N-acetyl derivative conjugated with glucuronic acid and sulphate. The extent of sulphate conjugation corresponds to 32 % of the dose.

3. The metabolism of *m*-acetotoluidide is similar to that of the ortho isomer; 25% is oxidized to the corresponding *N*-acetamidobenzoic acid, and the only other metabolite detected was 5-amino-*o*-cresol which has been isolated. This is probably excreted as its *N*-acetyl derivative conjugated with glucuronic acid and sulphate; 10% of the dose is excreted as an ethereal sulphate.

4. *p*-Acetotoluidide is virtually completely oxidized to *p*-acetamidobenzoic acid, 10 % being excreted as ester glucuronide. There is no conjugation with sulphuric acid.

5. *o*- and *m*-acetotoluidides cause an abnormally high excretion of reducing material in urine. The reason for this has not been determined.

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Studies in the Biochemistry of Micro-organisms

78. THE MOLECULAR CONSTITUTION OF MYCOPHENOLIC ACID, A METABOLIC PRODUCT OF *PENICILLIUM BREVI-COMPACTUM* DIERCKX

PART 2. POSSIBLE STRUCTURAL FORMULAE FOR MYCOPHENOLIC ACID

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Mycophenolic acid, $C_{17}H_{20}O_6$, was first named and isolated from cultures of *Penicillium stoloniferum* Thom by Alsberg & Black (1913), although Gosio (1896) had reported an unnamed crystalline metabolic product of '*P. glaucum*', to which he assigned the formula $C_9H_{10}O_3$, which is probably identical with mycophenolic acid. Clutterbuck, Oxford, Raistrick & Smith (1932) isolated mycophenolic acid, together with other phenolic compounds, from the culture filtrates of twelve species or strains in the *P. brevi-compactum* series, to which *P. stoloniferum* Thom belongs, and Clutterbuck & Raistrick (1933) reported an extensive chemical investigation of this substance.

Gosio (1896) demonstrated that his substance, $C_9H_{10}O_3$, suppresses the growth of the anthrax bacillus, so that it appears probable that mycophenolic acid, although of relatively low potency, was the first antibiotic of fungal origin to be obtained

in crystalline form. More recently, Florey, Gilliver, Jennings & Sanders (1946) investigated its antibacterial action against a number of animal and plant pathogens and also against a number of saprophytic and pathogenic fungi. They showed that it is more effective against Gram positive than against Gram negative bacteria and that its activity against staphylococci varies greatly with the number of organisms used for the inoculum. This 'inoculum size' phenomenon was thoroughly investigated by Abraham (1945).

Clutterbuck & Raistrick (1933) established, *inter alia*, the following facts bearing on the constitution of mycophenolic acid:

- (1) It is optically inactive.
- (2) The six oxygen atoms are present in the molecule in the following forms: (a) One as a OCH_3 group. On demethylation, normycophenolic acid, $C_{16}H_{18}O_6$, is formed. (b) One as a phenolic OH group, re-

sponsible for the blue-violet FeCl_3 colour. (c) Two as a COOH group. (d) Two as a lactone ring,

(3) It contains the aliphatic grouping

(4) On fusion with KOH 1:5-dihydroxy-3:4-dimethylbenzene is formed.

(5) Its monomethyl ether, on protracted oxidation with alkaline KMnO_4 , gives the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene (I) in good yield.

On the basis of these facts, and on others for which the original paper should be consulted, the structure (II) for normycophenolic acid was advanced.

No evidence was available as to which of the two phenolic groups in (II) is methylated in mycophenolic acid itself.

We now present experimental evidence which necessitates modification and amplification of structure (II) and may be summarized as follows.

I. Ozonization of mycophenolic acid, its monomethyl ether, and its monomethyl ether methyl ester

Chloroform solutions of these three substances were ozonized, and, after decomposing the resulting ozonides with water, the following breakdown products were isolated.

(a) From mycophenolic acid ($\text{C}_{17}\text{H}_{20}\text{O}_6$, with 1 OCH_3 group): levulinic acid, $\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOH}$; and an aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$, containing 1 OCH_3 group, giving the same FeCl_3 reaction as the parent substance, and not extractable from chloroform with aqueous NaHCO_3 .

(b) From mycophenolic acid monomethyl ether ($\text{C}_{18}\text{H}_{22}\text{O}_6$, with 2 OCH_3 groups): levulinic acid; an aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$, containing 2 OCH_3 groups, which will be dealt with more fully later; and a small

amount of the corresponding carboxylic acid, $\text{C}_{13}\text{H}_{14}\text{O}_6$.

(c) From mycophenolic acid monomethyl ether methyl ester ($\text{C}_{19}\text{H}_{24}\text{O}_6$, with 3 OCH_3 groups): methyl levulate, $\text{CH}_3 \cdot \text{CO} \cdot \text{CH}_2 \cdot \text{CH}_2 \cdot \text{COOCH}_3$; and the same aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$, containing 2 OCH_3 groups, as was obtained by ozonization of mycophenolic acid monomethyl ether.

These findings clearly prove that mycophenolic acid contains: (1) a hydroxyl group, directly attached to the nucleus, which can be methylated by diazomethane and is therefore phenolic in nature; and (2) a free carboxyl group in a side chain which gives rise to levulinic acid on ozonolysis of mycophenolic acid or its monomethyl ether and to methyl levulate on similar treatment of its monomethyl ether methyl ester.

The free COOH group attached directly to the nucleus in structure (II) must, therefore, now be transferred to the end of a side chain of six or more carbon atoms including the C_6H_{12} residue, the nature of which was left undetermined by Clutterbuck & Raistrick (1933).

II. Molecular constitution of the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$

The substance, $\text{C}_{13}\text{H}_{14}\text{O}_5$, obtained by the ozonolysis of both the monomethyl ether and the monomethyl ether methyl ester of mycophenolic acid, is a neutral substance and is a true aldehyde and not a ketone. It gives typical aldehyde reactions, is oxidized by alkaline iodine to a carboxylic acid, $\text{C}_{13}\text{H}_{14}\text{O}_6$, and on heating with acetic anhydride and anhydrous sodium acetate it gives a mono-enol acetate. The important conclusion follows that in the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$, the CHO group is not attached directly to the nucleus but through an intervening $-\text{CH}_2-$ link, the acetylated product having the form $R \cdot \text{CH} : \text{CH} \cdot \text{O} \cdot \text{CO} \cdot \text{CH}_3$. This conclusion is confirmed by the fact that ozonolysis of the enol acetate yields an aldehyde, $\text{C}_{12}\text{H}_{12}\text{O}_5$, fission having occurred at the double linking.

Of the thirteen carbon atoms present in the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$, six are present in a benzene ring, since permanganate oxidation of mycophenolic acid monomethyl ether yields the anhydride of 1:5-dimethoxy-2:3:4:6-tetracarboxybenzene, structure (I), two as OCH_3 groups and two as the group $-\text{CH}_2 \cdot \text{CHO}$, leaving three carbon atoms to be accounted for. It follows from the structure of the above anhydride (I) that each of these three carbon atoms must be attached singly to the benzene nucleus.

The nature of the groups in which each of these three carbon atoms is present may now be considered. The aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$ (A) behaves as a neutral substance on titration with NaOH in the cold, but as a monobasic acid on heating. It is thus

probably a monolactone. Alkaline iodine converts it into a compound $C_{13}H_{14}O_6$ (B) which still retains two methoxy groups but has lost its aldehydic properties. It titrates in the cold as a monobasic acid and on heating as a dibasic acid. It is therefore a monobasic acid monolactone. The disodium salt of (B), on oxidation with cold neutral aqueous $KMnO_4$, yields a substance which, after sublimation in a high vacuum, has the empirical formula $C_{13}H_{12}O_7$ (C). This substance still retains both OCH_3 groups, and titrates sharply in the cold as a tribasic acid. These facts can be most simply explained by postulating that (A) contains the groups $HCO.CH_2-$ and

that in (B) the $HCO.CH_2-$ group becomes $HOOC.CH_2-$ and the lactone grouping remains unchanged; that (C) still retains the group $HOOC.CH_2-$ while the lactone ring is opened in the sodium salt to

and oxidized and the ring reclosed on sublimation to give the anhydride ring

Thus we may conclude that two of the three carbon atoms in (A), whose nature is in doubt, are vicinal and in the form

The third carbon atom is present as a CH_3 group, since the aldehyde $C_{13}H_{14}O_5$ (A) was shown by analysis to contain one carbon-methyl, $CH_3(C)$, group. Mycophenolic acid itself was shown to contain two carbon-methyl groups, the second one being the branched CH_3 group in the side chain in structures (IX) and (X).

Since the two methoxyl groups are attached directly to the benzene nucleus in the meta position to each other (Clutterbuck & Raistrick, 1933) the constitution of the $C_{13}H_{14}O_5$ aldehyde may therefore be written in the form

and since each of the four different substituent groups, two of them, viz.

vicinal, must be attached directly to the nucleus, there are only four possible isomers, (III), (IV), (V) and (VI).

Evidence as to which of these four structural formulae most probably represents the structure of the aldehyde $C_{13}H_{14}O_5$, and hence part of the structure of the monomethyl ether of mycophenolic acid from which it is derived by ozonization, is available from a number of sources, all of which lead to the same conclusion.

(a) On the addition of a suspension of 2:6-dichloroquinone chloroimide to a solution of normycophenolic acid in sodium borate buffer of pH 9.2, at which pH the phthalide ring will be opened and a $COOH$ group liberated, a blue colour with a tinge of green is rapidly formed. The rapid production of a blue colour by a substituted benzene compound is indicative, according to Gibbs (1927), of a free position para to a phenolic group, though according to Davidson, Keane & Nolan (1943) the reaction is also positive if the position para to OH is occupied by $COOH$. Examination of structures (III), (IV), (V) and (VI), assuming the OCH_3 groups to be replaced by OH groups as in normycophenolic acid, shows that only (III) and (IV) satisfy the requirement of $COOH$ para to OH .

(b) Clutterbuck & Raistrick (1933) showed that, on fusion with KOH , mycophenolic acid yields 1:5-dihydroxy-3:4-dimethylbenzene. Hence, if one ignores the possibility of migration of substituents during potash fusion, two of the three substituents, CH_3 , CH_2OH and $CH_2.CHO$, likely to give rise to the two CH_3 groups in the fusion product, must be vicinal. This requirement also is satisfied only by (III) and (IV).

(c) One of the groups shown as methoxyl in (V) and (VI) is represented by a free (phenolic) OH in mycophenolic acid itself. As both methoxyl groups of (V) and (VI) are meta to the potential carboxyl group, the OH group of mycophenolic acid must also

be meta to the potential carboxyl if either (V) or (VI) is correct. This orientation would be unlikely to give rise to the intense *violet* coloration which mycophenolic acid gives with ferric chloride, whereas the possible *o*-OH group derivable from (III) and (IV) would be expected to give such a colour.

(d) Of the four known phthalides:

(A) (Fritsch, 1897) and (B) (Charlesworth & Robinson, 1934) are readily oxidizable to the corresponding phthalic acid by means of alkaline permanganate, whereas (C) (Charlesworth, Rennie, Sinder & Yan, 1945) and (D) (Mittar, Sen & Paul, 1927; Charlesworth *et al.* 1945) are not so oxidized. It appears that the presence of a substituent methyl group ortho to the potential carboxyl of the phthalide grouping prevents oxidation. If this is the case it would rule out formulae (V) and (VI) as possibilities for the C_{13} aldehyde, since (V) has a methyl group and (VI) a substituted methyl group ortho to the potential carboxyl, yet the product, after oxidation of $-CHO$ to $-COOH$ was oxidized by permanganate to the phthalic acid.

The two most probable formulae for normycophenolic acid therefore become (VII), from (III), and (VIII), from (IV).

We have no experimental evidence capable of deciding between (VII) and (VIII), but we are inclined to favour (VII), since this structure contains a preformed CH_3 group oriented towards the two OH groups as in 1:5-dihydroxy-3:4-dimethylbenzene, the potash fusion product of mycophenolic acid.

Finally, there arises the question as to which of the two OH groups in (VII) or (VIII) is methylated in mycophenolic acid itself. We have stated previously that normycophenolic acid rapidly gives a blue colour with 2:6-dichloroquinone chloroimide. Under the same conditions mycophenolic acid gives only a very faint dirty yellow colour even on standing. We are aware of the dangers of interpreting a negative 2:6-dichloroquinone chloroimide reaction (see Calam, Clutterbuck, Oxford & Raistrick, 1947), but, with this reservation, the choice of the hydroxyl group to be represented as methylated in mycophenolic acid has fallen on that para to a potential carboxyl group. This choice is strengthened by the fact that an aqueous solution of mycophenolic acid gives with aqueous $FeCl_3$ a strong violet colour somewhat similar to, but admittedly bluer than, that given by salicylic acid. Hence we believe that the more probable structure for mycophenolic acid is (IX) and the less probable (X).

It remains now to show that the experimental evidence obtained by Clutterbuck & Raistrick (1933) can be explained by the favoured constitution for mycophenolic acid. In most cases this is self-evident, but two points call for further discussion.

Titration values. On titration to phenolphthalein in water with aqueous NaOH in the cold, mycophenolic acid and its derivatives gave the following titration equivalents:

(a) Normycophenolic acid, 155.6. Theor. as a dibasic acid, 153.

(b) Mycophenolic acid, 169. Theor. as a dibasic acid, 160.

(c) Mycophenolic acid monomethyl ether, 332. Theor. as a monobasic acid, 334.

(d) Monoacetylmycophenolic acid, 361. Theor. as a monobasic acid, 362.

On heating the cold titrated solutions with excess of NaOH and back titrating, mycophenolic acid and its monomethyl ether became fully dibasic, while acetylmycophenolic acid became tribasic.

The interpretation of these findings appears to be as follows:

(1) Monoacetylmycophenolic acid is completely hydrolyzed with the formation of one molecule each of acetic and mycophenolic acids.

(2) Mycophenolic acid monomethyl ether. Since there is no free phenolic OH group in this compound either before or after hydrolysis, the ether being recovered unchanged from the acidified hydrolysis solution, it is clear that the free carboxyl group at the end of the long substituent chain titrates in the cold and that, on heating, the phthalide ring is opened and the COOH group thus liberated then titrates. The formation of a disilver salt,

involving the addition of the elements of water confirms this view.

(3) Mycophenolic acid. Two explanations seem possible. (a) One equivalent of acid is due to the free terminal COOH group. The second (partial) equivalent in the cold is due to the partial, but almost complete, opening of the phthalide ring in the cold. This, however, is contrary to the usual behaviour of the phthalide grouping. (b) An alternative explanation is that the second (partial) equivalent in the cold is due to the acidity of the phenolic OH group. It must then be assumed that, on hot alkaline hydrolysis, the phthalide ring is opened, and that the resulting formation of a nuclear carboxyl group has suppressed the ionization of the phenolic OH group, so that the latter has no longer a titratable acidity. This view is confirmed by the fact that according to Alsberg & Black (1913) mycophenolic acid forms two barium salts having the empirical formulae $(\text{C}_{17}\text{H}_{19}\text{O}_6)_2\text{Ba}$ and $\text{C}_{17}\text{H}_{18}\text{O}_6\text{Ba}$. The latter dibasic salt clearly arises without the addition of the elements of water, and hence, inferentially, without the opening of the phthalide ring.

The constitution of the alkaline permanganate oxidation product of mycophenolic acid monomethyl ether (Clutterbuck & Raistrick, 1933, p. 659)

This substance, which was isolated in good yield, has the empirical formula $\text{C}_{16}\text{H}_{14}\text{O}_6(\text{OCH}_3)_2$. It has, therefore, the same number of carbon atoms as the ether from which it is formed according to the equation

Since, unlike the parent ether, it does not immediately decolorize KMnO_4 in the cold, it probably arises by oxidation at the double bond to the glycol form (requiring one O), followed by lactonization on subsequent isolation in acid solution, coupled with oxidation of the phthalide to the phthalic anhydride (requiring two O), the product having the constitution (XI) from structure (IX) or the corresponding structure derived from (X):

It was found that the oxidation product, when dissolved in ethanol, titrated in the cold as a monobasic acid, but after alkaline hydrolysis as a tribasic acid. A product of structure (XI), when dissolved in ethanol, should yield the mono-acid mono-ester derived from the acid anhydride ring. This would then titrate in the cold as a monobasic acid, whereas two new acidic groups would be revealed on alkaline hydrolysis, one by hydrolysis of the ester grouping and the other by the opening of the lactone ring. This would also explain the fact that the substance was recovered unchanged after acidifying the hydrolysis solution.

EXPERIMENTAL

A. Action of ozone on mycophenolic acid and its derivatives

In this series of experiments ozonized O_2 was passed through a chloroform solution of the substance until a sample of the solution no longer decolorized a dilute solution of Br_2 in chloroform. The chloroform solution of the ozonide was well shaken with its own volume of water, warmed to c. 50° and again thoroughly shaken to decompose the ozonide. After cooling, the water fraction was separated from the chloroform fraction and the two were worked up separately.

I. *Action of ozone on mycophenolic acid.* Mycophenolic acid (0.5 g.) in chloroform (25 ml.) was ozonized for 2.5 hr.

Water fraction. The solution was treated with NaHCO_3 (0.5 g.) and continuously extracted with ether. On removal of the solvent only a trace of whitish residue remained. The extraction was continued after acidification with $2\text{N-H}_2\text{SO}_4$. The oil (0.18 g.) thus obtained was treated with 50 ml. of Brady's reagent (0.3% 2,4-dinitrophenylhydrazine in 2N-HCl). An orange-yellow crystalline precipitate soon formed, m.p. $203\text{--}205^\circ$, raised to 205° after recrystallization from methanol, and not depressed on admixture with authentic levulinic acid 2,4-dinitrophenylhydrazone, m.p. $205\text{--}206^\circ$. The substance is acid to litmus, and is readily soluble in aqueous NaHCO_3 to a golden yellow solution from which it separates on acidification as an orange flocculent precipitate.

Chloroform fraction. The chloroform solution was washed with aqueous NaHCO_3 . The acids were liberated by acidification with $2\text{N-H}_2\text{SO}_4$ and extracted with ether, yielding 0.06 g. of colourless crystalline solid which was not further examined. The neutral fraction, after removal of chloroform, remained as a mixture (0.21 g.) of crystals and oil. It was washed with a little ether to remove the oil, and the remaining crystals, after recrystallization first from ether and then from water, gave 0.07 g. of short colourless rods, m.p. $152\text{--}153^\circ$. (Found: C, 61.23, 61.14; H, 5.12, 5.40; OCH_3 , 12.7, 13.3. $\text{C}_{11}\text{H}_{12}\text{O}_4(\text{OCH}_3)$ requires C, 61.01; H, 5.12; OCH_3 , 13.1%.) The substance gave a strong aldehyde reaction (Schiff), and gave in water and in ethanol with FeCl_3 the same colour reactions as mycophenolic acid. It dissolved readily in cold 2N-NaOH giving an immediate yellow colour which became somewhat darker on standing. The substance is therefore an aldehyde, $\text{C}_{12}\text{H}_{12}\text{O}_5$, with one methoxyl group, and evidently the grouping, a phenolic OH group, responsible for the FeCl_3 reaction with mycophenolic acid, persists in its ozonolysis product.

II. *Action of ozone on mycophenolic acid monomethyl ether.* Mycophenolic acid monomethyl ether (1.0 g., m.p. 112° ; see Clutterbuck & Raistrick, 1933, p. 658) in chloroform (50 ml.) was ozonized for 2.25 hr. A total of 5.30 g. of the ether was ozonized.

Water fraction. The combined water fractions were treated with an excess of Brady's reagent giving an orange crystalline precipitate, 1.16 g., m.p. $201\text{--}203^\circ$ of levulic acid 2:4-dinitrophenylhydrazone. A sample recrystallized from methanol in orange needles, m.p. $204\text{--}205^\circ$, was analyzed. (Found: C, 44.38; H, 4.63; N, 19.05. $\text{C}_{11}\text{H}_{12}\text{O}_6\text{N}_4$, i.e. the dinitrophenylhydrazone of $\text{C}_6\text{H}_6\text{O}_3$, requires C, 44.60; H, 4.08; N, 18.9%.)

Chloroform fraction. The combined chloroform extracts were washed twice with 50 ml. aqueous NaHCO_3 , and this extract on acidification yielded 0.39 g. of almost colourless crystals with some oil. On recrystallization from chloroform—light petroleum colourless needles were obtained, m.p. 153° , identical with the alkaline iodine oxidation product of the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_6$. (Found: C, 59.05, 59.20; H, 5.17, 5.10. $\text{C}_{13}\text{H}_{14}\text{O}_6$ requires C, 58.64; H, 5.30%.)

The bicarbonate-extracted chloroform solution, on removal of the solvent, yielded an oil which soon crystallized. On recrystallization from water a colourless crystalline solid, 2.05 g., of constant m.p. 112° was obtained. Concentration of the aqueous mother liquors gave a further 0.4 g., m.p. 107° . The substance gives a strong Schiff reaction and is the same aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_6$, m.p. 112° as was obtained by the ozonization of mycophenolic acid monomethyl ether methyl ester (see section AIII).

Mono-2:4-dinitrophenylhydrazone of the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_6$. An ethanol solution of the $\text{C}_{13}\text{H}_{14}\text{O}_6$ aldehyde on treatment with Brady's reagent gave an immediate precipitate which on recrystallization from methanol yielded deep-yellow needles, m.p. 214° . (Found: C, 53.42; H, 4.34; N, 14.0, 13.3; OCH_3 , 14.25. $\text{C}_{17}\text{H}_{12}\text{O}_6\text{N}_4(\text{OCH}_3)_2$ requires C, 53.02; H, 4.21; N, 13.0; OCH_3 , 14.4%.) The substance is insoluble in aqueous NaHCO_3 , Na_2CO_3 , cold NaOH or dilute ammonia, but dissolves slowly in NaOH on heating producing a brown solution. The substance, therefore, does not contain a free carboxyl group, although it may have a lactone group.

Oxidation of the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_6$, with alkaline iodine. Formation of a monobasic acid monolactone, $\text{C}_{13}\text{H}_{14}\text{O}_6$. The aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_6$ (1.0 g.), was dissolved in methanol (5 ml.) by warming, cooled and treated with 0.1 N-iodine (200 ml.)

with vigorous shaking. 0.1 N- NaOH (250 ml.) was added, and the whole was well shaken until all the oil disappeared. The liquid became colourless. After 30 min. $\text{N-H}_2\text{SO}_4$ (25 ml.) was added and the liberated I_2 was titrated with 0.5 N- $\text{Na}_2\text{S}_2\text{O}_3$. The colourless mixture was made acid to Congo red and extracted four times with an equal volume of ether. The ethereal extract was shaken with aqueous NaHCO_3 (20 ml.), and the bicarbonate solution on acidification gave colourless crystals, 0.24 g., m.p. 147° , which on recrystallization from ether-light petroleum gave colourless feathery needles, m.p. $151\text{--}152^\circ$. (Found: C, 58.72, 58.60; H, 5.14, 5.20; OCH_3 , 23.4. $\text{C}_{11}\text{H}_8\text{O}_4(\text{OCH}_3)_2$ requires C, 58.64; H, 5.30; OCH_3 , 23.3%.) 0.2023 g. of the acid was titrated in the cold to phenolphthalein with 0.1 N- NaOH requiring 7.52 ml. corresponding to an equivalent of 269. $\text{C}_{13}\text{H}_{14}\text{O}_6$ titrating as a monobasic acid requires 266. A further 20.00 ml. of 0.1 N- NaOH was added and the solution was boiled under reflux, with exclusion of CO_2 , for 2.5 hr. A back-titration used 12.48 ml. of 0.1 N- H_2SO_4 . Thus the additional acid groups unmasked were equivalent to 7.52 ml. 0.1 N-alkali. A second acid group has thus been revealed by alkaline hydrolysis; the substance behaves as a mono-acid monolactone.

In a second oxidation experiment, in which a proportionately much larger amount of methanol was used ($\text{C}_{13}\text{H}_{14}\text{O}_6$ aldehyde, 0.25 g.; methanol, 7 ml.; 0.1 N- I_2 , 60 ml.; 0.1 N- NaOH , 75 ml.), the products extracted by ether, after acidification, were separated into acidic and neutral fractions by means of NaHCO_3 . The acidic fraction yielded the acid $\text{C}_{13}\text{H}_{14}\text{O}_6$, m.p. $151\text{--}152^\circ$, referred to above. The neutral fraction yielded, after several recrystallizations from ether, large colourless prisms, m.p. $94\text{--}95^\circ$. (Found: C, 59.88; H, 5.92; OCH_3 , 33.1, 32.9. $\text{C}_{11}\text{H}_7\text{O}_3(\text{OCH}_3)_3$ requires C, 59.98; H, 5.75; OCH_3 , 33.2%.) This product thus appears to be the methyl ester of the acid lactone $\text{C}_{13}\text{H}_{14}\text{O}_6$, m.p. $151\text{--}152^\circ$. Its formation must be due to the use of methanol to dissolve the aldehyde prior to oxidation.

Oxidation of the monobasic acid monolactone, $\text{C}_{13}\text{H}_{14}\text{O}_6$, with KMnO_4 . Formation of a tribasic acid, $\text{C}_{13}\text{H}_{12}\text{O}_7$. The monobasic acid monolactone $\text{C}_{13}\text{H}_{14}\text{O}_6$ (0.5 g.) was boiled for 1.5 hr. under reflux with N-NaOH (10 ml.) and water (50 ml.) to open the lactone ring. When cold the solution was treated with a cold solution of KMnO_4 (0.4 g., equivalent to 2 atoms O) in water (50 ml.). The mixture was left for 3 days at room temperature. The precipitated MnO_2 was removed by filtration. The pale-green filtrate was acidified to Congo red with H_2SO_4 and extracted with ether. On removal of the solvent a solid residue was obtained, 0.56 g., which proved to be a mixture of substances. It was fractionated by solution in water, neutralization with NaOH , and precipitation with aqueous neutral lead acetate (10 ml. of 0.5 N). The white precipitate thus obtained was collected, washed with water, acidified with H_2SO_4 and extracted with ether. On removal of the ether the dry product (0.19 g.) obtained was recrystallized from ether-light petroleum, giving 0.10 g. of colourless slender prisms, m.p. $161\text{--}162^\circ$, raised to 162.5° by sublimation *in vacuo*. (Found: C, 55.80; H, 4.37; OCH_3 , 23.0; neutralization equivalent in cold 96.8. $\text{C}_{11}\text{H}_6\text{O}_3(\text{OCH}_3)_3$ requires C, 55.71; H, 4.32; OCH_3 , 22.1%; equivalent, titrating as a tribasic acid, 93.4.)

III. *Action of ozone on mycophenolic acid monomethyl ether methyl ester.* Mycophenolic acid monomethyl ether methyl ester (5.6 g.), m.p. 58° (Clutterbuck & Raistrick, 1933, p. 657), in chloroform (250 ml.) was ozonized for 10 hr.

Water fraction. This fraction contained very little material giving a precipitate with Brady's reagent. Its ether extract

was split as in sections AI and AII, by extraction with aqueous NaHCO_3 , into (a) neutral fraction, 0.2 g. oil; (b) acid fraction, 0.15 g. oil. Neither fraction was examined further.

Chloroform fraction. The acid fraction, 0.12 g. of crystals, was not further examined. The neutral fraction, 5.4 g. of oil, was distilled *in vacuo* and gave the following fractions:

Fraction 1, b.p., $45-55^\circ/1$ mm.; 0.86 g.; mobile liquid.

Fraction 2, b.p., $190^\circ/1$ mm.; 0.96 g.; viscous oil, depositing crystals.

Fraction 3, b.p., $195^\circ/<1$ mm.; 0.96 g.; viscous oil, depositing crystals.

Fraction 4, undistilled residue. Some decomposition. Not further examined.

Fraction 1: 0.1 g. treated with Brady's reagent (50 ml.) gave crystalline orange needles, 0.16 g., m.p. 137° . It recrystallized readily from ethanol in bright-orange flattened needles, m.p. $137-138^\circ$, which did not depress the melting point of the mono-2:4-dinitrophenylhydrazone of synthetic methyl levulate, m.p. 139° . (Found: C, 46.56, 46.59; H, 4.65, 4.45; N, 18.3; OCH_3 , 10.1. $\text{C}_{11}\text{H}_{11}\text{O}_6\text{N}_4(\text{OCH}_3)$ requires C, 46.44; H, 4.55; N, 18.05; OCH_3 , 10.0%.)

Fractions 2 and 3 were combined and pressed on porous tile, giving 0.87 g. of colourless crystals, which after recrystallization from light petroleum, b.p. $40-60^\circ$, or water gave a strong Schiff reaction, and melted at 112° alone or in admixture with the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$ obtained by ozonization of mycophenolic acid monomethyl ether (see section AII). (Found: C, 62.15, 62.33; H, 5.75, 5.81; OCH_3 , 24.7; neutralization equivalent in hot solution, 240. $\text{C}_{11}\text{H}_8\text{O}_5(\text{OCH}_3)_2$ requires C, 62.40; H, 5.64; OCH_3 , 24.8%; neutralization equivalent, titrating as a monobasic acid, 250.) It also gave the same 2:4-dinitrophenylhydrazone, m.p. and mixed m.p. $214-215^\circ$. (Found: C, 53.10; H, 4.31; OCH_3 , 14.2. $\text{C}_{17}\text{H}_{12}\text{O}_8\text{N}_4(\text{OCH}_3)_2$ requires C, 53.02; H, 4.21; OCH_3 , 14.4%.)

B. Acetylation of the aldehyde, $\text{C}_{13}\text{H}_{14}\text{O}_5$.

Formation of an enol mono-acetate

The aldehyde, m.p. 112° (0.10 g.), was heated with anhydrous sodium acetate (0.2 g.) and acetic anhydride (0.4 ml.) until the mixture began to boil and was then maintained at 140° for 0.5 hr. There was only a slight discoloration. The mixture was poured into water and stirred. A white flocculent precipitate (0.08 g., m.p. $130-133^\circ$) separated. Recrystallization from water and light petroleum, b.p. $80-100^\circ$, gave colourless needles, m.p. 140° . (Found: C, 61.30; H, 5.70; CH_3CO , 17.0; $\text{CH}_3(\text{C})$, 10.6. $\text{C}_{11}\text{H}_7\text{O}_5(\text{CH}_3\text{CO})(\text{CH}_3)_2$ requires C, 61.65; H, 5.52; CH_3CO , 14.7; $2\text{CH}_3(\text{C})$, 10.3%.) The product gave no reaction with 2:4-dinitrophenylhydrazine (Brady's reagent) or with Schiff's reagent. It is thus clearly an enol mono-acetate of the $\text{C}_{13}\text{H}_{14}\text{O}_5$ aldehyde.

Ozonization of the enol acetate. Formation of an aldehyde, $\text{C}_{13}\text{H}_{12}\text{O}_5$. A solution of the enol acetate (0.2 g.) in chloroform (20 ml.) was ozonized for 45 min. After treatment with hot water the chloroform layer was separated, washed with a little aqueous NaHCO_3 , dried with Na_2SO_4 and evaporated. The oil (0.12 g.) remaining partly crystallized *in vacuo*. The whole was crystallized from light petroleum, b.p. $80-100^\circ$, and gave 25 mg. of colourless needles, m.p. 118° , which

were sublimed in a high vacuum giving a colourless crystalline sublimate m.p. $125-126^\circ$. (Found: C, 60.36; H, 4.92. $\text{C}_{13}\text{H}_{12}\text{O}_5$ requires C, 61.01; H, 5.12%.) The product gave a precipitate with Brady's reagent and a positive reaction with Schiff's reagent.

The residual oil (90 mg.), recovered from the light petroleum mother liquors, was treated with ethanol (2 ml.) and Brady's reagent (60 ml.). The crude red-orange 2:4-dinitrophenylhydrazone (115 mg.) was recrystallized from benzene, and gave 60 mg. of red-brown glistening needles containing one molecule of benzene of crystallization. On heating there was slight softening at $212-214^\circ$, with fairly sharp melting at $228-230^\circ$ with slight effervescence. (Found, on air-dried crystals: C, 58.60; H, 4.51; N, 11.8; loss in high vacuum at $100-110^\circ$, 19.7. $\text{C}_{15}\text{H}_{14}\text{O}_6\text{N}_4\cdot\text{C}_6\text{H}_6$ requires C, 58.30; H, 4.49; N, 11.3; loss 15.8%. Found, on crystals dried to constant weight: C, 51.76; H, 3.96; N, 13.3. $\text{C}_{15}\text{H}_{14}\text{O}_6\text{N}_4$, i.e. dinitrophenylhydrazone of $\text{C}_{13}\text{H}_{12}\text{O}_5$, requires C, 51.94; H, 3.88; N, 13.45%.)

C. Colour reactions of mycophenolic and normycophenolic acids with 2:6-dichloroquinone chloroimide

A small amount of each acid was dissolved in 2-3 ml. of sodium borate buffer, pH 9.2. To each solution was added two drops of a suspension of 2:6-dichloroquinone chloroimide in water. Mycophenolic acid gave a very faint dirty yellow colour, unchanged after 40 min. Normycophenolic acid rapidly gave a colour, blue with a tinge of green, inclining after 40 min. to purple. A control experiment using buffer solution and quinone alone gave a very faint pink unchanged after 40 min.

D. Carbon-methyl group estimations

(a) On the aldehyde $\text{C}_{13}\text{H}_{14}\text{O}_5$. Found: $\text{CH}_3(\text{C})$, 4.75%. $\text{C}_{13}\text{H}_{14}\text{O}_5$ with one $\text{CH}_3(\text{C})$ group requires 6.00%. This corresponds to 79% of the theoretical for one $\text{CH}_3(\text{C})$ group.

(b) On mycophenolic acid. Found: $\text{CH}_3(\text{C})$, 6.57%. $\text{C}_{17}\text{H}_{20}\text{O}_6$ with two $\text{CH}_3(\text{C})$ groups requires 9.38%. This corresponds to 140% of the theoretical for one $\text{CH}_3(\text{C})$ group and to 70% of the theoretical for two $\text{CH}_3(\text{C})$ groups.

SUMMARY

1. Further degradative experiments have been carried out on mycophenolic acid, a metabolic product of species and strains in the *Penicillium brevicompactum* Dierckx series. These experiments consist mainly of a study of the products formed by the ozonolysis of mycophenolic acid and some of its methylated derivatives and breakdown products.

2. They lead to two possible structural formulae for mycophenolic acid, one being more probable than, and preferred to, the other. Mycophenolic acid may be described as 6-hydroxy-4-methoxy-3(or 5)-methyl-5(or 3)-(5'-carboxy-3'-methylpent-2'-enyl) phthalide.

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The Oxidation of Diiodotyrosine Derivatives

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It is now well established that when diiodotyrosine is incubated under mildly alkaline conditions, thyroxine is formed (von Mutzenbecher, 1939; Block, 1940; Johnson & Tewkesbury, 1942; Harington & Pitt-Rivers, 1945); under the most favourable conditions, however, the yields of thyroxine are small, and a large proportion of the diiodotyrosine is lost, giving rise to tarry by-products, inorganic iodide and ammonia. So far, no compound has been isolated after such an incubation which would indicate through what intermediate step thyroxine is synthesized; nor has the side chain, which is removed from one molecule of diiodotyrosine during the coupling of two molecules to give thyroxine, or a recognizable derivative thereof, been isolated, although Johnson & Tewkesbury (1942) claim to have detected pyruvic acid in the reaction mixture when thyroxine is formed under these conditions.

It seemed possible that further investigation of the products of alkaline oxidation of diiodotyrosine might reveal some compound which would give a clue to the mechanism of the reaction. It had been shown by Harington & Pitt-Rivers (1945) that the pH at which diiodotyrosine is incubated exerts a marked effect both on the amount of thyroxine formed and on the extent to which diiodotyrosine itself is oxidized. At a high pH (13 or 14) no thyroxine was obtained by these authors, but instead another crystalline iodine-containing compound was isolated, which was not characterized at the time. This compound has now been re-examined and has been identified as 3:5-diiodo-4-hydroxybenzaldehyde. Further examination of the products of incubation of diiodotyrosine at pH 10 has shown that at this pH the aldehyde is formed at the same

time as thyroxine; at lower pH (7.8–8.8) none of the aldehyde is obtained, while the yields of thyroxine are very small.

The simultaneous formation of 3:5-diiodo-4-hydroxybenzaldehyde and thyroxine from diiodotyrosine led first to the hypothesis that the aldehyde might be an intermediate in thyroxine synthesis, and attempts were made to demonstrate this by incubating diiodotyrosine in the presence of the aldehyde, in the hope that increased yields of thyroxine might result. These attempts have proved abortive, and in all the experiments done the yield of thyroxine was unchanged and the aldehyde was recovered at the end of the reaction. Further, Dakin's (1909) observation that 3:5-diiodo-4-hydroxybenzaldehyde is unaffected by alkaline hydrogen peroxide, while the dichloro and dibromo analogues give more or less good yields of the corresponding quinols, has been confirmed. Only under the most drastic conditions may the diiodo aldehyde be oxidized, and the product of oxidation is 2:4:6-triiodophenol. These facts have, therefore, led to the abandonment of the hypothesis that the aldehyde is an intermediate in thyroxine synthesis, and it is now thought that its formation from diiodotyrosine is due to a different mechanism from that which leads to thyroxine.

The mode of formation of 3:5-diiodo-4-hydroxybenzaldehyde from diiodotyrosine is itself of some interest. It has been found that, when the aldehyde is obtained by incubating diiodotyrosine at pH 14, an amount of oxalic acid can be isolated as the calcium salt from the reaction mixture which is molecularly equivalent to the amount of aldehyde isolated; this leads to the suggestion that the