LXIV. STUDIES IN THE BIOCHEMISTRY OF MICRO-ORGANISMS.

XLVIII. PENICILLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM PUBERULUM* BAINIER AND *P. CYCLOPIUM* WESTLING.

BY JOHN HOWARD BIRKINSHAW, ALBERT EDWARD OXFORD, AND HAROLD RAISTRICK.

From the Division of Biochemistry, London School of Hygiene and Tropical Medicine, University of London.

(*Received January 31st, 1936.*)

DURING an investigation of the possible connection between the incidence of pellagra and mould deterioration of maize Black and Alsberg [1910] and Alsberg and Black [1913] isolated a hitherto undescribed mould metabolic product which they named penicillic acid, $C_8H_{10}O_4$. The constitution of this substance, which was separated from cultures of *Penicillium puberulum* Bainier, isolated from mouldy maize and grown on Raulin's medium, and which was somewhat toxic to mice, was not investigated. Through the courtesy of Dr Charles Thom, we obtained Alsberg and Black's culture of *P. puberulum* about seven years ago and were able to repeat their isolation of penicillic acid, though in diminished yield. It was found later [Birkinshaw and Raistrick, 1932] that the yield of penicillic acid had become very small indeed even on Raulin's medium, so that work on its molecular constitution had to be abandoned. Fortunately we have recently discovered during an investigation of the metabolic products of *Penicillium cyclopium* Westling [Oxford and Raistrick, 1935] that this organism produces relatively large amounts of penicillic acid, and hence we have been enabled to complete the investigation. It is worthy of note that *P. puberulum* and *P. cyclopium* are not closely related, morphologically.

The significant properties of penicillic acid are as follows.

It crystallises from light petroleum as $C_8H_{10}O_4$, and from water in the hydrated form $C_8H_{12}O_5$ and is optically inactive.

The anhydrous acid yields only one active hydrogen atom (Zerewitinoff), it contains one acetylatable hydroxyl group and one methoxyl group (Zeisel).

The presence of at least one double bond is indicated by formation of a dibromo-derivative $C_8H_{10}O_4Br_2$ and of a dihydro-derivative separating from non-aqueous solvents as $C_8H_{12}O_4$ and from water as $C_8H_{14}O_5$ (*cf.* the analogous behaviour of penicillic acid). A second molecule of hydrogen is absorbed more slowly, but no crystalline tetrahydro-derivative could be isolated.

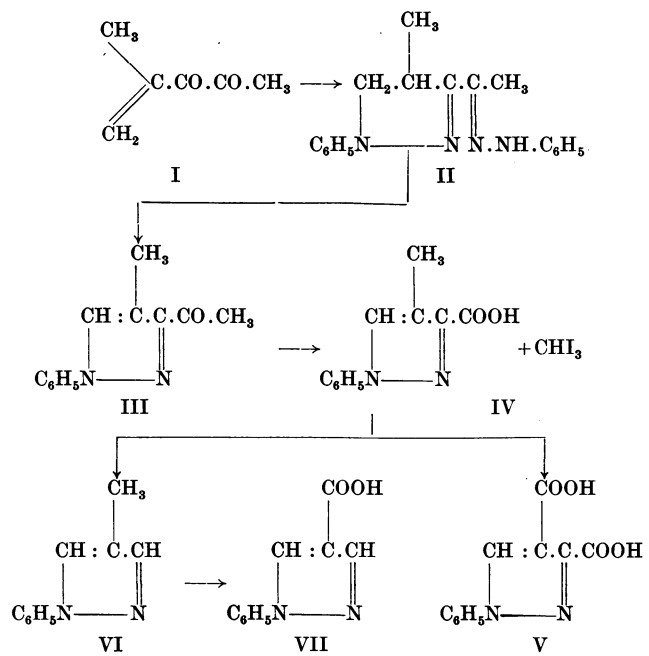
Penicillic acid probably contains a carboxyl or lactone group since it titrates as a monobasic acid and CO_2 is lost with comparative ease. Methylation of dihydropenicillic acid (diazomethane) gives an oily neutral methyl ester, $C_9H_{14}O_4$, whilst similar treatment of either anhydrous or hydrated penicillic acid leads to brisk evolution of nitrogen with formation of a neutral compound $C_{10}H_{14}O_4N_2$. The constitution of this compound is dealt with later (p. 397) but it is evident that during the reaction penicillic acid has combined with a molecule of diazomethane at a double bond in addition to gaining a methoxyl group by esterification of the acidic group.

Ozonisation of penicillic acid gives, in addition to other products to be discussed later, formaldehyde, indicating the presence of a terminal methylene grouping. Dihydropenicillic acid gives no formaldehyde with ozone indicating that the linkage of the terminal methylene group is reduced on hydrogenation.

Neither penicillic nor dihydropenicillic acid gives direct ketonic reactions in aqueous solution. On the other hand hydroxylamine titration of penicillic acid shows a rapid uptake of 1 mol. and a much slower uptake of a second mol. of hydroxylamine, whereas dihydropenicillic acid absorbs no hydroxylamine under the same conditions.

Considerable light was thrown on the constitution of penicillic acid by a study of the yellow crystalline derivative obtained by the action of phenylhydrazine on penicillic acid and originally described by Alsberg and Black. This product has the empirical formula $C_{18}H_{20}N_4$ (II) and gives a strong Knorr's reaction for pyrazolines (an intense purple colour with sulphuric acid and potassium dichromate). It is evidently a compound of 2 mols. of phenylhydrazine with a breakdown product $C_6H_8O_2$ (I), which is derived from penicillic acid $C_8H_{10}O_4$ by loss of CH_2 (*i.e.* a methoxyl group) and CO_2 (*i.e.* the carboxyl group). The empirical formula $C_{18}H_{20}N_4$ (II) agrees with the initial production of a bisphenylhydrazone of a diketone (or ketone-aldehyde or dialdehyde) containing one double bond, with subsequent rearrangement involving one phenylhydrazone grouping and leading to the formation of a pyrazoline ring to give compound (II). On oxidation of (II) with lead dioxide in acetic acid and subsequent treatment with sulphuric acid in the cold there is formed a colourless pyrazole $C_{12}H_{12}ON_2$ (III). Hydrolysis has thus eliminated 1 mol. of phenylhydrazine which must therefore have been combined with an aldehyde or ketone group (now liberated) in the form of a phenylhydrazone.

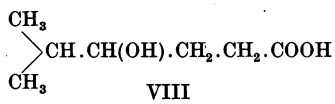
The pyrazole (III) on oxidation with alkaline iodine yields iodoform and a pyrazolemonocarboxylic acid $C_{11}H_{10}O_2N_2$ (IV), which is further oxidised by



potassium permanganate to a dicarboxylic acid, $C_{11}H_8O_4N_2$. This compound was shown by synthesis to be 1-phenylpyrazole-3:4-dicarboxylic acid (V). The substituents in the original pyrazole (III) must therefore be $\cdot CH_3$ and $\cdot CO \cdot CH_3$, and hence this pyrazole must be either 3-acetyl-4-methyl- or 4-acetyl-3-methyl-1-phenylpyrazole. The position to be allotted to each substituent was settled by decarboxylation of the pyrazolemonocarboxylic acid (IV). The resulting 1-phenylmethylpyrazole (VI) on oxidation with potassium permanganate gave a 1-phenylpyrazolecarboxylic acid (VII), M.P. 219–221°. This M.P. is assigned in the literature (*cf.* Wislicenus and Bindemann, [1901]) to the 1-phenylpyrazole-4-carboxylic acid, the 3- and 5-carboxylic acids having M.P. 146° and 183° respectively. Thus the original methyl group in (III) is in the 4-position, and hence (III) must be 3-acetyl-1-phenyl-4-methylpyrazole. The breakdown product $C_6H_8O_2$ (I) from which it arises is therefore $\beta\gamma$-diketo-δ-methylenepentane. The reactions are formulated above.

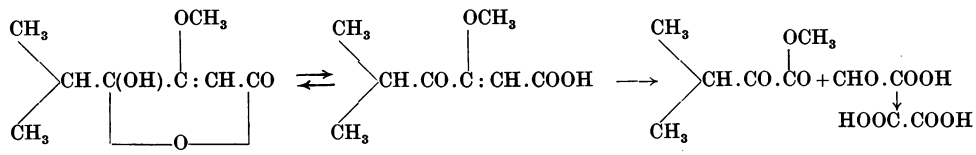
It now remains to decide the positions of attachment in the $\beta\gamma$-diketo-δ-methylenepentane of the methoxyl and carboxyl groups which are eliminated from penicillic acid by treatment with phenylhydrazine.

The carboxyl group would be expected to occupy a position β to one of the keto-groups in order to explain its ready removal. This was confirmed and its position was fixed by a study of the reduction products obtained by the action of hydriodic acid on dihydropenicillic acid. A lactone was obtained in small yield giving a crystalline phenylhydrazide, which was shown by synthesis to be the phenylhydrazide of γ-hydroxy-δ-methylhexanoic acid (VIII).



VIII

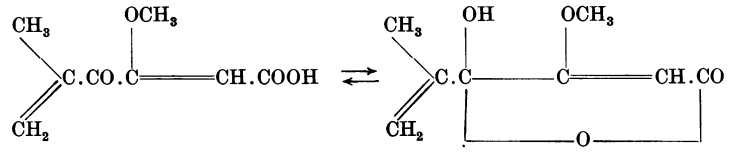
The position of the methoxyl group in penicillic acid was decided from a study of the oxidation of dihydropenicillic acid (IX and X) with potassium permanganate and with ozone to methyl dimethylpyruvate (XI) which was isolated as its 2:4-dinitrophenylhydrazone. Permanganate yields oxalic acid in addition. The methoxyl group is therefore attached to the β-carbon atom in the $\beta\gamma$-diketo-δ-methylenepentane structure (I). We must therefore suppose that the β-keto-group is methylated in dihydropenicillic acid (IX and X) in its enol form which would give a double linking between the α- and β-carbon atoms. Hence dihydropenicillic acid (keto-form X) contains both a potential β-keto- and a true γ-keto-group, so that any reaction which would demethylate penicillic acid would thereby render it susceptible to decarboxylation. The other product of the oxidation should be glyoxylic acid; assuming that this is further oxidised to oxalic acid by the excess of oxidising agent present, we may formulate the reaction



IX X XI

The course of the oxidation is good evidence for a double linking between the α- and β-carbon atoms. Further, since oxidation of penicillic acid with ozone yields formaldehyde the parent substance must contain a terminal $=CH_2$ group.

The constitution of anhydrous penicillic acid may therefore be written, in its simplest form, as a substituted γ-keto-acid of structure (XII). In this form penicillic acid is thus γ-keto-β-methoxy-δ-methylene-Δ^{α}-hexenoic acid. In order to explain its many properties we must also postulate that it can exist as a γ-hydroxylactone of structure (XIII).



XII Penicillic acid XIII

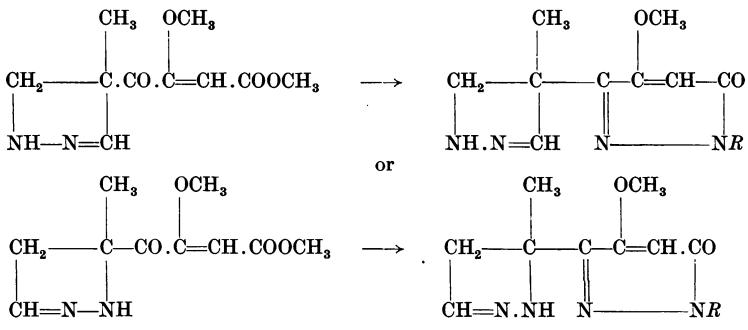
Chemical evidence for the existence of the keto-form (XII) with a free carboxyl group is afforded by the ready esterification of anhydrous penicillic acid with diazomethane and by the ready reaction of penicillic acid with one molecule of free hydroxylamine in the cold in aqueous solution.

Evidence for the lactone form (XIII) is found in the production of a neutral acetyl derivative by the action of acetic anhydride on penicillic acid.

Analogies for the existence of γ-keto-acids in tautomeric forms are to be found in the behaviour, *e.g.*, of laevulic acid, maleic aldehydo-acid and the trialkyl-succinaldehydo-acids (for discussion see Houben-Weyl [1930]). We therefore feel justified in postulating that penicillic acid is capable of existence in two tautomeric forms, (XII) and (XIII).

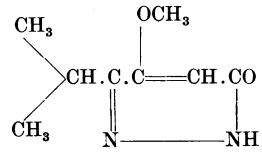
In the case of dihydropenicillic acid we again have evidence for both carboxylic and lactone forms (IX and X). This compound reacts with diazomethane to give a methyl ester but in aqueous solution is an extremely weak acid and does not react with hydroxylamine in the cold. Dihydropenicillic acid, unlike penicillic acid, apparently exists almost entirely in the lactone form in aqueous solution.

The action of diazomethane on penicillic acid, giving rise to the compound $C_{10}H_{14}O_4N_2$ referred to on p. 394, is readily explained from formulae (XII) and (XIII). A pyrazoline ring has been formed by the addition of CH_2N_2 at the double bond in the δ-methylene group to give (XIV). When (XIV) is treated with hydrazine or phenylhydrazine, crystalline products are obtained having the empirical formulae $C_9H_{12}O_2N_4$ and $C_{15}H_{16}O_2N_4$ (XV) respectively, which correspond with the addition of the constituents and loss of methyl alcohol. We therefore postulate that an orthodiazine ring is formed, the product being a pyridazone. This reaction is characteristic of γ-ketocarboxylic acids and is the analogue of pyrazolone formation by β-keto-esters.



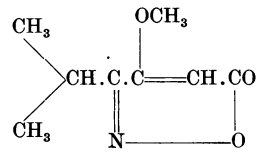
XIV (R=H or C_6H_5) XV

The position of attachment of diazomethane to penicillic acid follows from the fact that dihydropenicillic acid (IX and X) reacts normally with diazomethane to give a methyl ester containing no nitrogen. This ester reacts with hydrazine hydrate with the loss of 1 mol. each of CH_3OH and H_2O and the formation of a ring compound of structure (XVI).



XVI

On heating penicillic acid with hydroxylamine, demethylation and decarboxylation of the acid occur and a compound, possibly the dioxime of $C_6H_8O_2$ (I), is formed, a reaction which would be analogous to the first stage in the reaction with phenylhydrazine. Under the same conditions dihydropenicillic acid and hydroxylamine yield a crystalline product $C_8H_{11}O_3N$ (XVII) which gives no aldoxime or ketoxime reactions. Since it contains all the C atoms of dihydropenicillic acid including the methoxyl group, it almost certainly arises by ring closure between an oxime group formed initially and the carboxyl group, to give an orthoxazine ring structure (XVII).



XVII

This reaction is typical of γ-keto-acids and indicates that dihydropenicillic acid can behave as though it possessed such a structure.

Penicillic acid when written in the lactone form (XIII) bears a superficial resemblance to the tetronic acid structure, which has been shown to occur [Clutterbuck *et al.*, 1935] in several metabolic products obtained from *P. Charlesii*. In tetronic acid however the acidity is derived from the hydroxyl group attached to the β-carbon atom, whilst in penicillic acid this group is methylated and cannot therefore be acidic. Hence we must suppose that the acidity of penicillic acid is due to a true carboxyl group obtained by the opening of the lactone ring as indicated in the foregoing argument.

Penicillic acid is almost unique among natural products in that it is an aliphatic open-chain compound, which, although not a methyl ester, yet contains a methoxyl group. The only similar natural products appear to be the sugars digitalose and cymarose. The latter, which occurs in the cardiac glycosides cymarin and periplocymarin, has definitely the constitution

$$CH_3.CHOH.CHOH.CH(OCH_3).CH_2.CHO$$

[Elderfield, 1935], which bears some resemblance to penicillic acid. Finally, one other mould metabolic product containing a terminal $=CH_2$ group, *viz.* itaconic acid, has recently been obtained from *Aspergillus itaconicus* by Kinoshita [1931].

Experimental.

Preparation of penicillic acid.

(*a*) *From* P. puberulum *Bainier.*

Culture. A subculture of Alsberg and Black's strain of *P. puberulum* Bainier was received in October 1928 from Dr Charles Thom—Ardeer Catalogue No. Ad 113.

35 l. of Raulin medium of the following composition were made up and distributed equally between 100 one-litre conical flasks: glucose, 75 g.; tartaric acid, 4 g.; NH_4NO_3, 4 g.; $(NH_4)_2HPO_4$, 0·6 g.; K_2CO_3, 0·6 g.; $MgCO_3$, 0·4 g.; $(NH_4)_2SO_4$, 0·25 g.; $ZnSO_4$, $7H_2O$, 0·07 g.; $FeSO_4$, $7H_2O$, 0·07 g.; distilled water, 1500 ml. The flasks were sterilised, inoculated with a spore suspension of the organism and incubated at 24° for 19–23 days. At the end of the incubation period the metabolism solution was filtered, evaporated *in vacuo* to small bulk, acidified and extracted with ether. The crystalline residue remaining after evaporation of the ether was recrystallised 2 or 3 times from hot water + blood charcoal.

The yield of penicillic acid was at first of the order of 5 g. per 100 flasks, but over a period of 3 years the yield fell until in 1931 no penicillic acid could be obtained. In its place a small yield of succinic acid was isolated.

Large colourless rhombic or hexagonal plates, M.P. 64–65°. (Alsberg and Black report the same M.P.) Loss in weight on drying *in vacuo* over P_2O_5, 9·69, 9·63 %. Theory for $C_8H_{10}O_4$, $H_2O \rightarrow C_8H_{10}O_4$, 9·58 %. M.P. of dehydrated material, 87°, which is in agreement with Alsberg and Black's figure. Alsberg and Black, however, report that penicillic acid, crystallised from water, contains $2H_2O$.

(Found (Schoeller) on anhydrous material: C, 56·39, 56·31; H, 6·09, 6·09 %. Equiv. by titration, 169, 172. $C_8H_{10}O_4$ requires C, 56·44; H, 5·93 %. Equiv. titrating as a monobasic acid, 170. Found OCH_3 17·76 %; $C_7H_7O_5.OCH_3$ requires 18·24 %.) The general properties and reactions of the substance, which will be discussed later, are in agreement with those described by Alsberg and Black for penicillic acid, and hence we consider that no reasonable doubt exists as to the identity of the two substances.

(*b*) *From* P. cyclopium *Westling.*

Culture. The culture of *P. cyclopium* used (L.S.H.T.M. Catalogue No. P. 123) was purchased from the Centraalbureau voor Schimmelcultures, Baarn, in August 1931. It was derived from Westling's original strain.

A Raulin-Thom medium was used identical in composition with the Raulin medium used for *P. puberulum* except that 4 g. diammonium tartrate were used in place of 4 g. ammonium nitrate. Cultural conditions were also the same, 10–14-day old cultures on Czapek-Dox agar slopes being employed for inoculation. The flasks were incubated at 24° for 18–23 days. The course of metabolism and details of large scale preparations are summarised in Table I, from which it is evident that this mould has maintained its activity in full for a year.

Although penicillic acid does not appear to be metabolised by this mould, as is evident from a consideration of the bromine absorption figures, the metabolism solutions were worked up when about 0·2 % of glucose still remained, because of the danger of destruction of penicillic acid during evaporation at the alkaline reaction produced after a longer incubation period. Growth of the mould was fairly rapid, a good white surface felt being formed in 5 days which later became much wrinkled and covered with green patches. The reverse was brown and the metabolism solution became progressively darker in colour as incubation continued.

Table I.

Date of inoculation	Incubation period in days	% glucose by polarimeter	p_H	Titratable acidity ml. $N/10$ NaOH per 10 ml.	Br absorption mg./ml.
29. x. 34	—	5·35	3·9	2·5	0·10
,,	5	4·00	Below 3	5·1	0·72
,,	10	1·68	,,	6·5	4·96
,,	14	1·06	,,	5·9	6·4
,,	28	Nil	6·2	Negligible	8·8
,,	33	Nil	Alkaline	—	8·3
10. xii. 34 (Average of 100 flasks)	23	0·10	4·8	—	7·6
6. vi. 35 (Average of 100 flasks)	18	0·20	Below 3	—	7·54
18. ix. 35	20	0·20	,,	—	8·0

The metabolism solution from 100 flasks was evaporated *in vacuo* to 500 ml. The penicillic acid crystallising out overnight was collected and recrystallised from 300 ml. boiling water (norite). The crystals, which still contained some potassium hydrogen tartrate, were filtered, washed and completely dried *in vacuo*. They were then extracted with a minimum volume of hot chloroform (100–200 ml.) and 20 volumes of light petroleum were added. The various aqueous filtrates still contained some penicillic acid which could be recovered by extraction with chloroform. Yield of pure hydrated penicillic acid, 73 g. per 100 flasks, corresponding to 52 % of the amount calculated from the bromine absorption figures and 4·2 % of the glucose metabolised.

On crystallisation from light petroleum, B.P. 60–80°, penicillic acid crystallised in needles, M.P. 83–84°, alone or mixed with anhydrous penicillic acid obtained from *P. puberulum*.

(Found (Schoeller): C, 56·46, 56·43; H, 5·93, 5·91; OCH_3, 18·17, 17·82 %. Mol. wt. cryoscopic in dioxan, 176. $C_8H_{10}O_4$ requires C, 56·44; H, 5·93; OCH_3, 18·24 %. Mol. wt. 170.)

In a Zerewitinoff estimation (Roth) it gave 0·94, 1·09 mols. of CH_4 in pyridine and 0·98, 1·08 mols. in anisole at 18° and 95° in each case respectively.

The above product separated from water in rhombic or hexagonal plates, M.P. 58–64°, alone or mixed with hydrated penicillic acid from *P. puberulum*. Equiv. by titration, 188. $C_8H_{12}O_5$ requires 188. Mol. wt. cryoscopic in dioxan, 97·5. The hydrated acid is evidently almost completely dissociated into $C_8H_{10}O_4$ and H_2O in dioxan solution since the theoretical mol. wt. is 188.

In a Zerewitinoff estimation (Roth) it gave 1·91, 2·05 mols. of CH_4 in pyridine, and 1·65, 1·96 mols. in anisole at 18° and 95° in each case respectively.

This strain of *P. cyclopium* yields no penicillic acid when grown on Czapek-Dox medium with glucose as sole source of carbon and $NaNO_3$ as sole source of nitrogen. Further, a strain of *Penicillium* believed to be *P. cyclopium* Westling, freshly isolated from infected tulip bulbs by our colleague Mr G. Smith, failed to yield penicillic acid even when grown on Raulin-Thom medium.

Penicillic acid is however undoubtedly a product of the metabolism of glucose and not of tartaric acid, since Birkinshaw and Raistrick [1932] obtained it with Alsberg and Black's strain of *P. puberulum* when grown on Czapek-Dox medium containing glucose as the sole source of carbon.

General properties of penicillic acid.

Penicillic acid is soluble in cold water to about 2 %; it is readily soluble in hot water, alcohol, ether, benzene and chloroform and insoluble in cold light petroleum. An aqueous solution just blues Congo red paper and decomposes

carbonates. It gives no colour with aqueous $FeCl_3$ in the cold and only an orange brown colour on warming. As stated by Alsberg and Black it gives a reddish-purple colour with ammonia on standing. This reaction is fairly delicate since a 0·05 % solution gives a perceptible pink colour with 2–3 vols. of strong ammonia. It gives only a yellow colour with primary amines. It gives no colour with concentrated H_2SO_4 in the cold and chars on heating. It gives no colour with $NaNO_2$ (γ-methyltetronic acid and certain of its derivatives give a blue colour with this reagent). It gives no colour with Fearon's nitrochromic acid reagent [Fearon and Mitchell, 1932] and hence does not contain a primary or secondary alcoholic group. A neutral aqueous solution of the sodium salt gives only a green precipitate with $CuSO_4$ solution and no reduction to Cu_2O takes place on boiling. Hence penicillic acid does not contain the —CHOH·CO— or —CO·CO— grouping.

If penicillic acid is a $\beta\gamma$-unsaturated lactone it should reduce Tollens's reagent and give an immediate reaction with sodium nitro-prusside [Jacobs and Hoffmann, 1926]. Actually, it reduces ammoniacal silver in $\frac{1}{2}$–1 hour in the cold, but the reaction with nitroprusside (under the conditions given by Jacobs and Hoffmann, *viz.* in aqueous pyridine solution to which a few drops of dilute caustic soda have been added) is not immediate, but a deep, reddish brown colour develops in a few minutes. An almost immediate deep red colour is however obtained under the following modified conditions; before adding the nitroprusside, the alkaline pyridine solution of penicillic acid is kept for 5 min. or longer. The red colour then obtained with nitroprusside fades to yellow on long standing. Penicillic acid is therefore probably not a $\beta\gamma$-unsaturated lactone but may be changed into one by the action of alkali. One of the two isomeric structures we have assigned is that of an $\alpha\beta$-unsaturated lactone.

Ketonic reactions. Neither penicillic acid nor its dihydro-derivative dissolved in 2*N* HCl gives any precipitate with 2:4-dinitrophenylhydrazine in 2*N* HCl, except on long standing. When penicillic acid was treated with an excess of hydroxylamine hydrochloride and the solution titrated at intervals with *N*/10 NaOH (bromophenol blue as indicator) 2 equiv. of hydroxylamine were absorbed in 291 hours. The reaction proceeds much more quickly in presence of free hydroxylamine. One mol. of the latter rapidly combines at room temperature, as shown in a number of experiments in which penicillic acid hydrate (0·113 g.) in 5 ml. H_2O was added to 5 ml. of 25 % hydroxylamine hydrochloride and 20 ml. of *N*/10 NaOH. The HCl liberated, as determined by back-titration with *N*/10 HCl, after 10, 20, 30, 60, 180 and 360 min. was 3·7, 5·7, 7·0, 8·4, 10·2 and 10·8 ml. *N*/10 respectively, the theoretical value for the uptake of 1 mol. of hydroxylamine being 6·0 ml. This titration is possible since the sodium salt of penicillic acid behaves as free NaOH towards bromophenol blue. The slow uptake of a second mol. of hydroxylamine is probably due to demethylation with formation of a second keto-group.

In corresponding experiments with dihydropenicillic acid there was no uptake of hydroxylamine during 360 min.

Penicillic acid gives the iodoform reaction with hypoiodite solution.

We have confirmed the observation of Alsberg and Black that penicillic acid is optically inactive.

Derivatives of penicillic acid.

Dihydropenicillic acid. When hydrogenated in aqueous solution using a Pd-norite catalyst, penicillic acid rapidly absorbed 1 mol. of hydrogen (in 2–3 min.) and then gradually absorbed a second mol. in about 7 hours. With the

addition of $2H_2$ only non-crystallisable oils were obtained but the addition of 1 mol. of hydrogen gave a homogeneous crystalline product.

Hydrated penicillic acid (5 g.) was dissolved in ethyl alcohol (60 ml.) and about 30 ml. of water were added together with the catalyst prepared from 0·3 g. of $PdCl_2$. The mixture was allowed to absorb 596 ml. (corr. N.T.P.) of hydrogen (theoretical for 1 mol.). It was filtered from the catalyst and evaporated to dryness *in vacuo*. The crystalline residue was taken up in ether and treated with 5 volumes of light petroleum (B.P. 50–60°). Colourless needles, 3·7 g., M.P. 86°.

(Found (Schoeller): C, 56·06, 55·93; H, 7·14, 7·02 %. $C_8H_{12}O_4$ requires C, 55·80; H, 7·03 %.)

Dihydropenicillic acid crystallises from water in colourless flat needles, M.P. 62–64°, which contain 1 H_2O. (Found: loss on drying *in vacuo* over concentrated H_2SO_4, 9·64 %. $C_8H_{12}O_4.H_2O$ requires H_2O, 9·48 %.) The anhydrous substance so obtained melts at 83–85°.

Dihydropenicillic acid hydrate is less soluble in cold water than penicillic acid hydrate and is a much weaker acid, having no action on Congo red paper but turning blue litmus red.

Penicillic acid dibromide. A solution of Br (2·5 g. ≡ 2 atoms Br) in glacial acetic acid (100 ml.) was slowly added at room temperature to a solution of hydrated penicillic acid (2·834 g.) in the same solvent (30 ml.). The reaction, slow at first, later became very rapid. No HBr was evolved. The mixture was evaporated to dryness in a vacuum desiccator over solid KOH. Yield almost theoretical, M.P. 154°. Recrystallised from CCl_4, colourless slender needles, M.P. 154–5°.

(Found (Schoeller): C, 29·25, 29·24; H, 3·19, 3·20; Br, 48·85, 48·80 %. Equiv. by titration 326. $C_8H_{10}O_4Br_2$ requires C, 29·10; H, 3·05; Br, 48·44 %. Equiv. (monobasic) 330.)

On bromination in aqueous or 50 % aqueous acetic acid solution penicillic acid absorbs four atoms of Br, but complex reactions take place and result in partial breakdown.

Acetylpenicillic acid. Hydrated penicillic acid (0·5 g.), anhydrous sodium acetate (1·0 g.) and acetic anhydride (2 ml.) were heated together in a bath at 140° for 10 min. and the product was poured into water. An oil separated. After 24 hours the product was extracted with ether, the ether removed and the residue dried *in vacuo* over KOH and H_2SO_4. On standing it set to a crystalline mass, which was recrystallised twice from light petroleum.

Colourless elongated prisms, M.P. 72°. (Found (Schoeller): C, 56·81, 56·68; H, 5·95, 5·72; OMe, 14·01, 14·18 %. $C_{10}H_{12}O_5$ requires C, 56·58; H, 5·71; OMe, 14·63 %.) The product is therefore a monoacetyl derivative of anhydrous penicillic acid.

Action of diazomethane on penicillic acid.

(*a*) *The pyrazoline methyl ester of penicillic acid* (*XIV*). Anhydrous penicillic acid (1 g.) was treated with excess of diazomethane in ether. A vigorous effervescence occurred. After removal of the ether the residue set to a mass of colourless crystals (prisms) which were recrystallised from light petroleum (50–60°); M.P. 74°, not raised on further recrystallisation; yield 1·0 g.

(Found (Schoeller): C, 53·26, 53·04; H, 6·21, 6·03; N, 12·29, 12·20; macro-Zeisel, OCH_3, 27·25, 27·29 %. $C_{10}H_{14}O_4N_2$ requires C, 53·08; H, 6·24; N, 12·40; $2OCH_3$, 27·45 %.)

(*b*) *Pyridazone* (*XV*). The pyrazoline ester (XIV) (0·5 g.) in ethyl alcohol (2 ml.) was treated with 0·5 ml. of 50 % aqueous hydrazine hydrate. Considerable

heat was evolved. Colourless crystals separated overnight, 0·15 g., M.P. 174° (decomp.). After recrystallisation from alcohol they appeared to disintegrate without sintering at 175° and melted at 181° with decomposition.

(Found (Schoeller): C, 51·77, 51·87; H, 5·88, 5·78; N, 27·25, 27·19; OCH_3, 14·71, 14·87 %. $C_9H_{12}O_2N_4$ requires C, 51·88; H, 5·81; N, 26·92; one OCH_3, 14·90 %.)

(*c*) *Phenylpyridazone* (*XV*). The pyrazoline ester (XIV) (0·6 g.) was heated with phenylhydrazine hydrochloride (0·9 g.) and 1 drop of conc. HCl in a few ml. of alcohol for about 15 min. on the water-bath. There was some action, indicated by the production of a yellow colour. On dilution of a test sample with water practically all dissolved. Therefore 1 g. of crystalline sodium acetate was now added and the heating was continued for 15–20 min. longer. On cooling, 0·58 g. of somewhat dark-coloured crystalline material was obtained, which on warming with about 30 ml. of alcohol partly dissolved, yielding a greenish solution changing to brown. A lemon-yellow crystalline substance remained undissolved. This was recrystallised from alcohol in which it was not very soluble. It was observed that on boiling with alcohol, the solution, at first lemon-yellow in colour, rapidly became emerald-green. The recrystallised material had M.P. 198–199°.

(Found (Schoeller): C, 63·06, 63·21; H, 5·59, 5·67; N, 19·84, 19·94; OCH_3, 10·53, 10·34 %. $C_{15}H_{16}O_2N_4$ (1 MeO) requires C, 63·34; H, 5·68; N, 19·72; OCH_3, 10·91 %.)

Action of diazomethane on dihydropenicillic acid.

(*a*) *Dihydropenicillic acid methyl ester.* Dihydropenicillic acid (0·65 g.) was treated with excess of diazomethane in ether for several hours. The solution was filtered and the ether evaporated. The product (0·68 g.) was a colourless, somewhat viscid oil with an ester-like smell. It was insoluble in H_2O but readily soluble in cold light petroleum. It contained no nitrogen. A Zeisel determination on the material after drying *in vacuo* gave 32·24 % OCH_3. Calc. for $C_9H_{14}O_4$ (2 methoxyl), 33·32 %. The material is therefore a true methyl ester of dihydropenicillic acid.

(*b*) *Action of hydrazine hydrate on the above ester.* The methyl ester (0·33 g.), alcohol (1·0 ml.) and 50 % hydrazine hydrate (0·40 ml.) were mixed and kept for several hours. Crystals (0·16 g.) separated. These were collected and washed with a little alcohol and recrystallised from absolute alcohol. Colourless prisms, M.P. 207–208°, of structure XVI.

(Found (Schoeller): C, 57·05, 57·22; H, 7·12, 7·15; N, 16·86, 16·75; macro Zeisel OCH_3, 19·02 %. $C_8H_{12}O_2N_2$ requires C, 57·11; H, 7·20; N, 16·66; OCH_3, 18·45 %.)

Action of dimedon on penicillic acid.

Penicillic acid (1 g.), dimedon (2 g.) and anhydrous sodium acetate (2 g.) were mixed with H_2O (25 ml.). The mixture, which gave a clear solution on warming, was heated at 100° for 1½ hours. An oil soon separated which crystallised on keeping. The product was recrystallised by solution in alcohol and cautious addition of water. M.P. 201–203°, with decomposition at 204°.

(Found (Weiler): C, 62·20, 62·33; H, 7·24, 7·41; macro-Zeisel, OCH_3, 10·09 %. $C_{16}H_{22}O_6$ requires C, 61·89; H, 7·15; 1 OCH_3, 10·00 %.) The product is thus formed by combination of 1 mol. of (anhydrous) penicillic acid, $C_8H_{10}O_4$ with 1 mol. of dimedon, $C_8H_{12}O_2$. It is not the condensation typical of aldehydes in which 2 mols. of dimedon are employed, and 1 mol. of water is lost.

Action of hydroxylamine on penicillic acid.

Hydrated penicillic acid (5 g.) was heated with hydroxylamine hydrochloride (5 g.) anhydrous sodium acetate (10 g.) and water (50 ml.) for 75 min. in boiling water. Colourless needles separated overnight, 2·35 g.; M.P. 205°, raised to 210–212° (decomp.), after recrystallisation from alcohol.

(Found (Weiler): C, 50·94, 50·99; H, 6·72, 6·78; N, 19·63, 19·65 %. $C_6H_{10}O_2N_2$ requires C, 50·66; H, 7·09; N, 19·72 %.) This would correspond with a dioxime of $C_6H_8O_2$ (I of introduction), derived by the decarboxylation and demethylation of penicillic acid.

Action of hydroxylamine on dihydropenicillic acid.

Dihydropenicillic acid (3 g.) was heated at 100° for 2 hours with hydroxylamine hydrochloride (3 g.), anhydrous sodium acetate (6 g.) and water (50 ml.). An oil separated which was extracted with ether. The ether solution was washed with aqueous sodium bicarbonate and was evaporated. The residue set to a solid crystalline mass which was again dissolved in a little ether and chilled rapidly by a current of air. The crystals separating were collected and washed with chilled ether. M.P. 54–56°.

(Found (Weiler): C, 56·78; H, 6·41; N, 8·21; OCH_3, 18·09 %. $C_8H_{11}O_3N$ requires C, 56·76; H, 6·56; N, 8·28; OCH_3, 18·34 %.) The product gave no reaction for aldoxime or ketoxime by oxidation with monopersulphuric acid and was not attacked by acetic anhydride or by PCl_5 suspended in ether. Hence it does not contain a hydroxyl group. It probably has the structure XVII.

Action of phenylhydrazine on penicillic acid. Investigation of resulting product.

Hydrated penicillic acid (20 g.) and crystallised sodium acetate (30 g.) were dissolved by gentle warming in 100 ml. of water. Phenylhydrazine (25 g.) and glacial acetic acid (15 ml.) were then added. A vigorous reaction occurred with considerable rise in temperature and much CO_2 was evolved. The reaction appeared to be complete in about 5 min. and a yellow tarry mass separated. After about an hour the aqueous liquid was poured off, the tar was washed once with water which was poured off and then 50 ml. of absolute alcohol were added. On warming, the whole of the tarry mass disappeared and was replaced by yellow crystals. The mixture was cooled and the crystals were collected and washed with a little alcohol. Yield, 13·5–14 g. of almost pure product. Yellow prisms, M.P. 176° (Alsberg and Black [1913] give 171°).

(Found (Schoeller): C, 73·96, 73·76; H, 6·89, 6·71; N, 19·21, 19·13 %. $C_{18}H_{20}N_4$ requires C, 73·94; H, 6·90; N, 19·17 %.)

The substance is a phenylpyrazoline phenylhydrazone (II of introduction) of $C_6H_8O_2$ (I). It gives an intense purple colour with H_2SO_4 and $K_2Cr_2O_7$ (Knorr's reaction for pyrazolines), and also with 50 % H_2SO_4 + aqueous $FeCl_3$. With H_2SO_4 alone it gives an intense green colour.

(*a*) *Oxidation of the pyrazoline (II) to a pyrazole (III).* 7 g. of (II) were dissolved by warming in glacial acetic acid (175 ml.) and the solution was cooled rapidly to 40° (incipient crystallisation). Lead dioxide (15 g.) was gradually added, the flask being well shaken and cooled after each addition so that the temperature did not rise appreciably above 40°. During the addition of PbO_2 the colour of the solution changed to a dark green, later becoming brown.

The solution was treated with 175 ml. of 50 % (by volume) H_2SO_4 and left overnight. Next day 350 ml. of water were added and the lead sulphate was

removed by filtration. The filtrate was treated with 500 ml. of water and chilled. Colourless crystals separated, 0·76 g., M.P. 67°. The solution was made slightly alkaline and extracted with ether. The ether extract (0·77 g.) crystallised on keeping. A further 2·65 g. of syrup, later crystallising, was obtained by extraction with ether of the lead sulphate precipitate. The three products were combined, recrystallised from light petroleum and sublimed *in vacuo* giving colourless crystals, M.P. 72–74°.

(Found (Weiler): C, 72·02; H, 5·83; N, 14·14 %. $C_{12}H_{12}ON_2$ requires C, 71·97; H, 6·08; N, 14·01 %.) This corresponds with a phenylpyrazole derived from the substance $C_6H_8O_2$ (I) and phenylhydrazine. One phenylhydrazine group, obviously present as a phenylhydrazone, has been removed by hydrolysis from (II), and the pyrazoline ring has been oxidised to a pyrazole ring to give (III). (III) gave a strong Zimmermann reaction for the $\cdot CO \cdot CH_2$ group but gave no Schiff or Angeli-Rimini reaction for aldehydes. On the other hand it readily yielded an oxime. The oxygen is therefore present as a keto-, not as an aldehyde, group.

(*b*) *Preparation of the oxime of the pyrazole* (*III*). 0·64 g. of (III) was dissolved in alcohol (5 ml.) and hydroxylamine hydrochloride (0·25 g.) and crystalline sodium acetate (0·5 g.) were added in 3 ml. of water. The clear solution almost immediately began to deposit crystals. The mixture was warmed on the water-bath for 20 min. The crystals obtained on cooling were collected and washed with 2 ml. of 50 % aqueous alcohol; yield 0·49 g.; M.P. 149° not raised on sublimation *in vacuo*.

(Found (Weiler): C, 67·03; H, 6·05; N, 19·41 %. $C_{12}H_{13}ON_3$ requires C, 66·95; H, 6·09; N, 19·54 %.)

The only possible substituents of the pyrazole ring (apart from the 1-phenyl group), assuming that a keto-group is present, are $\cdot CO \cdot CH_2 \cdot CH_3$, $\cdot CH_2 \cdot CO \cdot CH_3$ or $\cdot CO \cdot CH_3 + \cdot CH_3$. The first of these is excluded by the fact that (III) is readily oxidised by hypoiodite solution, with production of iodoform, to a pyrazole-carboxylic acid (IV) having one carbon atom less than the original pyrazole (III).

(*c*) *Oxidation of the pyrazole* (*III*) *with hypoiodite to a monocarboxylic acid* (*IV*). The sublimed pyrazole (0·44 g.) was dissolved in methyl alcohol (500 ml.). Water (500 ml.) was added, followed by *N*/10 iodine (500 ml.) and *N*/10 NaOH (600 ml.). The mixture was left overnight. The crystalline iodoform was removed by filtration and the filtrate was acidified with 2*N* H_2SO_4 (50 ml.). Thiosulphate solution was then added to remove all free iodine. The solution was extracted with ether and the ether solution shaken with aqueous Na_2CO_3, which was then acidified and extracted with ether. On removal of the solvent 0·43 g. acid was obtained, M.P. after sublimation 170°.

(Found (Weiler): C, 65·22; H, 4·98; N, 13·78 %. $C_{11}H_{10}O_2N_2$ (IV) requires C, 65·31; H, 4·99; N, 13·87 %.)

(*d*) *Oxidation of* (*IV*) *with alkaline* $KMnO_4$ *to give 1-phenylpyrazole-3:4-dicarboxylic acid* (*V*). 0·7 g. of (IV) was neutralised with aqueous *N*/10 NaOH and to the hot solution was added, drop by drop, $KMnO_4$ (1·5 g.) dissolved in water (200 ml.). The mixture was heated at 100°. The precipitated MnO_2 was separated by filtration and the filtrate acidified. An immediate precipitate (0·26 g.) proved to be unchanged (IV), which was separated. The filtrate on evaporation *in vacuo* yielded impure (V) mixed with a little (IV), which was removed by fractional vacuum sublimation. (V) was finally crystallised from water to give colourless elongated plates, M.P. 232°.

(Found (Schoeller): C, 56·98, 56·97; H, 3·67, 3·62; N, 12·09, 11·93 %. Equiv. by titration (end-point not sharp to phenolphthalein), 127–118. $C_{11}H_8O_4N_2$ (V) requires C, 56·87; H, 3·47; N, 12·07 %. Equiv. 116.)

The acid (V) gave no depression in M.P. when mixed with synthetic 1-phenylpyrazole-3:4-dicarboxylic acid (recorded M.P. 234°; Balbiano [1898]).

Synthesis of 1-phenylpyrazole-3:4-dicarboxylic acid. 1-Phenyl-3:4-dimethylpyrazole (2·2 g.) obtained from 1-phenyl-3:4-dimethylpyrazolone by the method of Stoermer and Martinsen [1907] was heated with $KMnO_4$ (13 g.) in water (400 ml.) under reflux at 100°. When all the $KMnO_4$ was reduced, the solution was filtered and evaporated on the water-bath to small volume. The dicarboxylic acid, mixed with 1-phenyl-3-methylpyrazole-4-carboxylic acid was separated by acidification and was purified by fractional vacuum sublimation and crystallisation from a mixture of ethyl acetate and light petroleum; colourless plates, M.P. 232° alone or mixed with the product (V) from penicillic acid. (Found (Schoeller): C, 56·85; H, 3·47; N, 12·32 %. $C_{11}H_8O_4N_2$ requires C, 56·87; H, 3·47; N, 12·07 %.) *Dimethyl ester.* The product obtained by the action of diazomethane on the above acid was recrystallised from ether-light petroleum; colourless plates, M.P. 89° alone or mixed with the corresponding product from penicillic acid.

(*e*) *Decarboxylation of 1-phenyl-4-methylpyrazole-3-carboxylic acid (IV) to give 1-phenyl-4-methylpyrazole (VI).* The acid (IV) (0·4 g.) was heated under reflux (air condenser) in a bath at 250–260° for 30 min. after which no more evolution of gas could be observed. On cooling the residue set solid. This was taken up in ether, washed with aqueous sodium carbonate and the ether was evaporated. The crystalline residue weighed 0·28 g. This was dissolved in light petroleum (B.P. 40–50°) in which it was extremely soluble. It was crystallised by cooling in solid CO_2. The substance had M.P. 43°, and analysed correctly for 1-phenyl-4-methylpyrazole. (In the literature this substance is given as a liquid.)

(Found (Schoeller): C, 75·78; H, 6·29; N, 17·83 %. $C_{10}H_{10}N_2$ (VI) requires C, 75·90; H, 6·37; N, 17·72 %.)

(*f*) *Oxidation of 1-phenyl-4-methylpyrazole (VI) with $KMnO_4$ to 1-phenylpyrazole-4-carboxylic acid (VII).* 0·16 g. of (VI) was oxidised at 100° with 0·5 g. (50 % excess) of $KMnO_4$ dissolved in water. When all the $KMnO_4$ had disappeared there was still unchanged (VI) present. The solution was filtered from manganese dioxide and unchanged material and was acidified. The crystalline precipitate was collected, 0·05 g.; M.P. on sublimed sample, 219–221°.

(Found (Schoeller): C, 63·96; H, 4·38; N, 14·69 %. Equiv. by titration = 190. $C_{10}H_8O_2N_2$ (VII) requires C, 63·80; H, 4·29; N, 14·89 %. Equiv. = 188.)

Action of HI on penicillic acid.

Anhydrous penicillic acid (5 g.) was heated with HI (sp. gr. 1·7, 50 ml.) and red phosphorus (2 g.) for 2½ hours under an air condenser; bath temperature 160°. The cooled reaction mixture was diluted, extracted with ether, free iodine removed by shaking with aqueous thiosulphate, and the ether solution evaporated. The residue was heated for 45 min. with zinc (20 g.) and 6*N* HCl (50 ml.) under reflux to remove combined iodine and then extracted with ether. Weight 1·10 g. of an oil which was neutralised with 6·25 ml. *N* NaOH and converted into the *p*-phenylphenacyl ester by heating with water (10 ml.), alcohol (30 ml.) and *p*-phenylphenacyl bromide (1·72 g.); colourless needles from light petroleum M.P. 63°.

(Found (Schoeller): C, 74·64, 74·74; H, 6·58, 6·65 %. OCH_3, nil. $C_{21}H_{22}O_4$ requires C, 74·50; H, 6·56 %.)

This represents the *p*-phenylphenacyl ester of an acid $C_7H_{12}O_3$ which in the light of the results obtained on the action of HI on dihydropenicillic acid (see next section) is believed to be $CH_3(CH_2:)C\cdot CHOH\cdot CH_2\cdot CH_2\cdot COOH$.

Action of HI on dihydropenicillic acid.

Dihydropenicillic acid (20 g.) was heated with HI (sp. gr. 1·7, 150 ml.) and red phosphorus (8 g.) for 2½ hours. The details of the isolation of the crude reduction product were the same as in the previous section.

The product was then divided into acid, lactone and neutral fractions. By shaking the ether solution with aqueous Na_2CO_3 the acids were removed. They were regenerated by acidification of the aqueous layer and weighed 1·71 g. The ethereal layer was evaporated and the product was heated for 30 min. on the water-bath with an excess of *N* NaOH. The neutral substances extracted by ether weighed 0·27 g. The lactones were regenerated by acidification of the aqueous layer and extracted with ether; weight 2·2 g. 2 g. of the lactones were distilled at ordinary pressure and yielded a trace of water and 0·86 g. of liquid distilling at 217–218°. There was a considerable amount of higher-boiling residue. The oil, B.P. 217–218°, was converted into the phenylhydrazide by heating under reflux with 0·75 g. of phenylhydrazine in 2–3 ml. of absolute alcohol for about 3 hours. The product yielded crystalline plates on keeping exposed to the air. These were treated with a little ether, collected and washed with ether and recrystallised by solution in a small amount of absolute alcohol and addition of several volumes of ether; plates, M.P. 126°. There was no depression in M.P. when this material (structure VIII) was mixed with synthetic γ-hydroxy-δ-methylhexanoic acid phenylhydrazide (*v. infra*).

(Found (Schoeller): C, 65·77, 65·94; H, 8·46, 8·64; N, 12·54 %. $C_{13}H_{20}O_2N_2$ requires C, 66·07; H, 8·50; N, 11·87 %.)

Synthesis of γ-hydroxy-δ-methylhexanoic acid phenylhydrazide. Ethyl δδ-dicarbethoxy-β-keto-αα-dimethylvalerate (20 g., B.P. 150°/1 mm.; *cf.* Conrad [1897]) was heated with 2*N* H_2SO_4 (250 ml.) under reflux, in an oil bath at 120°. The CO_2 was driven out by a current of CO_2-free air and absorbed in potash bubblers and weighed. The hydrolysis was practically complete in 24 hours. The product was extracted with ether and transferred to sodium carbonate solution. It was liberated by HCl and again extracted with ether. After removal of the ether it set to a solid mass of crystals, weight 9·3 g. This material dissolved in alcohol (70 ml.) was heated to boiling, and sodium (15 g.) was added during 10 min. [*cf.* Losanitsch, 1914]. Then 130 ml. of alcohol were gradually added so as to dissolve the remainder of the sodium in 20–30 min. The solution was cooled and treated with 130 ml. of water and alcohol was removed *in vacuo*. The residue was treated with 60 ml. of a mixture of 50 g. of sulphuric acid and 50 ml. of water (total volume of mixture 72 ml.). The product was boiled for 5 min., cooled and extracted with ether. The oil (7·15 g.) on distillation gave 6·35 g. of product of B.P. 227–230° at atmospheric pressure.

The lactone (3·25 g.), phenylhydrazine (2·7 g.) and alcohol (5 ml.) were heated together on a steam-bath under reflux for 2 hours. The product was cooled and treated with much ether. It gave 1·16 g. of crystals which, after recrystallisation from alcohol-ether, appeared as glistening plates, M.P. 126° alone or mixed with the corresponding compound from penicillic acid.

Oxidation of penicillic acid by ozone. Production of formaldehyde and a keto-acid $C_7H_8O_5$.

Ozonised oxygen was passed into a solution of hydrated penicillic acid (0·994 g.) in water (55 ml.) at 0–5° for 5–6 hours. A considerable amount of unchanged acid crystallised out during the experiment. The mixture was kept overnight, and a test on the filtered solution showed that an aldehyde was present

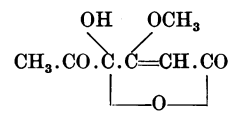
(colour restored to Schiff's reagent in less than 2 min.). An equal volume of Brady's reagent was added to the bulk of the solution, and after several hours the yellow crystalline precipitate was collected, washed, dried (0·2 g.), triturated with saturated Na_2CO_3 and filtered. The filtrate was only pale red in colour and gave no appreciable precipitate on acidification, but by repeated washing of the insoluble residue with water, a deep red solution was obtained which gave a good yellow precipitate (0·10 g.) on acidification with conc. HCl. The acidic hydrazone crystallised from benzene-light petroleum in groups of small, flat, pointed, yellow needles, M.P. 202–204° with some effervescence.

(Found (Weiler): C, 44·35; H, 3·58; N, 15·77; OCH_3, 8·93 %. $C_{13}H_{12}O_8N_4$ (monodinitrophenylhydrazone of $C_7H_8O_5$) requires C, 44·31; H, 3·44; N, 15·91; OCH_3, 8·81 %.)

The residual dinitrophenylhydrazone insoluble in dilute Na_2CO_3 was crystallised from boiling water in which it was almost completely soluble. It formed slender, brownish yellow needles, M.P. 162–164°. Mixed with an authentic specimen of formaldehyde dinitrophenylhydrazone (M.P. 164–165°) it melted at 162–165°. The action of ozone on penicillic acid therefore proceeds according to the following equation:

$$C_8H_{10}O_4, H_2O + O_3 \longrightarrow CH_2O + C_7H_8O_5 + H_2O_2.$$

It was desirable to determine whether the compound $C_7H_8O_5$ was an aldehydo- or keto-acid. To this end, the ozonisation experiment was repeated and formaldehyde removed from the resulting solution by repeated evaporation to small volume *in vacuo* at 35–40°. Eventually, a solution was obtained which, when made up to one-half of the original volume, did not restore the colour to Schiff's reagent, even on long standing, but gave a good precipitate of an acidic dinitrophenylhydrazone with Brady's reagent, which proved to be identical with the acidic dinitrophenylhydrazone described above. The oxidation product $C_7H_8O_5$ may therefore be the lactone of a keto-acid, *i.e.*



(Found on dinitrophenylhydrazone: equiv. by micro-titration, 318. $C_{13}H_{12}O_8N_4$ titrating as a monobasic acid requires equiv. 352.)

Oxidation of dihydropenicillic acid by aqueous potassium permanganate. Formation of oxalic acid and the methyl ester of dimethylpyruvic acid.

To a solution of dihydropenicillic acid (0·77 g.) in water (100 ml.) at 0°, was slowly added during 3 hours $N/10$ $KMnO_4$ (300 ml.) with stirring and cooling in ice. The liquid was kept for 30 min., after which the supernatant liquid was quite colourless. The oxidation was evidently not yet complete although 2 atoms of available oxygen had been used up. After filtration from MnO_2, the filtrate, which was practically neutral to litmus, was divided into two equal parts, to one of which was added an equal volume of Brady's reagent, and to the other, after making slightly alkaline to phenolphthalein, an excess of $N/2$ $CaCl_2$. The dinitrophenylhydrazone so obtained weighed 0·38 g. (55 % of theoretical, assuming that it is derived from $C_6H_{10}O_3$), whilst the precipitate of Ca oxalate weighed only 0·035 g., *i.e.* only 12 % of the theoretical for the expected formation of 1 mol. of oxalic acid. The oxalic acid was isolated by repeated extraction of a solution of the Ca salt in HCl with ether, removal of the solvent, and sublimation

of the residue at 80–90° in a high vacuum. The crystalline sublimate melted at 189–190° alone or mixed with authentic anhydrous oxalic acid.

The dinitrophenylhydrazone, which was insoluble in Na_2CO_3 solution, was purified by repeated crystallisation from light petroleum (B.P. 80–100°) in which it is sparingly soluble in the cold, but moderately so at the B.P. It formed fine flat, yellow needles and prisms, M.P. 176–178°, without decomposition.

(Found (Schoeller): C, 46·42, 46·39; H, 4·72, 4·55; N, 17·84, 17·94; OCH_3, 10·01, 9·99 %. $C_{12}H_{14}O_6N_4$ (monodinitrophenylhydrazone of $C_6H_{10}O_3$) requires C, 46·44; H, 4·55; N, 18·06; OCH_3, 10·00 %.) When triturated with *N* NaOH it slowly dissolved to give a deep brown solution, which yielded on acidification an acidic dinitrophenylhydrazone, crystallising from aqueous alcohol in yellow needles, M.P. 196–197° (decomp.), alone or mixed with the authentic dinitrophenylhydrazone of dimethylpyruvic acid, prepared by the method of Ramage and Simonsen [1935]. (These authors quote M.P. 194–195° for the hydrazone.)

The synthetic dimethylpyruvic acid (1·2 g.) was also esterified by excess of ethereal diazomethane. The solvent was removed and the residual oil was taken up in water (500 ml.) and the crude hydrazone thrown down by addition of Brady's reagent was purified by trituration with *N* NaOH for a few min., and finally by crystallisation from light petroleum; yellow needles and prisms, M.P. 178–180°. Mixed with the dinitrophenylhydrazone of the oxidation product of dihydropenicillic acid the M.P. was 176–179°.

Action of ozone on dihydropenicillic acid.

Methyl dimethylpyruvate was also produced when ozonised O_2 was passed into a solution of dihydropenicillic acid (0·8 g.) in water (50 ml.) at 0–10° for 12 hours. Absorption of O_3 was very slow indeed, even although the gas was distributed through a sintered glass plate. The resulting liquid did not restore the colour to Schiff's reagent even on long standing, but gave a slight yellow precipitate with Brady's reagent which proved to be absolutely insoluble in aqueous Na_2CO_3. Hence glyoxylic acid is not present in the solution. After crystallisation from light petroleum, the dinitrophenylhydrazone melted at 178–180° alone or mixed with the authentic dinitrophenylhydrazone of methyl dimethylpyruvate. The expected glyoxylic acid had evidently been oxidised to oxalic acid, since the neutralised reaction liquid gave a slight precipitate with $CaCl_2$ solution, insoluble in acetic acid, but soluble in nitric acid.

Acid hydrolysis of penicillic acid.

Hydrated penicillic acid (0·4921 g.) was heated with 50 ml. of boiling 2 *N* H_2SO_4 under reflux in a stream of nitrogen. The gaseous products were bubbled through standard baryta solution. Evolution of CO_2 ceased after 24 hours and was then equivalent to 0·0327 g. of C or 12·49 g. per g. mol. of penicillic acid. Thus 1 mol. of penicillic acid yields 1 mol. of CO_2 on prolonged acid hydrolysis. The reaction mixture was found by titration to contain no acid other than the mineral acid added. The only product of acid hydrolysis of 10 g. penicillic acid other than CO_2 which was isolated was obtained as a 2:4-dinitrophenylhydrazine derivative. It formed orange-brown crystals from aqueous dioxan, M.P. 254°.

(Found: C, 46·84; H, 4·19; N, 19·74 %. $C_{11}H_{12}O_5N_4$ requires C, 47·12; H, 4·32; N, 20·00 %.) This corresponds to the mono-dinitrophenylhydrazone of $C_5H_8O_2$ or the bis-dinitrophenylhydrazone of $C_{10}H_{16}O_4$. It gave a positive Neuberg's test for bis-dinitrophenylhydrazones (a purple colour on addition of KOH to its alcoholic solution). The parent substance is therefore $C_{10}H_{16}O_4$, possibly formed

by condensation of 2 mols. of acetylpropionyl, $C_5H_8O_2$, which as will be shown later is a product of the alkaline hydrolysis of penicillic acid.

The same product was obtained on hydrolysis of penicillic acid with boiling aqueous sodium acetate solution.

Acid hydrolysis of dihydropenicillic acid.

Dihydropenicillic acid (2 g.) was heated with 80 ml. of boiling $2N$ H_2SO_4 under reflux in a stream of nitrogen. 1 mol. of CO_2 was evolved in 20 hours. The volatile products were bubbled through Brady's reagent before absorption of CO_2 and from the precipitated 2:4-dinitrophenylhydrazones (2 g. crude) there were isolated three different products:

(*a*) Red prisms *ex* benzene, M.P. 226–227° (decomp.); insoluble in light petroleum (B.P. 60–80°). Gave a positive Neuberg's test for bis-dinitrophenylhydrazones. (Found (Schoeller) after drying at 110° in high vacuum: C, 45·62, 45·63; H, 3·92, 3·87; N, 23·31 %. $C_{18}H_{18}O_8N_8$ requires C, 45·55; H, 3·83; N, 23·63 %.)

(*b*) Golden needles *ex* light petroleum and finally from ether, M.P. 186–187°. Insoluble in boiling H_2O. Negative Neuberg's test. (Found (Schoeller): C, 48·91; H, 4·79; N, 19·03 %. $C_{12}H_{14}O_5N_4$ requires C, 48·97; H, 4·80; N, 19·08 %.)

(*c*) Lemon-yellow rhombic plates *ex* boiling water, light petroleum and ether. M.P. 121°. Negative Neuberg's test. (Found (Schoeller): C, 49·36, 49·28; H, 4·76, 4·89; N, 18·99 %. $C_{12}H_{14}O_5N_4$ requires C, 48·97; H, 4·80; N, 19·08 %.)

Hence (*a*) is the bis-dinitrophenylhydrazone, and (*b*) and (*c*) are the mono-derivatives of $C_6H_{10}O_2$, which from a consideration of the formula established for dihydropenicillic acid (IX and X) we believe to be acetyl*iso*butyryl, arising from the parent substance by decarboxylation and demethylation. Acetyl*iso*butyryl, with limited amounts of Brady's reagent could give rise to a mixture of one bis- and two different mono-dinitrophenylhydrazones.

Alkaline hydrolysis of penicillic acid.

Alsberg and Black [1913] record the formation (but not the analysis) of "delicate needles" when penicillic acid is treated with boiling aqueous baryta. This experiment was repeated. Penicillic acid (5 g.) was heated with $N/4$ baryta (500 ml.) under reflux in a current of nitrogen for 4 hours. The gaseous products were bubbled through Brady's reagent. A complex mixture of hydrolysis products was formed, and the following were isolated, mostly as derivatives either from the Brady's reagent or from the hydrolysis solution.

(1) CO_2.

(2) A substance $C_5H_{10}O_2$ isolated as the mono-dinitrophenylhydrazone; yellow needles *ex* alcohol, M.P. 193–194°. (Found (Schoeller): C, 46·63; H, 4·97; N, 19·99 %. Mol. wt. 274, 282. $C_{11}H_{14}O_5N_4$ requires C, 46·80; H, 5·00; N, 19·86 %; mol. wt. 282.) The constitution of this hydrolysis product is unknown but it was shown not to be either β-keto-γ-hydroxypentane or β-hydroxy-γ-ketopentane as described in the literature.

(3) Acetylpropionyl, $C_5H_8O_2$, isolated as the bis-dinitrophenylhydrazone; red crystals *ex* benzene. Insoluble in boiling alcohol. Gave positive Neuberg's test; M.P. 272–274° (decomp.). (Found (Schoeller): C, 44·87; H, 3·62; N, 24·84%; mol. wt. 372, 364. $C_{17}H_{16}O_8N_8$ requires C, 44·32; H, 3·50; N, 24·35 %; mol. wt. 460.) Gave no depression on mixing with a synthetic specimen prepared from diethylketone by the method of Diels and Stephan [1907].

(4) A substance $C_8H_{12}O$ isolated as the monodinitrophenylhydrazone; dark red needles *ex* alcohol, M.P. 152–154°. (Found (Schoeller): C, 55·32; H, 5·14;

N, 18·83%; mol. wt. 266, 279. $C_{14}H_{16}O_4N_4$ requires C, 55·24; H, 5·30; N, 18·43%; mol. wt. 304.) This substance probably arises by condensation of some of the hydrolysis products.

(5) A substance $C_{10}H_{10}O_2$, colourless irregular prisms, M.P. 98–102°, probably identical with the "delicate needles" of Alsberg and Black. (Found (Schoeller): C, 74·20, 74·11; H, 6·39, 6·43%; mol. wt. 154, 159. $C_{10}H_{10}O_2$ requires C, 74·05; H, 6·22%; mol. wt. 162.) The substance titrates as a lactone but sufficient was not available for an accurate titration value. It is obviously a secondary condensation product.

(6) Methyl alcohol identified as the *p*-nitrobenzoate.

(7) Formic acid isolated as the lead salt. (Found: Pb, 69·2%. $Pb(H.COO)_2$ requires Pb, 69·7%.)

SUMMARY.

Penicillic acid, $C_8H_{10}O_4$, a metabolic product of *Penicillium puberulum* Bainier, is also formed in considerably larger yield by *P. cyclopium* Westling. The study of its reactions and breakdown products establishes its constitution as γ-keto-β-methoxy-δ-methylene-Δ^{α}-hexenoic acid or the corresponding γ-hydroxylactone.

We tender our best thanks to the Research Council of Imperial Chemical Industries, Ltd., for a grant to one of us (J. H. B.).

REFERENCES.

Alsberg and Black (1913). *U.S. Dept. Agric. Bureau Plant Ind. Bull.* No. 270.
Balbiano (1898). *Gazzetta*, **28**, I, 382.
Birkinshaw and Raistrick (1932). *Biochem. J.* **26**, 441.
Black and Alsberg (1910). *U.S. Dept. Agric., Bureau Plant Ind. Bull.* No. 199.
Clutterbuck, Raistrick and Reuter (1935). *Biochem. J.* **29**, 300.
Conrad (1897). *Ber. deutsch. chem. Ges.* **30**, 856, 864.
Diels and Stephan (1907). *Ber. deutsch. chem. Ges.* **40**, 4337.
Elderfield (1935). *J. Biol. Chem.* **111**, 527.
Fearon and Mitchell (1932). *Analyst*, **57**, 372.
Houben-Weyl (1930). Die Methoden der Organischen Chemie. 3rd edition, vol. 3, p. 653.
Jacobs and Hoffmann (1926). *J. Biol. Chem.* **67**, 336.
Kinoshita (1931). *Acta Phytochimica*, **5**, 271.
Losanitsch (1914). *Monatsh. Chem.* **35**, 302.
Oxford and Raistrick (1935). *Biochem. J.* **29**, 1599.
Ramage and Simonsen (1935). *J. Chem. Soc.* 532.
Stoermer and Martinsen (1907). *Liebig's Ann.* **352**, 330.
Wislicenus and Bindemann (1901). *Liebig's Ann.* **316**, 37.