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On a New Methoxy-dihydroxy-toluquinone Produced from Glucose by Species of Penicillium of the P. spinulosum Series

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Studies in the Biochemistry of Micro-organisms.

PART XII.—*On a new methoxy-dihydroxy-toluquinone produced from glucose by species of* Penicillium *of the* P. spinulosum *series.*

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Reference to the carbon balance sheets given in Part IV for species of *Penicillium* shows that three species of *Penicillium*, diagnosed by Dr. CHARLES THOM as belonging to the *P. spinulosum* series, were included in Table I. The only salient feature of the carbon balance sheets of these three species is the large amount of acid, entirely of the non-volatile type, which they produce from glucose.

These three species—Ad. 74, Ad. 79 and Ad. 80—show, in addition, a very characteristic colour reaction when grown on a modified CZAPEK-DOX glucose medium containing twenty times the usual amount of ferrous sulphate given in Part I, p. 7.

When the above moulds were grown on this medium at 23° C. it was found that a purple ring first appeared immediately under the mycelium and gradually spread downwards till the whole medium became purple. In later stages of growth a yellow ring was formed below the mycelium and it also spread downwards till the purple colour had almost entirely disappeared. When the slightly yellow medium was exposed to the air, as for instance in filtration of the medium, the liquid was found to assume a much darker tint, almost comparable with the original purple.

On addition of ferric chloride solution to the filtered metabolism solution a characteristic deep brown colour was produced. Addition of dilute permanganate solution, in the cold, to the filtered metabolism solution resulted in an immediate reduction of the permanganate, the amount of reduction being roughly proportional to the depth of the purple colour and to the intensity of the ferric chloride reaction.

The work described in this paper is divided into two parts. The first part deals with the isolation and identification of the acid—citric acid—which is largely responsible for the abnormal acidity produced by this group of fungi. The second part deals with the isolation, properties and constitution of the compound responsible for the characteristic colour of the medium and for the ferric chloride and permanganate reactions. This substance, which is itself a strong acid and contributes in part to the acidity of the medium, is a methoxy-dihydroxy-toluquinone, crystallising in purple-black crystals similar in appearance to potassium permanganate.

PART I.—*Investigation of the nature of the acids produced from glucose by Ad.* 74, *Ad.* 79 *and Ad.* 80.

The salient features of the carbon balance sheets for the *Penicillium* species Ad. 74, Ad. 79 and Ad. 80, given on p. 56 of Part IV, are the following :—

(*a*) Titratable acidity = 29·1 c.c., 20·3 c.c., 21·7 c.c. N/1 acid per 250 c.c. medium.

(*b*) Carbon in non-volatile acids = 0·937 gm., 0·862 gm., 0·799 gm., corresponding respectively to 20·0 per cent., 18·3 per cent. and 22·6 per cent. of the glucose fermented.

The investigation of the nature of the acid chiefly responsible for these figures was carried out as follows :—

The mould Ad. 74 was grown in a series of 43 one litre conical flasks, plugged with cotton wool, each containing 350 c.c. of CZAPEK-DOX glucose medium, and incubated for 18 days at 24° C. The volume of the filtered metabolism solution was 13·8 litres. To this was added a small amount of washings from the mycelium.

The free acids were neutralised with sodium hydroxide and the solution was precipitated with basic lead acetate. The lead precipitate was well washed and then ground up with an excess of dilute sulphuric acid. The lead sulphate was filtered, the excess sulphuric acid was quantitatively removed from the filtrate with baryta and the filtrate evaporated *in vacuo* down to a syrup. This was twice taken up in methyl alcohol and re-evaporated so as to remove as much water as possible. The residue was finally treated with 400 c.c. of dry methyl alcohol and then saturated with dry hydrochloric acid gas. Crystals separated overnight and were filtered off, washed with methyl alcohol and dried at 60° C. The washings were mixed with the main portion of the filtrate and the passage of the hydrochloric acid gas continued. No further crystals were obtained, so the alcoholic solution was evaporated down to 100 c.c. and re-saturated with hydrochloric acid gas. A further crop of crystals obtained was filtered off and washed. Weight of 1st crop of crystals = 58 gm. Weight of 2nd crop of crystals = 10 gm.

10 gm. of the first crop of crystals were recrystallised from water. They now melted at 76°–78° C., which agrees with trimethyl citrate (M. Pt. 78·5° C.). For confirmation, 5 gm. of the above methyl ester and 25 c.c. of freshly saturated aqueous ammonia solution were mixed and allowed to stand overnight. A fair crop of crystals separated —2·6 gm.—which when recrystallised from water melted at 206°–207° C. Citramide from pure citric acid melted at 207°–208° C. The mixed melting point was 207°–208° C. For further confirmation 0·5 gm. of the amide from Ad. 74 was heated with 2 c.c. of 72 per cent. sulphuric acid in a test tube to 130° C. and poured into water. A yellow crystalline precipitate appeared, which was filtered and washed with water. This gave the characteristic reactions of citrazinic acid—blue fluorescence with ammonia, and a deep blue colour when dropped into boiling sodium nitrite solution. All the

above tests serve to identify completely the main acid product of Ad. **74** as citric acid.

The second crop of crystals, when recrystallised from water with the addition of a little charcoal, melted at 76°–78° C. and evidently also consisted of trimethyl citrate.

The alcoholic filtrate from the crystals was extracted with ether, after removal of alcohol *in vacuo*, and the extract was washed with dilute potassium hydroxide solution and then with a little water. After drying over anhydrous sodium sulphate the ether was distilled and the residue poured into a tube. It immediately began to crystallise and soon set solid. Weight = 1·89 gm. After recrystallisation from water it melted at 74°–76° C. and proved to be trimethyl citrate. Thus, the only esterifiable acid formed by Ad. **74** under the conditions adopted is citric acid.

The acids from Ad. 79 and Ad. 80 were also investigated and were found to consist chiefly of citric acid.

In the case of Ad. 80 the ethyl esters were prepared and the portion insoluble in ether appeared to be considerable. An attempt was made to distil it in a high vacuum using a mercury vapour pump, but this met with no success.

To sum up then, the acid which is mainly responsible for the high acidity and high "carbon in non-volatile acids" in each of the three species of *Penicillium*—Ad. 74, Ad. 79, Ad. 80—is citric acid.

PART 2.—*Preparation, properties and constitution of the substance produced by Ad.* 74, *Ad.* 79 *and Ad.* 80, *which gives the characteristic colour to the medium and the reactions with ferric chloride and permanganate.*

N.B.—For convenience, and in the absence of a name, this substance will be referred to briefly as "Z."

Preparation of Z.—Sixty litres of the CZAPEK-DOX glucose solution rich in iron was distributed between 12 trays and placed in the combined sterilizer-incubator used for the preparation of kojic acid and described on p. 136 (Part VII). After sterilization each tray was sown with an emulsion of spores of culture X. 80, prepared from a ROUX bottle culture on beer wort agar. (X. 80 is another strain of the same group as Ad. 74, Ad. 79 and Ad. 80, isolated at a later date. It was chosen in preference to any of the other strains as it gives a deeper colour reaction. Z has, however, been isolated from each of the species Ad. 74, Ad. 79 and Ad. 80.) The trays were incubated at room temperature—an average of about 22° C.—and an abundant supply of sterile air was passed through the incubator during the whole incubation period. The trays were sampled on two occasions with the following results (See Table I, Tank Expt. Z1.) :—

TABLE I.—*Tank Experiment Z 1.*

Tray No.	7 Days.				11 Days.			
	Titration, c.c. N/1 Alkali per 25 c.c.	c.c. N/10 $KMnO_4$ per 25 c.c.	Glucose by Polari-meter.	$FeCl_3$ Reaction.	Titration, c.c. N/1 Alkali per 25 c.c.	c.c. N/10 $KMnO_4$ per 25 c.c.	Glucose by Polari-meter.	$FeCl_3$ Reaction.
			Per cent.				Per cent.	
1	—	—	—	—	2·40	5·0	0·714	Strong reaction
2	1·35	2·8	2·55	+ +	2·20	5·8	0·953	,,
3	—	—	—	—	2·16	4·8	1·044	,,
4	—	—	—	—	2·30	6·0	0·777	,,
5	1·18	3·3	2·44	+ + +	2·27	7·2	0·601	,,
6	—	—	—	—	2·44	5·8	1·158	,,
7	—	—	—	—	2·42	6·3	0·787	,,
8	1·55	3·1	2·48	+ + +	2·35	6·0	0·822	,,
9	—	—	—	—	2·45	4·8	0·985	,,
10	—	—	—	—	2·70	5·3	0·855	,,
11	1·15	2·4	2·48	+ +	2·46	5·8	1·022	,,
12	—	—	—	—	2·52	5·0	1·061	,,

After 14 days' incubation the metabolism solution, which was dark purple in colour, was filtered and analysed. 44·13 litres were obtained, having the following characteristics :—

(1) *Titration*, 22·15 c.c. N/1 NaOH for 250 c.c.
(2) $KMnO_4$, 5·5 c.c. N/1 $KMnO_4$ for 250 c.c.
(3) *Glucose* (by polarimeter), 0·318 per cent.

The whole of this liquid was evaporated *in vacuo* to 1,520 c.c. Several methods for the isolation of Z were tried, but these were not very successful until it was realized that Z is present in solution, partly free, and partly as a complex which on hydrolysis with acids breaks down. The method finally adopted was as follows :—

Sufficient sulphuric acid was added to the evaporated metabolism solution to bring the solution to normality, and the mixture was boiled for several hours under a reflux condenser. The cooled solution was then thoroughly extracted with ether, which extracts all the Z present, together with large amounts of citric acid. A quantity of Z separated from the evaporated ether solution, and the remainder was separated by extraction of the mother liquors, after dissolving in water, with toluene. Most of the toluene was evaporated *in vacuo*, when Z crystallised out. The crude Z, whether from ether or toluene, was finally purified by two sublimations *in vacuo*. Z begins to sublime at 120° C. at about 1 mm., giving a pure product in purple-black crystals.

Properties of Z.—The substance Z forms dark coloured, almost black, crystals with a metallic lustre, having the appearance of brownish plates under the microscope. It is very slightly soluble in cold water, more soluble in hot water, giving an amethyst shade which on acidifying changes first to pink and then to yellow. The change in colour from purple to yellow on acidification explains the characteristic colour change in the medium observed with all strains of *Penicillium* in this group and previously given in detail. When potassium or sodium hydroxide is added to neutralise its acid reaction the solution becomes deep purple, changing to faint blue when the reaction is made more alkaline. With concentrated sulphuric acid Z gives an intense blue colour.

On heating, Z melts without decomposition at 202°–203·5° C. and sublimes to some extent on the M. Pt. tube.

Z has a definite aromatic odour, reminiscent of the quinones.

It immediately decolorises acidified permanganate solution in the cold, and gives a deep rich brown colour with ferric chloride.

It is reduced by zinc dust and hydrochloric acid to a colourless compound. This on extraction with ether gave colourless microscopic crystals, but these very quickly darkened owing to atmospheric oxidation. The reduced Z is readily soluble in water giving a colourless solution which, on standing, quickly absorbs oxygen and becomes pink in colour. A freshly prepared solution of reduced Z in water gives an intense blue-green colour with ferric chloride.

A re-sublimed sample of Z gave the following results on combustion :—

Weight of Z analysed.	Weight of H_2O.	Weight of CO_2.	Percentage of Hydrogen.	Percentage of Carbon.
	Gm.	Gm.		
0·1759 gm.	0·0702	0·3372	4·47	52·28
0·1726 gm.	0·0661	0·3299	4·29	52·14
Calculated for $C_8H_8O_5$	—	—	4·38	52·16

The empirical formula for Z is therefore $C_8H_8O_5$.

Determination of molecular weight of Z.—(*a*) A determination of the molecular weight of Z was carried out by RAST's camphor method (1922). 0·0266 gm. of Z mixed with 0·1409 gm. of camphor lowered the melting point of the latter 41° C. This corresponds to a molecular weight of 184. (Theoretical for $C_8H_8O_5 = 184$.)

(*b*) *By titration with sodium hydroxide.*—0·1141 gm. of Z was weighed out and titrated with N/10 sodium hydroxide to phenolphthalein. In order to ensure complete solution an excess of sodium hydroxide was added and then the solution was titrated back with N/10 hydrochloric acid. Owing to the dark permanganate colour of the solution the end-point was difficult to determine, and the figure obtained can only be regarded as a rough approximation.

22·0 c.c. N/10 NaOH were added and 8·8 c.c. N/10 HCl were required for back titration, giving a net value of 13·2 c.c. N/10 NaOH.

Assuming that the substance is a monobasic acid this corresponds to a combining weight of 86·4. Since the molecular weight of Z has been shown to be 184 it is obvious that Z titrates as a dibasic acid to phenolphthalein.

Preparation of the diacetyl compound of Z, $C_8H_6O_3\,(O.CO.CH_3)_2$.—0·5 gm. of Z was mixed with 5 c.c. of acetic anhydride and three drops of concentrated sulphuric acid. The mixture was heated for a few seconds, when the original purple colour had completely disappeared. The mixture was then poured into cold water and crystals quickly separated which were filtered and dried. Yield of crude product = 0·65 gm. of yellow needles. This was recrystallised from absolute alcohol in yellow needles having a melting point of 139·5° C.

This compound gave the following results on combustion :—

Weight of Acetyl Compound Analysed.	Weight of H_2O.	Weight of CO_2.	Percentage of Hydrogen.	Percentage of Carbon.
	Gm.	Gm.		
0·1543 gm.	0·0624	0·3057	4·53	54·02
0·1508 gm.	0·0592	0·2956	4·39	53·57
Theoretical for $C_8H_6O_3\,(O.CO.CH_3)_2$	—	—	4·51	53·71

The number of acetyl groups present was estimated in the following manner :—

0·2705 gm. of the acetyl compound was hydrolysed by heating under a reflux with 25 c.c. of N/1 sulphuric acid in an oil bath at 130° C. for 7 hours. The mixture was then made up to about 500 c.c. with distilled water, and evaporated *in vacuo* to about 25 c.c., the operation being repeated until a constant titration figure was obtained for the distillate. This was taken as the blank for each distillation and the total corrected titration figure was 20·37 c.c. of N/10 sodium hydroxide.

This corresponds to a CH_3CO content of 32·38 per cent.

Calculated for two acetyl groups in $C_8H_6O_3\,(O.CO.CH_3)_2$ = 32·10 per cent. These figures indicate clearly that the compound is a diacetyl derivative, and prove the presence in Z of two acetylatable hydroxyl groups.

During the distillation a very small amount of Z came over and tinged the distillate pink. This would not affect the result, however, as the amount would be practically the same in each distillation and would be included in the blank.

Preparation of the tetra-acetyl compound of reduced Z, $C_8H_6O\,(O.CO.CH_3)_4$.—As it had previously been shown that the reduction product of Z, formed by treating Z with zinc dust and hydrochloric acid, was easily oxidized by atmospheric oxygen it was thought that it might be a matter of some difficulty to prepare the substance in a sufficiently pure state for combustion.

It was therefore decided to prepare the reduction product of Z and to acetylate it in one operation as in the method of FICHTER and WILLMANN (1904) for dialkylated dihydroxyquinones.

0·5 gm. Z, 2 c.c. acetic acid, and 1 gm. zinc dust, were therefore heated to boiling for about 1 minute. Then 10 c.c. of acetic anhydride were added and 4 gm. of sodium acetate. As the reduction was not quite complete, more zinc dust was added until the solution was practically colourless, this requiring 3 to 4 minutes heating. The product was poured into water, well stirred and filtered. A crystalline acetyl derivative was obtained mixed with zinc dust. The mixture was extracted with boiling absolute alcohol and filtered hot. There was a good separation of crystals on cooling. Yield = 0·7 gm. of an almost colourless product. When recrystallised from absolute alcohol it formed colourless rhombic prisms melting at 192·5°–194° C.

This compound gave the following results on combustion :—

Weight of Acetyl Compound Analysed.	Weight of H_2O.	Weight of CO_2.	Percentage Hydrogen.	Percentage Carbon.
	Gm.	Gm.		
0·1506 gm.	0·0664	0·3011	4·93	54·52
0·1250 gm.	0·0549	0·2476	4·91	54·03
Theoretical for $C_8H_6O(O.CO.CH_3)_4$	—	—	5.10	54·23

The number of acetyl groups present was estimated as follows :—

0·2695 gm. of the acetyl compound was hydrolysed by boiling with 25 c.c. of N/1 H_2SO_4 in the same manner as was described above for diacetyl Z. The separation of the acetic acid formed on hydrolysis was also carried out in the same way.

30·00 c.c. of N/10 sodium hydroxide were required to neutralise the acetic acid formed, corresponding to 47·87 per cent. of $CH_3.CO$ in the acetyl compound. The calculated value for four acetyl groups in $C_8H_6O.(O.CO.CH_3)_4$ is 48·59 per cent.

The reduced Z evidently contains four acetylatable hydroxyl groups. Since Z ($C_8H_8O_5$) itself contains two OH groups and gives rise to another two OH groups on reduction, the nature of four out of the five oxygen atoms in the molecule of Z has been determined. The nature of the only remaining oxygen atom was elucidated by carrying out an estimation of the alkoxyl groups present in Z.

Determination of alkoxyl groups in Z.—A ZEISEL estimation was carried out on Z with the following result :—

0·1948 gm. Z gave 0·2497 gm. silver iodide.
This expressed as methoxyl group = 16·92 per cent.
Calculated for 1 methoxyl group in $C_8H_8O_5$ = 16·85 per cent.

In order to decide whether the alkoxyl grouping is really a methoxyl group and not an ethoxyl or higher group, the products from a second ZEISEL operation were passed into freshly distilled dimethylaniline. Crystals separated which were left overnight, and were then filtered off. They appeared to be plates, and on heating, sintered (in a sealed tube) at 210° C. and at about 220° C. they had completely volatilised. With quick heating they showed signs of melting at 230° C.

A little trimethylphenylammonium iodide was prepared from methyl iodide and dimethyl-aniline. It showed the same phenomena as the product from Z when an attempt was made to determine its melting point.

An iodine estimation was then carried out on the product from Z by precipitating the iodine with silver nitrate in aqueous solution in presence of HNO_3. The silver iodide was collected on a Gooch crucible and weighed.

0·0833 gm. of the trialkylphenylammonium iodide from Z gave 0·0739 gm. of AgI, corresponding to 47·9 per cent. iodine.

Calc. for methyl compound, 48·3 per cent.
Calc. for ethyl compound, 45·8 per cent.
Calc. for propyl compound, 43·6 per cent.

The result agrees most closely with the methyl compound. Further support was obtained for the belief that the compound is really a methyl compound from the fact that a mixture of ethyl iodide and dimethylaniline could not be induced to form crystals. Methyl iodide gives crystals immediately as did the product from Z.

Z therefore contains one methoxyl group.

Action of hydroxylamine on Z.—0·2 gm. of Z, 0·5 gm. of hydroxylamine hydrochloride, and 15 c.c. of absolute alcohol were boiled under a reflux condenser for 1½ hours. There was no separation of an oxime and 0·12 gm. of Z was recovered unchanged. It may be concluded, therefore, that Z does not form an oxime under these conditions.

Constitution of Z.—The evidence presented as to the constitution of Z may be summarized as follows :—

(1) Z has the empirical formula $C_8H_8O_5$ and a molecular weight of 184 corresponding to this formula.
(2) Z titrates as a dibasic acid.
(3) Z contains in its molecule 5 oxygen atoms.

Of these :—

(*a*) One oxygen atom is present as a methoxyl group.
(*b*) Two other oxygen atoms are present as acetylatable hydroxyl groups, and, as Z titrates as a dibasic acid, these two OH groups are presumably phenolic OH groups attached directly to a benzene nucleus.
(*c*) The remaining two oxygen atoms are present as CO groups since, on reduction,

they become acetylatable hydroxyl groups, reduced Z giving a tetra-acetyl compound.

(4) Z is purplish-black in colour, but becomes colourless on reduction. Z and its reduction product give an intense red-brown and blue-green colour with ferric chloride respectively.

(5) Z does not give an oxime with hydroxylamine.

(6) Z has a definite aromatic odour reminiscent of the quinones.

From all the above considerations we are of the opinion that Z is a hydroxyquinone derivative, with which class of bodies it agrees in all particulars. Further, since it titrates as a dibasic acid, both hydroxyl groups must be in the nucleus and not in a side chain, and Z is thus a dihydroxyquinone. This accounts for four carbon atoms of the six present in the benzene ring, *i.e.*, two as CO groups and two as C . (OH) groups. The other two carbon atoms must then be represented as either (*a*) CH and $C . CH_2OCH_3$ or (*b*) $C . CH_3$ and $C . OCH_3$.

The evidence available points to (*a*) as being very improbable for the following reasons :—

(1) It is known that hydroxyquinones containing an unsubstituted CH group in the nucleus behave in an abnormal manner when acetylated by acetic anhydride in the presence of concentrated sulphuric acid. Under these conditions the CH group as well as the CO groups and OH groups are acetylated to $C . O . CO . CH_3$. Thus a hydroxyquinone having a free CH group gives a tetra-acetyl derivative (THIELE and WINTER, 1900) whereas Z, which contains two OH groups gives only a diacetyl derivative under these conditions (see p. 250).

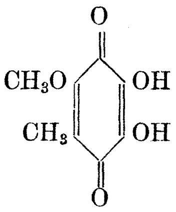
(2) It is known that hydroxyquinones are only " oximated " by hydroxylamine if they contain a CH group. Thus HOUBEN-WEYL, " Die Methoden der organischen Chemie " (2nd edition, Vol. 3, p. 611), say : " According to KEHRMANN the CO of halogen- and alkyl-substituted quinones can only be ' oximated ' by hydroxylamine if there is at least one CH group in the *ortho* position to the CO group. The hydroxylated quinones conform to this rule in so far as hydroxylamine in hydrochloric acid solution is used. With alkaline hydroxylamine they show deviations." Since it has been shown that Z is not " oximated " by treatment with hydroxylamine hydrochloride the absence of a CH group in the nucleus is indicated.

The six carbon atoms in the ring of the molecule of Z are thus arranged as follows :— Two carbon atoms as CO groups, two carbon atoms as C.(OH) groups, one carbon atom as a $C . CH_3$ group, and one carbon atom as a $C . O . CH_3$ group.

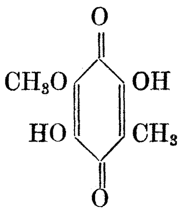
Z is thus a methoxy-dihydroxy-toluquinone.

There is at present no positive proof as to whether Z is a *para*- or *ortho*-quinone derivative, though the available evidence points to it being a *para*-derivative. The relative positions of the methyl, methoxyl, and two hydroxyl groups in the quinonoid nucleus is also unknown since no compounds of this type have been described in the literature.

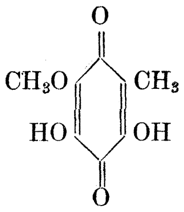
If it is allowed that Z is a *para*-quinone derivative it must have one of the following formulæ :—



(1)



(2)



(3)

Work is at present in progress having as its aim the synthesis of each of these compounds.

Note by Dr. CHARLES THOM, mycologist to the United States Department of Agriculture, on cultures Ad. 74, Ad. 79 and Ad. 80 :—

" Our transfers of Ad. 74, Ad. 79 and Ad. 80 put them together and in the group with *P. spinulosum*, or, in deference to the belief that Dr. WEHMER's *Citromyces Pfefferianus* belonged somewhere in this group, to the *P. Pfefferianum* series. Our notes indicate that they approach *P. viridi-dorsum* of BIOURGE which, in our cultures from the transfer he furnished us, belongs here."

Summary.

The products arising from the growth on a glucose medium of three strains of a species of *Penicillium* of the *P. spinulosum* series—Ad. 74, Ad. 79 and Ad. 80—have been investigated. All three strains produce large amounts of citric acid from glucose, but in addition give rise to smaller amounts of a substance " Z " which is responsible for the colour changes in the medium which are characteristic of this species of *Penicillium*. The preparation, properties and constitution of substance "Z" are described. Substance " Z," which was separated as purplish-black permanganate-like crystals of the formula $C_8H_8O_5$, is a methoxy-dihydroxy-toluquinone.

This is the first recorded instance of the production from glucose of a quinone derivative by fungi.