus, all the 14 carbon atoms in penicillin I had been accounted for and the equation $C_{14}H_{20}O_4N_2S + 2H_2O = C_5H_{11}O_2NS + CO_2 + C_8H_{13}O_2N$ could be written. The constitution of the aldehyde $C_8H_{13}O_2N$ was elucidated as follows :

Oxidation with $Ag_2O$ of penillo-aldehyde gave a crystalline acid $C_8H_{13}O_3N$. Information about the nature of the nitrogen in this acid was obtained by hydrolysis at 100⁰ with N HCl: the hydrolysate gave a strong ninhydrin reaction for α-amino acids and about 70% of the nitrogen present in it was detectable as $NH_2$-nitrogen by the Van Slyke procedure. Hence it was con-

cluded that the acid $C_8H_{13}O_3N$ contained a peptide linkage. Its exact con-
stitution was deduced from information about the composition and behav-
iour on degradation of the American penicillin. The empirical formula of
this penicillin was telegraphed to the M.R.C. in July 1943; it was $C_{16}H_{18}O_4$-
$N_2S$. Now we know that on acid hydrolysis the English penicillin decom-
posed according to the equation

$$C_{14}H_{20}O_4N_2S \rightarrow \underset{\text{penicillamine}}{C_5H_{11}O_2NS} + \underset{\text{penilloaldehyde}}{C_8H_{13}O_2N} + CO_2 \qquad (1)$$

As we were informed that the American penicillin afforded the same amino
acid penicillamine on acid hydrolysis, we assumed that its hydrolysis pro-
ceeded according to the equation

$$C_{16}H_{18}O_4N_2S \rightarrow C_5H_{11}O_2NS + C_{10}H_{11}O_2N + CO_2 \qquad (2)$$
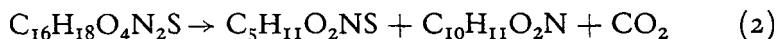
We had heard that the American workers had isolated phenylacetic acid
from acid hydrolysis of their penicillin. This is an easily recognizable sub-
stance which we never encountered among our own degradation products.
when we were informed that the American workers had isolated phenyl-
acetic acid from a crystalline degradation product of penicillin, we then
knew for certain that the American penicillin differed in chemical com-
position from our own penicillin and that the difference could only be in the
penilloaldehyde moiety. Assuming that the American penilloaldehyde con-
tained a phenylacetyl group and, like ours, a peptide linkage, its structure
could only be $C_6H_5CH_2CONHCH_2 \cdot CHO$, that is phenylacetylamino-
acetaldehyde.

If this assumption were correct our $C_8$ aldehyde should have the structure
$C_5H_9CO \cdot NH \cdot CH_2 \cdot CHO$, of a hexenoylamino-acetaldehyde, and our $C_8$
acid, derived from the aldehyde by oxidation should have the structure
$C_5H_9CONH \cdot CH_2 \cdot COOH$, of a hexenoylglycine. The presence of glycine
in this acid was proved by its isolation in the form of the naphthalene-sul-
phonyl derivative. The structure of the unsaturated fatty acid $C_5H_9COOH$
was established by oxidation with cold permanganate which gave propional,
locating the double bond in the $\beta, \gamma$-position, and proving the structure as
$CH_3H_2CH=CH \cdot CH_2 \cdot COOH$. This structure was also confirmed by
work at the Imperial College, where caproic acid, in the form of its $p$-
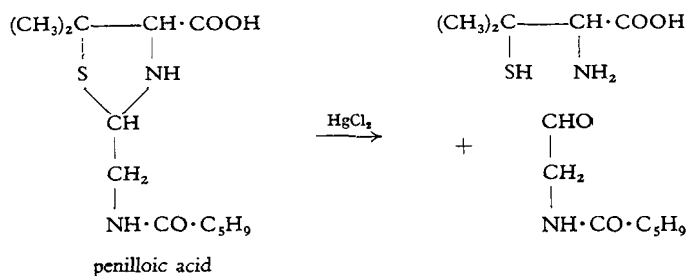bromophenyl-phenacyl derivative was isolated after hydrogenation of the

acid and subsequent acid hydrolysis. The structure of penillo-aldehyde-I was thus proved to be da-hexenoylamino-acetaldehyde, $CH_3CH_2CH=CHCH_2$-$CONHCH_2 \cdot CHO$. The acetal of this aldehyde was synthesized from the acetal of aminoacetaldehyde and $\Delta^2$-hexenoylchloride. Treatment of the products with 2,4-dinitrophenylhydrazine in $2N H_2SO_4$ gave a 2,4-dinitro-phenylhydrazone identical with that obtained from natural penillo-alde-hyde-I.

The hexenoyl group in the penilloaldehyde moiety of the penicillin I mol-ecule is responsible for the uptake of one molecule of $H_2$ when penicillin I is treated with $H_2$ and Pt or Pd. Later we obtained the information that the American penicillin II did in fact yield a penilloaldehyde of the constitution we had postulated, namely phenylacetylamino-acetaldehyde. It had thus been established that the penicillin molecule is built up from three parts: (1) the thiolamino acid penicillamine, (2) a labile carboxyl group that readily yields $CO_2$ on heating free penicillin to $60^o$, or on treating alkali-inactivated penicillin with $HgCl_2$, and (3) an acylated aminoacetaldehyde termed pen-illoaldehyde. The first two components, penicillamine and the labile car-boxyl group, are common to all penicillins. The penilloaldehyde moiety varies in the different penicillins. In penicillin I it is $\beta,\gamma$-hexenoylamino-acet-aldehyde, in penicillin II phenylacetaldehyde.

The two other penicillins that have been obtained in crystalline state yielded, on degradation, penilloaldehydes which were recognized as $p$-hy-droxyphenylacetylamino-acetaldehyde in penicillin III, and $n$-heptoylami-no-acetaldehyde in penicillin IV. The question now remaining to be an-swered was the manner in which the three components were linked together in the penicillin molecule. It was hoped to obtain information on this point by obtaining larger breakdown products of penicillin. The study of the reac-tions occurring during the inactivation of penicillin by various reagents led to the isolation of such products.

Let us consider first the product that is obtained on inactivation of peni-cillin with alkali. We have seen that after alkali inactivation and subsequent addition of $HgCl_2$ the mercury complex of penicillamine is precipitated and the supernatant solution contains the free penilloaldehyde. Penilloaldehyde appears only after the addition of $HgCl_2$; before this, no precipitate is ob-tained with 2,4-nitrophenylhydrazine. Similarly, no reaction for SH or $NH_2$-N is given by alkali-inactivated penicillin, thus showing that no free penicillamine is present in solution; the latter is formed only through the action of $HgCl_2$ on the alkali inactivation product of penicillin. From these

facts we concluded that penicillamine and penilloaldehyde were bound in solution in a thiazolidine ring which was broken by $HgCl_2$, in a manner characteristic of thiazolidines:

$$(CH_3)_2C\text{——}CH \cdot COOH$$
$$| \qquad |$$
$$S \qquad NH$$
$$\diagdown \diagup$$
$$CH$$
$$|$$
$$CH_2$$
$$|$$
$$NH \cdot CO \cdot C_5H_9$$

penilloic acid

$$\xrightarrow{HgCl_2}$$

$$+$$

$$(CH_3)_2C\text{——}CH \cdot COOH$$
$$| \qquad |$$
$$SH \qquad NH_2$$

$$CHO$$
$$|$$
$$CH_2$$
$$|$$
$$NH \cdot CO \cdot C_5H_9$$

Very soon after the precipitation of penicillamine with $HgCl_2$ from alkali-inactivated penicillin, $CO_2$ development sets in and finally one molecule of $CO_2$ is liberated. The ease of liberation of $CO_2$ could best be explained by the assumption that it derived from a carboxyl group in the β-position to the potential aldehyde carboxyl group of penilloaldehyde. The most probable structure of the alkali-inactivation product of penicillin was therefore a thiazolidine with the formula:

$$(CH_3)_2C\text{——}CH \cdot COOH$$
$$| \qquad |$$
$$S \qquad NH$$
$$\diagdown \diagup$$
$$CH$$

$$CH \cdot COOH$$

$$NH \cdot CO \cdot C_5H_9$$
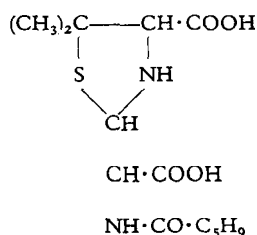
This compound has been isolated in the form of its crystalline sodium salt and various crystalline derivatives, such as different esters, amides, and N-acylated derivatives of these (Merck group). It is one of the most important degradation products of penicillin and has been given the name penicilloic acid. Its structure was proved by degradation and synthesis. The information leading to the certain elucidation of its structure was obtained from the study of the reaction products of penicillin with methyl alcohol and with benzylamine, reagents which, as I mentioned before, readily inactivate penicillin. When the sodium salt of penicillin I is dissolved in methyl alcohol its antibacterial activity is lost in a few hours. The product of the reaction, a monobasic acid like the original penicillin with roughly the same solubility

properties, contains one $CH_3O$ group. This group is easily split off by mild alkaline hydrolysis, e.g. at pH 10 at room temperature, with the appearance of a new acid group. The resulting dicarboxylic acid behaves in every respect like alkali-inactivated penicillin (penicilloic acid), giving an identical electrometric titration curve and, on decomposition with $HgCl_2$, yielding penicillamine, penilloaldehyde and $CO_2$. This suggested that the product of methanol inactivation of penicillin was a mono-methyl ester of penicilloic acid of the structure (III).

This structure was proved (Merck group) by degradation of penicillin II



(III)                              (IV)
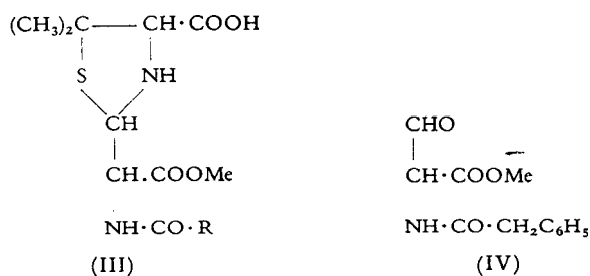
with $HgCl_2$ which produced penicillamine and the methyl ester of a $\beta$-aldehydic acid, termed penaldic acid, which was obtained as the crystalline 2,4-dinitrophenylhydrazone and as the amide (IV).
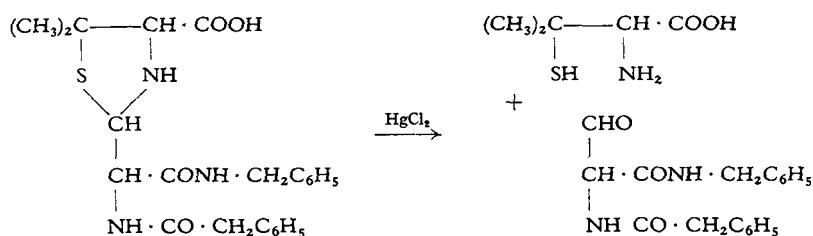
The structure of this aldehydic acid was proved by catalytic reduction to hexahydrophenylacetylalanine.
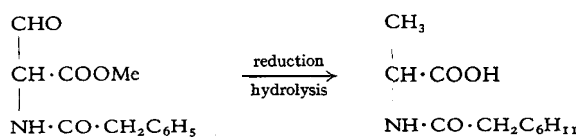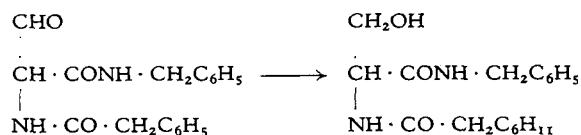


In a similar manner benzylamine reacts with free penicillin II in ether to give a crystalline compound which was shown to be the benzylamine salt of the benzylamide of penicilloic-II-acid (Merck group):
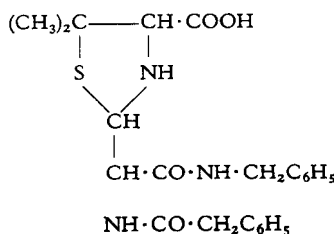
This compound is decomposed by $HgCl_2$ into penicillamine and the benzylamide of II-penaldic acid.

$$(CH_3)_2C\!\!-\!\!-\!\!-\!\!CH \cdot COOH \qquad\qquad (CH_3)_2C\!\!-\!\!-\!\!-\!\!CH \cdot COOH$$
$$S \qquad NH \qquad\qquad\qquad\qquad SH \qquad NH_2$$
$$CH \qquad\qquad\qquad + $$
$$CH \cdot CONH \cdot CH_2C_6H_5 \qquad\qquad CHO$$
$$NH \cdot CO \cdot CH_2C_6H_5 \qquad\qquad CH \cdot CONH \cdot CH_2C_6H_5$$
$$NH \ CO \cdot CH_2C_6H_5$$

with $\xrightarrow{HgCl_2}$ above

The structure of the benzylamide of penaldic acid was proved by reduction to the benzylamide of hexahydrophenylacetylserine.

$$CHO \qquad\qquad\qquad\qquad CH_2OH$$
$$CH \cdot CONH \cdot CH_2C_6H_5 \longrightarrow CH \cdot CONH \cdot CH_2C_6H_5$$
$$NH \cdot CO \cdot CH_2C_6H_5 \qquad\qquad NH \cdot CO \cdot CH_2C_6H_{11}$$

The latter compound was synthesized by phenylacetylating serine, esterifying the product with $CH_2N_2$, treatment of the methyl ester of phenylacetylserine with benzylamine, and catalytic reduction. The isolation of penicillamine after $HgCl_2$ degradation of the benzylamine inactivation product of penicillin (benzylamide of penicilloic acid) has also proved conclusively that the free carboxyl group in penicillin belongs to the penicillamine moiety.

It may be helpful to say a few words about the nomenclature of the penicilloic acids. The two carboxyl groups are termed a and $\beta$. The two ester groups are hydrolyzed by alkali with different velocities, the a-group coming off very easily at pH 10, the $\beta$-group remaining untouched under these conditions. It is thus possible to carry out a stepwise hydrolysis of penicilloic acid di-esters. Four stereo-isomers are theoretically possible and three of them have been synthesized in the form of the N-benzoyl derivatives of the α-methylesters.Their melting points and specific rotations differ considerably as shown in Table 1. The fourth has been obtained in the form of its crystalline copper salt by the action of copper sulphate on sodium penicillin II or the α-isomer of penicilloic-II-acid. The isomers were prepared by mutarotating the synthetic material (which is predominantly the γ-form) in methanol, benzoylating the crude mixture and fractionating the N-benzoyl

derivative by crystallization from ether. Frequent use was also made of the benzylamine salts and the perchlorates.

Table 1.

|  | Melting point of N-benzoyl derivatives | $[\alpha]_D^{22}$ (alcohol) |
|---|---|---|
| α-Methyl d-α-II-penicilloate | 171–173° | + 60.0 |
| α-Methyl d-β-II-penicilloate | 236–237° | − 13.2 |
| α-Methyl d-γ-II-penicilloate | 190–192° | + 123.0 |

Before we turn to discussing possible formulae for penicillin it is necessary to consider the structure of another degradation product obtained in good yield on acid inactivation. When free penicillin acid, obtained-for example by treating the barium salt with one equivalent of $H_2SO_4$, is left in aqueous solution at room temperature for about 30 minutes, about 80% of the material becomes insoluble in organic solvents, and on evaporation of the aqueous phase a nicely crystalline compound is obtained in good yield. This compound has been termed penillic acid and was one of the first degradation products of penicillin obtained in the crystalline state (Duffin and Smith, *Nature* 151 (1943) 251). Its composition is the same as that of penicillin, but its chemical and physical properties are totally different. It is thus a product of some intramolecular re-arrangement of the penicillin molecule. Penillic acid contains two acid groups and one basic group (Fig. 4). It is more strongly dextro-rotatory than penicillin, having $[\alpha]_D + 529°$, and it has a characteristic u.v. absorption spectrum, with a maximum at 2350 Å. On heating with acid it yields the same products as are obtained on acid hydrolysis of penicillin, i.e. penicillamine, penilloaldehyde and $CO_2$.

The structure of penillic-I-acid was deduced from a crystalline degradation product obtained by the action on it of $HgCl_2$. When $HgCl_2$ is added to a solution of penillic acid, one molecule of $CO_2$ is liberated and the mercury complex of a base $C_{13}H_{20}O_2N_2S$ precipitates. The hydrochloride of this base is obtained on decomposition of the mercury complex with $H_2S$. The base was termed penillamine. It gives a strong SH test with ferric chloride and sodium nitroprusside. Electrotitration (Fig. 5) shows up the SH group indicated by the colour tests, and reveals in addition the presence of one carboxyl group and one basic group. On heating it with 2,4-dinitrophenyl-hydrazine, there is obtained the dinitrophenylosazone of glyoxal. On oxida-

tion with bromine it yields penicillaminic acid. The only possible formula which could be constructed on the basis of these facts was (V). This formula was later proved by synthesis (Abraham, Baker, Chain, and Robinson).
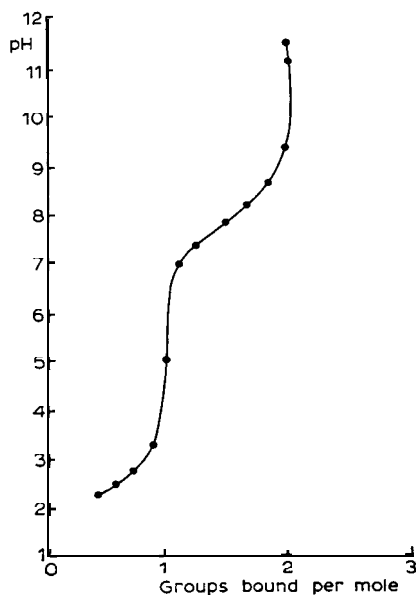
Fig. 4. Electrometric titration curve of 2-pentenylpenillic acid (25°C).
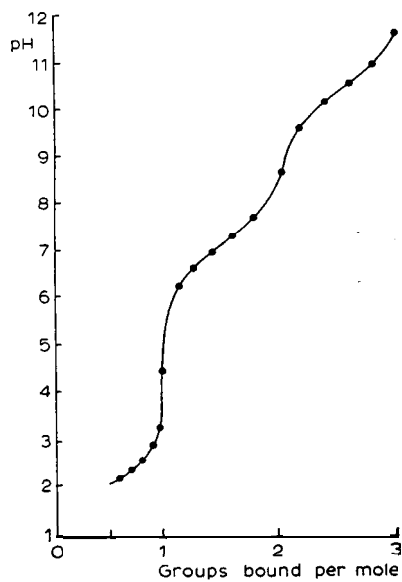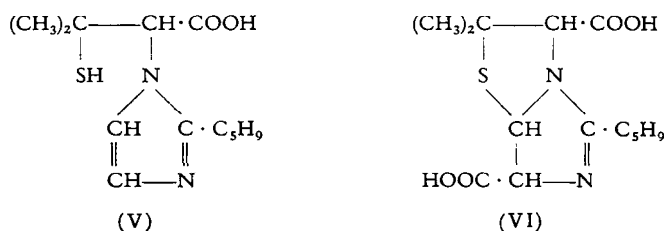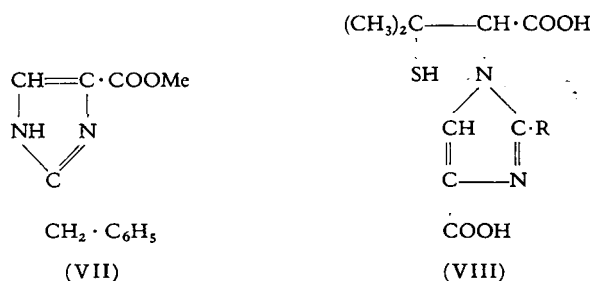
Fig. 5. Electrometric titration curve of 2-pentenylpenillamine hydrochloride (25°C).

The construction of a formula for penillic-I-acid on the basis of the formula for I-penillamine was a matter of placing into the right position the labile carboxylic group that appears in the form of $CO_2$ when penillic acid is treated with $HgCl_2$ or is heated with aqueous HCl. The most reasonable as-

$$(CH_3)_2C\text{——}CH \cdot COOH \qquad\qquad (CH_3)_2C\text{——}CH \cdot COOH$$

$$\begin{array}{cc} | & | \\ SH & N \\ & \diagup\diagdown \\ CH & C \cdot C_5H_9 \\ \| & \| \\ CH\text{——}N \end{array} \qquad\qquad \begin{array}{cc} | & | \\ S & N \\ \diagdown & \diagup \\ CH & C \cdot C_5H_9 \\ | & \| \\ HOOC \cdot CH\text{——}N \end{array}$$

$$\text{(V)} \qquad\qquad\qquad\qquad \text{(VI)}$$

sumption was that this carboxylic group was in B-position to a potential aldehydic carbonyl group, and on this assumption penillic acid could only be formulated as (VI). This formula for penillic-I-acid was in accord with all its known properties. It accounted for its two carboxylic groups and the basic group revealed in the electrotitrations, for its solubility properties and its easy transformation into penillamine by $HgCl_2$. Treatment with $HgCl_2$ involves opening of the thiazolidine ring by hydrolysis, loss of $CO_2$ from the aldehyde-ammonia compound formed and subsequent or simultaneous elimination of $H_2O$ through the tendency of this compound to go over into the very stable imidazole ring system. Further evidence for this formula was obtained much later from the mild thermal decomposition of dimethyl pen-illate- at 115° *in vacuo,* which gave (VII), a base which was considered to be 2-benzyl-4-carbomethoxy-imidazole. This assumption was proved correct by synthesis.

$$\begin{array}{c} CH\!=\!\!=\!C \cdot COOMe \\ | \qquad | \\ NH \quad N \\ \diagdown \ \ \diagup \\ C \end{array} \qquad\qquad \begin{array}{c} (CH_3)_2C\text{——}CH \cdot COOH \\ \ | \qquad | \\ SH \quad N \\ \diagup\diagdown \\ CH \quad C \cdot R \\ \| \qquad \| \\ C\text{——}N \end{array}$$

$$CH_2 \cdot C_6H_5 \qquad\qquad\qquad COOH$$

$$\text{(VII)} \qquad\qquad\qquad\qquad \text{(VIII)}$$

The formula of penillic acid was finally confirmed by total synthesis (Merck group).

When penillic acid 1 or II is treated with alkali or simply heated in meth-

anol, the thiazolidine ring is opened and a new isomeric crystalline compound, termed isopenillic acid, is obtained. This contains a free SH group, as indicated by the colour reaction with nitroprusside and $FeCl_3$, and has the structure (VIII) (Oxford workers). This structure has also been obtained by synthesis (Merck group).

Knowing the structure of penicilloic and penillic acids it is possible to construct formulae for the penicillin molecule. In the attempt to do this the main considerations to be borne in mind are the following:

(1) Penicillin is a monobasic acid, but the degradation products are dibasic acids. Penicillin must therefore have in bound form one carboxyl group that is easily liberated by alkali, methanol and primary amines at room temperature.

(2) The free carboxylic acid in penicillin is the penicillamine-carboxylic group.

(3) Penicillin has no basic group, not even of the weakest type.

(4) The penicillin molecule is capable of undergoing a facile rearrangement to an imidazoline derivative.

On the basis of these considerations our group at Oxford proposed two formulae for penicillin later known as the "β-lactam" structure (IX) and the "thiazolidine-oxazolone" structure(X).

The main guiding principle leading to the construction of Formula (IX) was the non-basicity of penicillin, and the only feasible manner in which the penicilloic NH could be rendered non-basic was to connect it with the labile carboxyl group, producing a peptide linkage. This linkage produced an admittedly very unusual four-membered ring which has not previously been observed in any natural product, but we were prepared to accept its existence because we could not find any other reasonable way of producing two non-basic nitrogens in the penicillin molecule. Furthermore we thought that
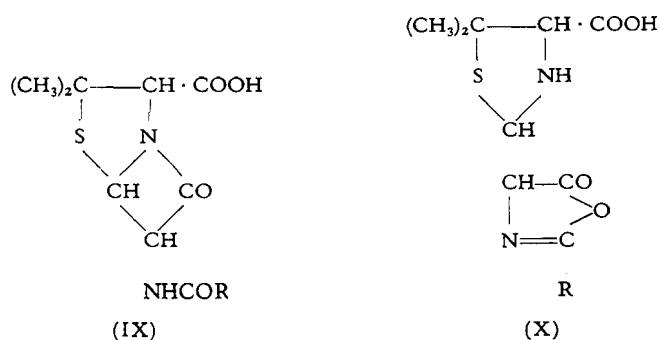
the strain inherent in four-membered rings might account for its reactions with methanol, primary amines, etc. Two arguments of purely chemical character were advanced against Formula (IX). Firstly the possibility of the existence of the four-membered ring system was considered unlikely; secondly, no reaction mechanism could be conceived which could explain in a satisfactory manner the penillic acid rearrangement since the NH-CO-R linkage as assumed in the $\beta$-lactam structure would be expected to be relatively non-reactive and would certainly not be expected to pass over into an imidazoline derivative on treatment with very dilute acid at room temperature.

The oxazolone-thiazolidine formula contained the well-known five-membered azlactone ring and appeared to explain very well the reactivity of penicillin towards $CH_3OH$, etc. Furthermore, a plausible reaction mechanism for the penillic acid re-arrangement on the bais of the electronic theory of Sir R. Robinson was suggested.

At the same time when these formulae were proposed, very little was known about thiazolidines or azlactones, but even then it was difficult for some of us to see any reason for the non-basicity of the NH nitrogen in the thiazolidine ring of structure (X). A nitrogen atom can only be made non-basic by being    bound to a strong electron-accepting centre, such as the C=O group,  and such centre was not present in Formula (X). It was argued that the basic strength of the NH could be depressed by intraspatial interaction of the carbonyl group in the azlactone group or by other factors of hitherto unknown nature, but this argument appeared unacceptable for quantitative reasons. The pK of penicilloic acid was known to be about 5 and to explain the non-basicity of penicillin a shift of about 4 pK units would have to be postulated to occur through the influence of unknown factors. However, despite these considerations the oxazolone-thiazolidine structure was for a long time most favoured by the majority of workers. In general, the attitude of the investigators to the two formulae varied according to whetherthey attached more weight to physico-chemical considerations, or to purely chemical arguments, based on the likelihood of reaction mechanisms that could be derived from the two formulae, to explain the various rearrangements.

Apart from Formulae (IX) and (X) many other formulae for penicillin can be constructed on paper from the elements of penaldic acid and penicillamine by the elimination of two molecules of water. Most of these formulae can be excluded *a priori* on account of their obvious disagreement with the

properties of penicillin. Two of these (XI and XII) have, however, received more serious attention in various quarters, in particular by those workers who saw the difficulties inherent in the thiazolidine-oxazolone structure, but were not prepared to accept the $\beta$-lactam structure.



(XI)

(XII)
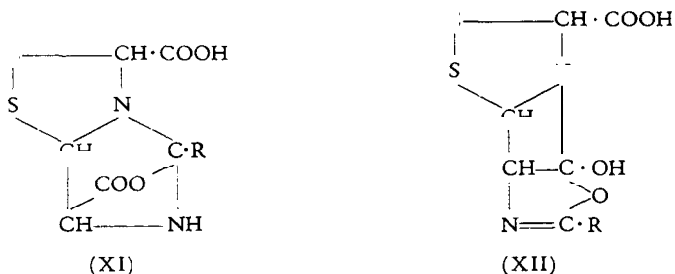
Formula (XI) (Imperial College workers) contains the penillic acid skeleton performed, and explains thus in an easy way the facile formation of penillic acid on acid inactivation of penicillin. It does not, however, explain the reaction with methanol or primary amines, which in fact gives penicilloic acid derivatives whereas Formula (XI) would be expected to lead to penillic acid derivatives. Furthermore, Formula (XI) gave no satisfactory explanation of the non-basicity of the thiazolidine nitrogen, which appears in penillic acid as a fairly strong basic group. One would, in fact, expect a compound of structure (XI) to possess two basic centres; Formula (XI) was finally eliminated by X-ray diffraction analysis, and the same applies to Formula (XII), an intermediate between the azlactone and $\beta$-lactam formulae. On chemical grounds Formula (XII) (Stodola, Northern Regional Research Laboratory) seemed unacceptable because it contained a carbinolamine group; these groups are known to be strong bases and thus could not explain the non-basicity of penicillin. Furthermore, carbinol-amines react with primary alcohols readily to give alkyl ethers, and Formula (XII) therefore provided no satisfactory explanation for the formation of penicilloic acid derivatives under the influence of primary alcohols.

Formulae (IX) and (X) remained strong rivals for a long time, and were the object of many spirited discussions. As the work progressed, more and more evidence came in which was quite incompatible with Formula (X), but in good agreement with Formula (IX). This evidence was derived partly from synthetic model compounds, and partly from degradation studies. Let us first consider the evidence derived from synthetic work. As I have mentioned before, at the outset of this work very little was known about either
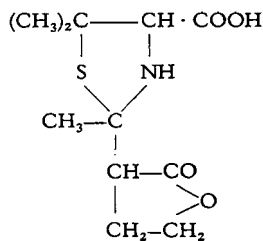
azlactones or thiazolidines. What was known was not compatible with structure (X). The few known thiazolidines derived from cysteine and a few simple aldehydes and ketones all showed a definitely basic group, having a pK of about 7. During the course of the work, a great number of thiazolidines deriving from penicillamine and from cysteine by condensation with a large variety of aldehydes and ketones of widely differing chemical constitutions were synthesized, as well as N-acyl derivatives of these. The thiazolidines are formed very easily by simple fusion of the thiolamino acid and the corresponding aldehyde and ketone at 80-100°C, with or without solvents, or by heating the acetals of the aldehydes and ketones with the hydrochlorides of the thiolamino acids at temperatures from 80° to 110°. In most cases they crystallize easily, or crystalline derivatives are formed readily.

The investigation of the properties of the newly synthesized thiazolidines has given the following general result:

All tbiazolidines with non-acylated amino groups, without a Single exception, have properties which are widely different from those of penicillin.

The N-acylated thiazolidines, on the other hand, resemble penicillin in many of their properties. In particular the following facts are of interest in this respect:

(1) All thiazolidines containing a non-acylated NH group possess a basic group which manifests itself clearly through facile formation of salts and in electrotitrations. The p$K$ of the thiazolidines deriving from penicillamine is somewhere near 5. The imido group of the thiazolidines can readily be acylated, and the N-acyl thiazolidines, as is to be expected, possess no basic group. Penicillin cannot be acylated, even by the most active acylating reagents, such as ketene or acid chlorides and pyridine; its biological activity is unimpaired by these reagents. In order to investigate whether a carbonyl group in the same position as the carbonyl group of the oxazolone postulated in the thiazolidine-oxazolone structure could depress significantly the basicity of the imido group of thiazolidines the following compound was synthesized from α-acetyl-butyrolactone and penicillamine (Abbott group) :

$$
\begin{array}{c}
(CH_3)_2C\text{——}CH\cdot COOH \\
|\qquad\quad| \\
S\qquad NH \\
\diagdown\quad\diagup \\
CH_3\text{——}C \\
| \\
CH\text{——}CO \\
|\qquad\quad\diagdown O \\
CH_2\text{–}CH_2
\end{array}
$$

The p$K$ of the imino group of this substance was very similar to that of other thiazolidines, being about 4 (Eli Lilly group). Thus the carbonyl group of the lactone ring had no significant influence on the basicity of the thiazolidine nitrogen atom and the hypothesis of an intraspatial influence of the carbonyl group on the basic strength of the thiazolidine N had been rendered untenable.

During one stage of the discussion on the formula of penicillin the formation of hydrogen bonds between NH and CO of structure (X) was considered by some workers as another possible explanation for the non-basicity of the thiazolidine nitrogen. To test this inherently unlikely hypothesis, thiazolidines derived from penicillamine and *p, m-* or *o* -sahcylaldehyde  were made. All these compounds readily formed hydrochlorides and, when titrated electrometrically, showed no significant differences in the p$K$ values of their NH groups thus excluding possibility that there was any influence by the formation of hydrogen bonds on the p$K$'s of the thiazolidine NH.

 (2) All thiazolidines  containing non-acylated NH groups react easily with I$_2$ to form the corresponding S-S compounds. This is because in solution there exists an equilibrium between the thiazolidine and the free thiolamino acid and the carbonyl compound; this equilibrium is displaced by oxidation of the SH compound to S-S. N-acylated thiazolidines are much more stable and do not react with I$_2$. Penicillin behaves like an N-acylated thiazolidine in that it does not react easily with I$_2$. Its degradation products, penillic or penicilloic acid react readily, however, with iodine, as is to be expected from their structures.

 (3)  All thiazolidines  with free imino groups invariably poison Pt or Pd hydrogenation catalysts. Not only is it impossible to reduce catalytically unsaturated groups in N-non-acylated thiazolidines, but the presence of even small amounts of such thiazolidines prevents completely the catalytic hydrogenation of easily reducible substances, such as cinnamic acid. The reason for this is the formation of free SH groups, well-known catalyst poisons. N-acylated thiazolidines, on the other hand, are inert towards hydrogenation catalysts because of the greater stability of the thiazolidine ring (Eli Lilly group). Penicillin I, which contains an unsaturated hexenyl side chain, is easily reduced by Pt or Pd catalysts and behaves thus also in this respect like an N-acylated thiazolidine. Again, the breakdown products penillic and penicilloic acids behave like all other ordinary thiazolidines towards catalytic hydrogenation; i.e. they poison the catalysts irreversibly.

 (4) On oxidation with KMnO$_4$, thiazolidines with free imino groups are

oxidized to the corresponding sulphonic acids while N-acylated thiazolidines yield the corresponding sulphones. Penicillin methyl ester on oxidation with KMnO$_4$ gives a sulphone (Merck group), a fact which is incompatible with structure (X) but in agreement with structure (IX).

To sum up, the following can be said: The examination of the properties of many model thiazolidines has shown unequivocally that penicillin behaves in no way like a normal thiazolidine with a non-acylated free imino group; its behaviour is therefore not compatible with the thiazolidine oxazolone structure (X). It resembles much more a N-acylated thiazolidine, which is in accordance with the $\beta$–lactam structure (IX). No evidence could be found for the hypothesis that the basicity of the thiazolidine NH can be depressed through the intraspatial influence of neighbouring groups.

So much about thiazolidines. Apart from these, a great deal of effort has been expended in the preparation of oxazolones and the study of their properties. The outcome of this work, which time does not permit me to discuss in detail, has given the following main results:

In accordance with earlier works in the literature no oxazolone of the type (XIII) is stable in water at any pH; all are hydrolyzed more or less rapidly to

$$
\begin{array}{c}
CH_2\!\!-\!\!CO \\
|\qquad\quad >\!\!O \\
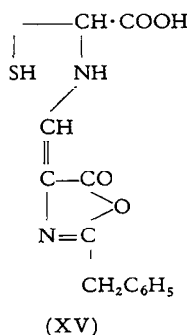N\!\!=\!\!=\!\!C \\
\\
\dot{R} \\
\text{(XIII)}
\end{array}
$$
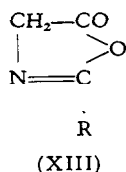
the corresponding acylated amino acids. Penicillin salts are, in contradistinction, stable in water for an indefinite time.

4-Hydroxymethylene oxazolones are stable in water in the form of their alkali salts, but are rapidly decomposed in acid medium. All oxazolones, even the most stable ones, react with liquid ammonia to give the-corresponding amide with ring opening. Penicillin, on the other hand, is quite inert towards liquid ammonia. Particular attention has been given to the preparation and study of 2-benzyl-4-hydroxymethyleneoxazolone (XIV), and various methods of preparation of this compound have been worked out.

$$
\begin{array}{c}
CHOH\!\!=\!\!C\!\!-\!\!CO \\
|\qquad\quad >\!\!O \\
N\!\!=\!\!C \\
| \\
CH_2C_6H_5 \\
\text{(XIV)}
\end{array}
$$
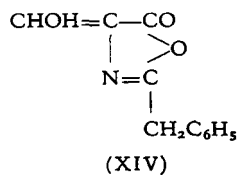
A few words about the properties of 4-hydroxymethylene oxazolones. Their discussion is necessary for the understanding of certain degradation reactions of penicillin which will be considered shortly. The hydroxymethylene oxazolones give a strong blue colouration with $FeCl_3$ and have no pronounced aldehydic character. Their reactivity is more like that of acid chlorides than of aldehydes. Thus they react with diazomethane to give methoxymethylene compounds and combine readily with amines to give the corresponding aminomethylene compounds. With ammo acids a similar reaction occurs. Thus crystalline aminomethylene derivatives have been obtained by combining 2-benzyl-4-ethoxymethyleneoxazolone with the amino acids glycine, alanine, and valine. With thiolamino acids hydroxymethylene oxazolones do not form thiazolidines, like normal aldehydes, but both $NH_2$ and SH groups react separately and independently. When molecular proportions of thiolamino acids and hydroxy- or alkoxy-oxazolones are combined, the amino group reacts preferentially; thus penicillamine and 2-benzyl-4-hydroxymethyleneoxazolone give the compound (XV)

$$
\begin{array}{c}
{-\!-\!-\!-\text{CH·COOH}} \\
|\qquad\quad | \\
\text{SH}\quad\text{NH} \\
\diagup \\
\text{CH} \\
\parallel \\
\text{C}\!-\!\text{CO} \\
|\qquad\diagdown\!\!\text{O} \\
\text{N}\!=\!\text{C} \\
| \\
\text{CH}_2\text{C}_6\text{H}_5
\end{array}
$$

(XV)

Compounds of this type are of interest because, as will be shown later, they are degradation products of penicillin; they have been given the name penicillenic acids. 4-Aminomethylene oxazolones are recognized easily by their characteristic absorption spectra; they have two absorption maxima, at 3,200 Å *(EM* 25,000) and (a weaker one) at 2,700 Å *(EM* 5,000).

The aminomethylene oxazolones tend to be more stable in acid solution than the alkoxy- or hydroxy-methylene oxazolones; in alkali they behave like the alkoxymethylene compounds, i.e. they are hydrolyzed to the sodium salt of the hydroxymethylene derivatives. Thus sodium penicillenate is hydrolyzed by alkali to the sodium salt of 2-benzyl-4-hydroxymethyleneoxazolone.

Let us now return to evidence based on the degradation studies. The Merck

workers found that when penicillin methylester in ether solution is treated with HgCl$_2$ and the resulting precipitate is decomposed with H$_2$S, an amorphous substance is obtained which exhibits the characteristic absorption spectrum of penicillenic acid with two maxima at 3,150 Å and 2,700 Å.

This finding attracted a good deal of interest. Ever since the thiazolidine oxazolone structure for penicillin was proposed, continuous attempts were made, particularly by the Merck group, to isolate the 2-benzyl-4-hydroxymethyleneoxazolone which formed one component of this structure. The most obvious way to obtain this oxazolone, which was known to be quite stable in alkali, was to try to split sodium penicillin by the action of HgCl$_2$. All normal N-non-acylated thiazolidines are instantly decomposed by HgCl$_2$ into the mercury complex of the thiolamino acid and the carbonyl component. However, when the effect of HgCl$_2$ on sodium penicillin was tried, it was found that - unlike the normal N-non-acylated thiazolidine - it did not react instantaneously, but only very slowly after an interval of many hours; and examination of the degradation products showed that it had been split into penicillamine and penaldic acid, but no trace of 2-benzyl-4-hydroxymethyleneoxazolone, easily detectable by its characteristic u.v. absorption spectrum, was ever observed. The behaviour of sodium penicillin towards HgCl$_2$ was in fact additional evidence against the thiazolidine-oxazolone structure and in favour of the $\beta$–lactam structure. Now, if the product obtained after reaction of HgCl$_2$ on methyl penicillin was really penicillenic acid, then it would have been definitely proved that 2-benzyl-4-hydroxymethyleneoxazolone can be obtained from penicillin by a mild degradation process and this finding would have to be taken into account in the considerations of the structure of the penicillin molecule. The HgCl$_2$ degradation product of methyl penicillin was therefore examined very carefully, and the result of the examination left no doubt that it was composed predominantly of penicillenic acid from $d$-penicillamine and 4-hydroxymethylene-oxazolone; it was therefore an easily available compound whose properties could be studied without difficulty. Penicillenic acid has two characteristic reactions:

(1) On addition of benzylamine to penicillenic acid the characteristic u.v. absorption disappears and the α-benzylamide of penicilloic acid is formed. The HgCl$_2$ degradation product of methyl penicillin behaves in the same manner; after addition of benzylamine, the a-benzylamide of penicilloic acid was isolated in the crystalline state and was shown to be identical with the synthetic material.

(2) Synthetic penicillenic acid is hydrolyzed by alkali to penicillamine and 2-benzyl-4-hydroxymethyleneoxazolone which can be isolated as the crystalline Na salt and in form of crystalline derivatives. The $HgCl_2$ degradation product of methylpenicillin behaves in an identical manner, and the Merck workers succeeded in isolating from its alkaline hydrolysates the sodium salt of 2-benzyl-4-hydroxymethyleneoxazolone in the crystalline state and characterized it by various crystalline derivatives which were identified with synthetic specimens.
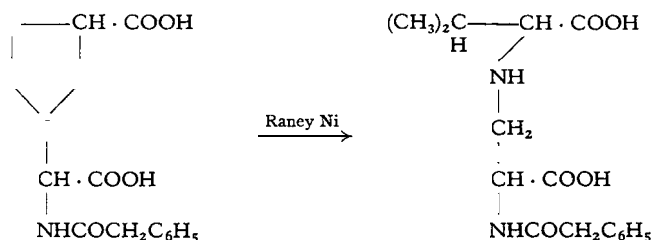
If we now consider the implications of the isolation of 2-benzyl-4-hydroxymethyleneoxazolone as a degradation product of penicillin for arguments concerning the structural formula of penicillin, it must be admitted that, in the absence of all other evidence, the isolation of one component of the postulated thiazolidine-oxazolone structure would naturally be considered as strong evidence in favour of this structure; in fact it is the strongest evidence that could be obtained from straightforward chemical degradation reactions. However, at the time when the oxazolone was isolated by the Merck workers, a great deal of very strong evidence against the thiazolidine-oxazolone structure had already been accumulated and this evidence could not simply be disregarded in front of the new finding. Consequently, the less simple explanation for the formation of the oxazolone during the degradation of penicillin had to be taken into consideration, namely that it was formed as the result of a novel type of intramolecular rearrangement of the four-membered ring of the $\beta$–lactam structure present in the original penicillin molecule, induced by the reaction of $HgCl_2$ with methyl penicillin. A distinct aversion to this assumption was noticeable among many chemists because no analogous reaction was known in the literature and no plausible reaction mechanisms for the rearrangement could be suggested.

While the discussion on the significance of the isolation of the oxazolone for the structure of the penicillins was still in full swing, the Merck group isolated several new crystalline degradation products of penicillin II, and the elucidation of their structure neutralized completely all the arguments in favour of the oxazolone-thiazolidine structure that could be advanced by the defenders of this structure from the isolation of the oxazolone. They succeeded, in fact, in isolating in good yield and by a very mild degradation procedure, a product which was shown to possess the $\beta$–lactam structure.

It will be remembered that Mozingo, of the Merck Institute, had developed a new method for desulphurization, by hydrogenolysis with Raney nickel, which led to an important advance in the elucidation in the structure

of biotin. This method consists in heating the sulphur-containing compound for a short time with a suspension of finely divided Raney nickel through which hydrogen had been passed. The sulphur is thereby removed as nickel sulphide and is replaced by two atoms of hydrogen in a very smooth reaction. This method has a wide applicability and has proved very useful in the studies aimed at elucidating the chemical structure of the penicillins.
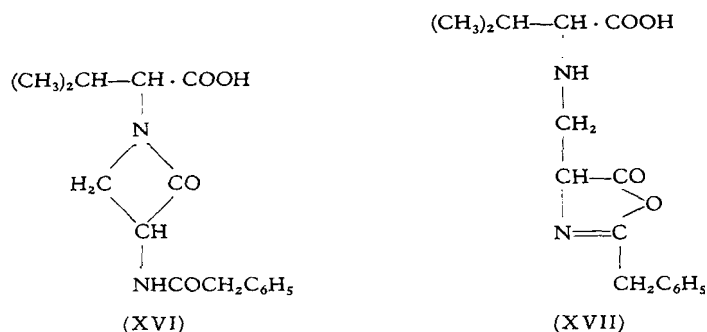
When sodium penicillin II is treated with Raney nickel at 90°C for 1 min, the sulphur is eliminated and two crystalline compounds $C_{16}H_{20}O_4N_2$ and $C_{16}H_{22}O_4N_2$ are obtained, the first in very good yield, the latter in smaller amounts. The first compound has the elementary composition of penicillin II except that the sulphur is removed and replaced by two hydrogen atoms, and it has accordingly been termed desthiopenicillin. The acid-base properties of desthiopenicillin are the same as those of penicillin, i.e. it is a monobasic acid with no detectable basic group. Chemically it is much more stable than penicillin; it does not react with acid, alkali, primary amines, or alcohols at room temperature. However, on heating for a short time in acid or alkali, desthiopenicilloic acid is obtained, a compound which was found to be identical with desthiopenicilloic acid prepared from natural penicilloic acid by Raney treatment.

$$
\begin{array}{ccc}
\text{——CH·COOH} & & \text{(CH}_3)_2\text{C——CH·COOH} \\
|\qquad | & & \text{H}\quad\diagup \\
& & \text{NH} \\
\diagdown\diagup & \xrightarrow{\text{Raney Ni}} & | \\
| & & \text{CH}_2 \\
\text{CH·COOH} & & \text{CH·COOH} \\
| & & | \\
\text{NHCOCH}_2\text{C}_6\text{H}_5 & & \text{NHCOCH}_2\text{C}_6\text{H}_5
\end{array}
$$

Electrometric titration of desthiopenicilloic acid shows that it possesses two acid groups and one basic group, p$K$ 8.2.

When desthiopenicillin is heated with benzylamine for three hours in refluxing dioxan, the benzylamide of desthiopenicilloic acid is obtained. This is identical with the compound obtained by hydrogenolysis of the penicilloic acid benzylamide derived from natural penicillin.

Only one formula (XVI) could be constructed for desthiopenicillin II that was in agreement with its properties and chemical reactions. This formula contained the four-membered ring postulated in the $\beta$–lactam structure for penicillin. An alternative formula (XVII) containing the oxazolone ring could be disregarded, firstly because of the stability of desthiopenicillin and

$(CH_3)_2CH-CH \cdot COOH$

$(CH_3)_2CH-CH \cdot COOH$

|
N
/ \
$H_2C$    CO
\ /
CH
|
$NHCOCH_2C_6H_5$

(XVI)

NH
|
$CH_2$
|
$CH-CO$
|        $>O$
$N=C$
|
$CH_2C_6H_5$

(XVII)

secondly because of the absence of a basic group which should certainly have been present if the oxazolone structure containing the NH group was correct.

Thus the rather curious situation had arisen that the degradation work had furnished apparent support both for the thiazohdine-oxazolone and the $\beta$-lactam structure for penicillin; constituents of both formulae, an oxazolone and a substance containing the four-membered $\beta$-lactam ring had been isolated from penicillin by very mild degradation procedures. It then remained to decide which of the two ring systems was originally present in penicillin, and which the result of a rearrangement process. Which of the two rearrangements could be considered the more plausible was largely a matter of personal opinion, and as might be expected the views on this question differed widely among the various investigators. The elucidation of the structure of the second degradation product which had been obtained by treatment of penicillin with Raney nickel did not add anything decisive. It appeared that this substance, $C_{16}H_{22}O_4N_2S$ (m-p. 206-2070°), was phenyl acetyl C (+) alanyl d (-) valine;

$(CH_3)_2CH-CH \cdot COOH$
|
NH

CO

$CH_3CHNHCOCH_2C_6H_5$
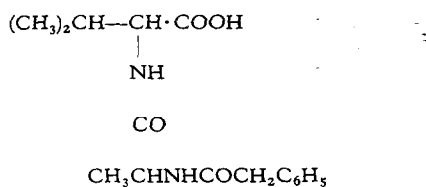
this was proved by synthesis (from the azide of phenylacetyl $l$ (+) alanine and $d$-valine). The importance of the isolation of phenylacetyl $l$ (+) alanyl $d$ (-) valine in respect to the structure of penicillin lies in the fact that it has settled the optical configuration of another of the three asymmetric carbon atoms in the penicillin molecule. The penicillamine radical had, as was point-

ed out before, the unnatural *d*-configuration; the alanine radical was now shown to possess the natural *l*-configuration. The simplest explanation for the appearance of this substance was that it derived by hydrogenolysis from the *β*-lactam which contained the two N-CO linkages preformed. It could, however, also have arisen from the oxazolone by an internal acylation followed by a rearrangement.

To make the situation somewhat more confused a new crystalline isomerization product of penicillin was found which differed from the two other isomers penillic and penicillenic acids. This new product, termed penillonic acid, is formed when methyl penicillin is heated in toluene in the presence of a small amount of $I_2$ (Merck group). As in the case of all the other crystalline degradation products, the structure of the new isomer penillonic acid was eagerly studied in the hope of finding a new line of approach to the problem of the structure of penicillin. With the formulae for penicillin, penillic acid and penicillenic acid already disposed of, the possibilities for new structural arrangements were becoming rather limited and it became quite difficult to think of yet another structural isomer of penicillin.
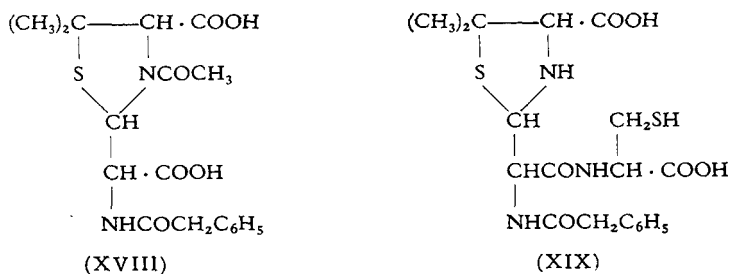
It was found that methyl penillonate was also obtained from methyl penicillenate, both natural and synthetic, by heating in toluene in the presence of a small amount of $I_2$; but in addition it was formed by simple sublimation *in vacuo* of methyl penicillin, whereas methyl penicillenate gave no penillonic acid under these conditions. Penillonic acid is therefore not merely a rearrangement product of methyl penicillenate, which would be of secondary interest to structural considerations concerning penicillin, but is also formed directly from penicillin, as the result of yet another rearrangement, in addition to the penillic and penicillenic acid rearrangements. The structure of penillonic acid, a monobasic acid with no basic centre, was finally elucidated by degradation of desthiopenillonic acid, $C_{16}H_{20}O_4N_2$, obtained by treatment of methyl penillonate with Raney nickel and subsequent saponification. This compound is isomeric with desthiopenicilhn, but quite different in its physical and chemical properties. It does not react with benzylamine, mercuric chloride or methanol, even under drastic conditions. It does not react with acid or alkali at room temperature or when heated to 110° for a short time. Prolonged hydrolysis with NaOH or conc. HCl at 100° yields phenaceturic acid, valine and one molecule of formaldehyde. These findings were best reconciled with the following structures for desthiopenillonic II acid and penillonic-II acid:

(CH₃)₂CH—CH · COOH

CH₂   CO

C₆H₅CH₂CO · N——CH₂

Desthiopenillonic acid

——CH · COOH
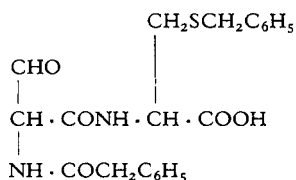
CO

C₆H₅CH₂CON——CH₂
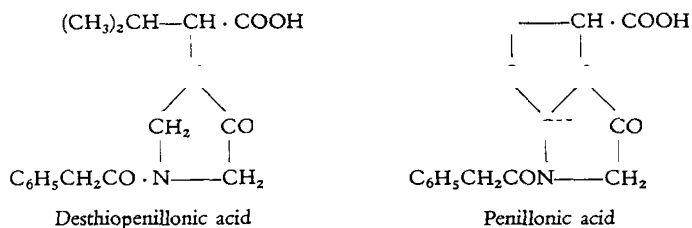
Penillonic acid

The elucidation of the structure of penillonic acid, like that of the other degradation products of penicillin, was thus of little use for obtaining unequivocal evidence about the structure of penicillin and was disappointing in this respect. All it showed was the occurrence of yet another extraordinary rearrangement involving a reaction mechanism which was difficult to explain on the basis of any formula. Ring expansion from the four-membered to a five-membered ring would have to occur on the basis of the β-lactam structure, a rearrangement of the oxazolone involving a migration of nitrogen on the basis of the oxazolone structure. The latter rearrangement does in fact occur, as penillonic acid is formed from synthetic penicillenic acid which has a known structure containing the oxazolone ring; but no satisfactory explanation for the reaction mechanism of this rearrangement has yet been put forward.

A great deal of most interesting degradation work on penicillin has been carried out in addition to that mentioned above. Time unfortunately does not permit me to give anything like an adequate account of this work, but I want to mention the broad results. These were quite as ambiguous as those obtained from the other degradation studies, and did not allow of a definite decision in favour of one or the other of the two formulae under discussion.
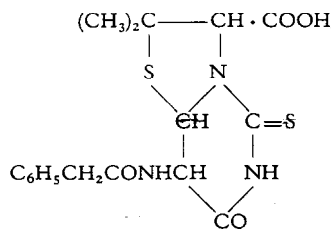
Thus, it was found, that free penicillin-II acid is easily inactivated by acetic acid in an organic solvent (Squibb group); N-acetyl-II-penicilloic acid (XVIII) is formed; the constitution of this compound was proved by synthesis. Sodium penicillin II is inactivated by cysteine at pH 7 ( Squibb group).

(CH₃)₂C——CH · COOH

S    NCOCH₃

CH

CH · COOH

NHCOCH₂C₆H₅

(XVIII)

(CH₃)₂C——CH · COOH

S    NH

CH    CH₂SH

CHCONHCH · COOH

NHCOCH₂C₆H₅

(XIX)

The compound formed is a peptide of penicilloic acid and cysteine, with the SH group free. The constitution of this compound (XIX) was proved by benzylation with benzylchloride and splitting with $HgCl_2$, which yielded the aldehyde (XX). The two reactions just mentioned could be explained on the basis of either structure (IX) or (X). With HN=C=S, penicillin-II methyl ester reacts to give in good yield a crystalline product on which a considerable amount of very ingenious degradation work was carried out by the Cornell and Squibb groups of workers. This cannot be reported in any detail but has led to the elucidation of the structure of this product (XXI). This
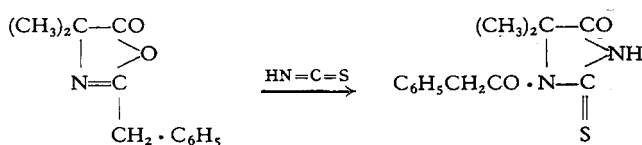
<div align="center">

$CH_2SCH_2C_6H_5$

CHO   |

|     |

$CH \cdot CONH \cdot CH \cdot COOH$

|

$NH \cdot COCH_2C_6H_5$

(XX)

</div>

<div align="center">

$(CH_3)_2C$———$CH \cdot COOH$

|    |

S    N

\  / \

CH  C=S
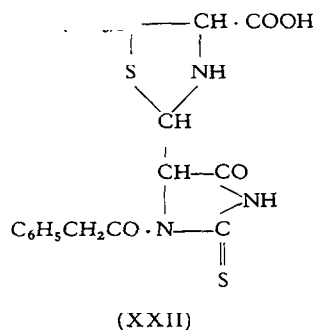
|   |

$C_6H_5CH_2CONHCH$ NH

\  /

CO

(XXI)

</div>

structure could be derived easily from structure (IX), but was not in good agreement with structure (X). It was known from the literature that N-acylated amino acids on heating with thiocyanic acid and acetic anhydride gave I-acyl-2-thiohydantoins and it was assumed that this reaction proceeds via the azlactones. A great deal of work on model oxazolones has shown that I-acyl-2-thiohydantoins are indeed easily formed when they are treated with thiocyanic acid at room temperature. Thus 2-benzyl-4,4-dimethyloxazolone gives I-phenylacetyl-2-thio-5,5-dimethyl-thiohydantoin.

<div align="center">

$(CH_3)_2C$—CO       $(CH_3)_2C$—CO

|  >O         |  >NH

N=C    $\xrightarrow{HN=C=S}$ $C_6H_5CH_2CO \cdot N$—C

|               ‖

$CH_2 \cdot C_6H_5$          S

</div>

If penicillin had an oxazolone ring as one of the constituents of its molecule, a thiohydantoin of the structure (XXII) would be expected to be formed on treatment of penicillin-II methyl ester with thiocyanic acid. The actual reaction product has a different constitution, as pointed out above, containing a thiouracil nucleus. Thus, the structure of the thiocyanic degradation product of penicillin is really against structure (X), though an intramolecular re-

$$\begin{array}{c} \text{———CH·COOH} \\ | \qquad | \\ \text{S} \qquad \text{NH} \\ \diagdown \diagup \\ \text{CH} \\ | \\ \text{CH——CO} \\ | \qquad \diagup\text{NH} \\ \text{C}_6\text{H}_5\text{CH}_2\text{CO·N———C} \\ \| \\ \text{S} \end{array}$$

(XXII)

arrangement from an originally present oxazolone to a $\beta$-lactam under the conditions of the reaction cannot, of course, be excluded.

I have now come to the end of my account of the degradation studies on penicillin. These certainly do not lack in variety and surprises and have led to the discovery of several entirely novel rearrangements.

Summarizing the evidence for the structure of penicillin obtained from the degradation work it can be stated that no absolutely unequivocal conclusion could be derived from it although the balance of the work was more in favour of the plactam structure than of the thiazolidine-oxazolone structure. In particular the formation of the sulphone and of the reaction product with thiocyanic acid was extremely difficult to explain except on the basis of the $\beta$-lactam formula.

The final solution of the problem of the structure of penicillin came from crystallographic X-ray studies. This work, in which Mrs. D. Crowfoot and her colleague Mrs. Barbara Rogers-Low have played a predominant rôle, has led to the definite exclusion of the thiazolidine-oxazolone structure and to the conclusive proof of the $\beta$-lactam structure. Through a series of Fourier analyses of electron diffraction densities, obtained by X-ray pictures of single crystals of the rubidium and potassium salts of penicillin II, Mrs. Crowfoot and Mrs. Rogers-Low succeeded in measuring all bond distances between the atoms in the penicillin molecule with an accuracy of 0-2 Å and in thus mapping out clearly the whole penicillin molecule. The alkali metal and the sulphur atom served as landmarks in the Fourier analyses. The measurements of the atomic distances show clearly and unequivocally that there exists a normal bond between the thiazolidine nitrogen and the carbonyl group of the labile carbonyl group, but no bond exists between this carbonyl group and the oxygen atom of the peptide side-chain. The four-membered $\beta$-lactam ring is clearly visible. These calculations were completely con-

firmed by an independent group of X-ray workers, Dr. C. W. Bunn and his colleagues of Imperial Chemical Industries Ltd. Thus the structure of penicillin was definitely proved to be the $\beta$-lactam structure (IX). The work of Mrs. Crowfoot and Mrs. Rogers-Low is a considerable achievement; for the first time the structure of a whole molecule has been calculated from X-ray data, and it is the more remarkable that this should have been possible in the case of a substance having the complexity of the penicillin molecule. The ever-increasing importance of crystallographic X-ray work for the elucidation of chemical structures in collaboration with the organic chemist has been demonstrated in an impressive manner by these investigations.

The elucidation of the chemical structure of the penicillins has been a most interesting and fascinating task in every respect from the very beginning. It became apparent very soon that the chemical behaviour of penicillin was in no way less interesting and original than its biological properties. Penicillin is a simple dipeptide, composed of two simple amino acids: $\beta$-thiol-valine and an acylated serine in which the alcohol group has been oxidized to the aldehyde group. Through the incorporation of a peptide linkage in a peculiar ring system so far not observed in any other natural product, this peptide linkage has acquired a high reactivity, and it is the fusion of thiazolidine and $\beta$-lactam rings which confers to the penicillin molecule its unique biological and biochemical properties. This is a fact which deserves attention beyond the limited field of penicillin chemistry. The question of the nature of the linkages by which naturally occurring peptides acquire their specific and often very pronounced biological properties has been, and is, one of the major problems in biochemistry. Now it has been shown in the case of the penicillin molecule that a simple dipeptide can acquire most characteristic and specific chemical and biological properties through a novel, but very simple type of linkage of the two amino acids. Naturally, one is tempted to ask immediately whether the occurrence of the $\beta$-lactam type of peptide linkage is limited to the penicillin molecule, and whether it does not occur also in other natural products, such as proteins. Perhaps the $\beta$-lactam structure is an important feature of many natural products and has escaped discovery up to the present time only because it does not manifest itself by some characteristic property serving as an indicator for its existence, as, in the case of penicillin, the striking bacterial action of this compound. This applies also to the various rearrangements which the penicillin molecule has been shown to undergo readily. These rearrangements, in particular the formation of

imidazole derivatives and oxazolones from amides under very mild conditions, are all of great general biochemical interest. The search for suitable reaction mechanisms for these entirely novel rearrangements will provide an attractive field of research for the theoretical organic chemist.

To end this lecture, just a few words may be said about the present state of the work on the synthesis of penicillin. Despite the apparent simplicity of the penicillin molecule and despite a tremendous effort on the part of many competent chemists, no workable method of synthesis has as yet been evolved. All feasible routes that could possibly lead to such synthesis have been explored, but have not given positive results. In attempts to synthesize the oxazolone-thiazolidine structures, traces of biologically active material have been obtained both by the Merck group and the Oxford workers through condensation of 2-benzyl-4-methoxymethyleneoxazolone, and similar compounds with *d*-penicillamine. *l*-Penicillamine, or *d*- and *l*-cysteine led to no activity. There can be no doubt that the active material synthesized in this manner is in fact penicillin. It acts against the same bacteria as does natural penicillin, and is inactivated by the same specific reagents that inactivate natural penicillin: acid and alkali, methanol and the specific enzyme penicillinase. Furthermore, penicillamine containing radioactive sulphur has been used for the condensation with 2-benzyl-4-methoxymethyleneoxazolone (Cornell group); when a large amount of natural crystalline penicillin II was added to the active product thus obtained and the mixture crystallized, it was found that on recrystallization, the radioactivity always followed the crystalline penicillin fraction, even after 14 recrystallizations of the sodium salt and a further 14 recrystallizations of the acid inactivation product penillic acid. This showed that the solubility properties of the synthetic active material were extremely similar to those of natural penicillin II, so that the identity of the synthetic with the natural material was made even more probable. All attempts to improve the very small yield of synthetic penicillin (about 0.1%) have failed, and it appears improbable that a synthetic process will be evolved that could compete successfully with the cheap biological production of penicillin.