CXCIV. THE CORRELATION OF THE OXIDATION OF CERTAIN PHENOLS AND OF DIMETHYL-*p*-PHENYLENEDIAMINE BY BACTERIAL SUSPENSIONS.

By FRANK CHARLES HAPPOLD.

From the Department of Pathology and Bacteriology, the University of Leeds.

(*Received October 30th, 1930.*)

Gordon and McLeod [1928] have shown that the colonies of most bacteria which grow well only in the presence of oxygen, when treated with a 1 % solution of dimethyl-*p*-phenylenediamine hydrochloride, assume a reddish tint which progressively darkens to black. This they attributed to the presence in such bacteria of a direct oxidase system the relative thermolability of which was shown when the bacteria were heated to 88° for 1 hour. Nishibe [1926], following Schultze's [1910] use of a mixture of dimethyl-*p*-phenylenediamine with *α*-naphthol in the study of the structure of the anthrax bacillus, used this same indophenol reagent in an investigation similar to that of Gordon and McLeod and obtained similar results.

During the course of some routine work a young culture of *B. pyocyaneus* was treated with the dimethyl-*p*-phenylenediamine reagent. Rapid pigmentation commenced but almost immediately a rapid fading process was superimposed and in a few minutes only a fringe of colour round the periphery of the culture plaque was discernible. The culture was again treated with the reagent and with a similar result except that a larger portion of the culture became irreversibly pigmented. This phenomenon was attributed to bacterial reduction, and, since Vernon [1911] and Batelli and Stern [1912] have shown that the oxidation of this reagent in the presence of *α*-naphthol may be greatly inhibited by reducing systems, it seemed possible that this "direct oxidase" reaction might be missed by the technique of Gordon and McLeod in bacterial systems where it was present but masked by reducing systems. This possibility has been indicated by Ellingworth, McLeod and Gordon [1929]. It was also felt that the argument of Gordon and McLeod would be strengthened if it could be shown that those organisms which give the dimethyl-*p*-phenylenediamine reaction could also catalyse the oxidation of certain phenols to substances which could be chemically isolated and identified as products of oxidation. Happold and Raper [1925 and unpublished results] have shown that tyrosinase in the presence of atmospheric oxygen and certain phenols which can be oxidised to form *ortho*-quinones causes the deamination and

decarboxylation of amino-acids, a reaction first reported by Chodat and Schweizer [1913] but attributed by them to the direct action of the enzyme on the amino-acid in the presence of oxygen. The aldehyde which contains one carbon atom less than the parent acid, ammonia and carbon dioxide are the final products of the reaction. They also showed that phenols which do not form *ortho*-quinones by oxidation are devoid of such action; *p*-benzoquinone can exert no such effect on amino-acids in the presence of oxygen but synthetic *o*-benzoquinone will cause deamination and decarboxylation of the amino-acid without the addition of the enzyme. Attempts to isolate *o*-benzoquinone from the system tyrosinase-catechol-oxygen were not made because of the instability of the quinone, but Pugh and Raper [1927] successfully proved that *o*-benzoquinone and homoquinone are products of the aerobic action of tyrosinase on phenol or catechol and *p*-cresol respectively. This they accomplished by isolating the dianilino-derivatives of the quinones and by establishing the chemical identity of the isolated derivatives.

In the present investigation the technique of these authors has been followed in most essentials in testing the ability of bacterial suspensions to catalyse the oxidation of phenols. Well washed, heavy bacterial suspensions buffered in phosphate at p_H 6·5 have been used throughout. The colour changes produced when solutions of catechol, guaiacol, orcinol, phenol, *p*-cresol and tyrosine are treated with washed bacterial suspensions have been noticed and compared with the results obtained by following the technique of Gordon and McLeod. None of the organisms tested has been capable of causing colour changes in solutions of phenol, *p*-cresol or tyrosine with the exception of a strain of *Vibrio tyrosinatica* obtained through the courtesy of Prof. Kluyver of Delft, but changes have been noticed with solutions of catechol, guaiacol and orcinol. Increased deamination of amino-acids in the presence of catechol appears to be restricted to suspensions of those organisms which produce colour changes in catechol and the production of *o*-benzoquinone in such systems has been demonstrated by the isolation of the dianilinoquinone from the system aniline, catechol and oxygen with suspensions of cholera and an anthracoid respectively. Guaiacol was oxidised in the presence of oxygen to a maroon-coloured compound by the anthracoids studied, but no deamination took place in the system anthracoid-guaiacol-alanine. A lemon-yellow colour is also produced with orcinol by certain organisms. The chemical nature of the coloured compounds produced is not known but there is evidence that compounds containing the *ortho*-quinone grouping are not produced in the oxidation.

Colour changes produced with phenolic substances in the presence of oxygen by bacterial suspensions. Cultures of organisms were made on agar plates or, in the case of delicate bacteria, on blood-agar plates. After 24 hours the cultures were suspended in sterile saline and the bacteria were then separated from the saline by centrifuging. The deposited bacteria were suspended in phosphate buffer at p_H 6·5 so that a suspension of milk-like opacity was

obtained and this suspension was divided and one-half was heated for 30 minutes in a boiling water-bath; the boiled and unheated suspensions were divided into 2 cc. portions and 1 cc. of a 1 % solution of phenol, *p*-cresol, catechol, or orcinol, or 1 cc. of a saturated solution of tyrosine or guaiacol, was added in duplicate to the two suspensions. The action of dimethyl-*p*-phenylenediamine hydrochloride on a plate culture of the organism was also tested for purposes of comparison. Colour changes were observed during the first 3 hours and after 18 hours and the latter results have been recorded. A further control was made by adding 2 cc. of buffer to 1 cc. of the solution containing the phenol.

Table I.

Suspension tested	Substrate: Dimethyl-*p*-phenylene-diamine	Catechol	Guaiacol	Orcinol	*p*-Cresol, phenol and tyrosine
Anthracoid	+ + +	+ + +	+ +	+ +	−
Do. boiled	.	+ +	−	Trace	−
B. subtilis	+ +	+ + +	+ +	.	−
Do. boiled	.	+ +	−	.	−
Cholera	+ + +	+ +	−	−	−
Do. boiled	.	−	−	−	−
Gonococcus	+ + +	+ +	−	+	−
Do. boiled	.	−	−	−	−
Meningococcus	+ + +	+ +	−	−	−
Do. boiled	.	−	−	−	−
Gram-negative diplococcus (throat)	+ + +	+ +	−	.	−
Do. boiled	.	−	−	.	−
B. pyocyaneus	+ ±	+ ±	−	−	−
Do. boiled	.	−	−	−	−
B. prodigiosus	+ ±	+	−	+ +	−
Do. boiled	.	−	−	−	−
Sarcina	+	+ +	−	+	−
Do. boiled	.	−	−	Trace	−
Staph. albus	−	+	−	+	−
Do. boiled	.	−	−	−	−
Vibrio tyrosinatica	+ +	+ + +	−	.	+ + +
Do. boiled	.	−	−	.	−

Negative results were obtained with the following, Streptococcus isolated from case of puerperal sepsis, *B. pfeiffer*, three strains of Sarcina, *B. welchii*, *B. sporogenes*, *C. diphtheriae*, *B. coli*, *B. aertryck* "Mutton" and "Newport."

The next stage in the investigation was to show that those organisms which gave the colour changes with the dimethyl-*p*-phenylenediamine and with the catechol could also cause deamination of amino-acids in the presence of the latter reagent with the liberation of ammonia. Previously this had been studied by following not only the liberation of ammonia in the system enzyme-catechol-amino-acid-buffer, but also the decrease in the amino-nitrogen content of the system as oxidation of the catechol to *o*-benzoquinone proceeded. In the present investigation the liberation of ammonia has been taken as sole evidence of deamination. Four test aeration bottles

were prepared as follows: (1) 30 cc. of phosphate buffer (p_H 6·5) 0·2 g. of alanine, 0·1 g. of catechol and 10 cc. of a washed suspension of the organism to be studied; (2) differed only from (1) in that the bacterial suspension had been immersed in a boiling water-bath for 30 minutes; (3) as (1) except that no catechol was added; (4) differed from (1) since no bacterial suspension had been added. Chloroform was now added to all four bottles to depress any possible bacterial growth in system (3). Ammonia-free air was aspirated for 24 hours through the four bottles and was then caused to pass through four wash-bottles which contained 25 cc. of $N/10$ H_2SO_4. After this period of aeration a few drops of capryl alcohol and 2–3 g. of sodium hydroxide were added to each reaction bottle and any ammonia which was liberated was rapidly aspirated into the bottles containing the standard acid. The ammonia could then be estimated. The first apparent difficulty lay in the observed fact that, though non-enzymic oxidation of catechol could not be observed at the reaction (p_H 6·5) at which the oxidations were being carried out, when the alkali was added for liberation and estimation of the ammonia the oxidation of catechol could proceed rapidly without the intervention of any enzyme system and presumably by a similar type of oxidation to that found in the enzyme system. Thus if the control bottle (4), containing no bacterial suspension, was taken and was aerated for 24 hours, there would be practically no trace of pigment, and the production of *o*-benzoquinone as stated by Pugh and Raper [1927] could not be demonstrated at this stage. If now the 2–3 g. of NaOH were added, a rapid pigmentation of the mixture proceeded accompanied by the deamination of the amino-acid. The rate of this non-enzymic deamination has been measured; the average ammonia liberated in such a system during the first 40 minutes of rapid aspiration was so slight that it was within the limit of experimental error. After 80 minutes it represented on an average the equivalent of 0·14 cc. of $N/10$ NaOH, after 160 minutes, 0·31 cc. and after the whole system had been left standing overnight and then aspirated rapidly for a further period of 40 minutes in the morning the total ammonia which had been liberated was equivalent to 1·21 cc. of $N/10$ NaOH. In view of these findings the period of rapid aspiration subsequent to the addition of the NaOH was limited to 60 minutes, a period which had been shown to be adequate for the liberation and estimation of such amounts of ammonia as were likely to be present, and in which the ammonia formed and liberated by the non-enzymic oxidation of the catechol averaged approximately 0·15 mg. NH_3.

Table II, column 1 represents the difference between the average estimated ammonia content of the system (amino-acid-buffer-bacterial suspension-catechol) or of bottle (1) and that of bottle (4) which represents the ammonia liberated by non-enzymic action (0·15 mg.). Column 2 gives the same difference between bottle (2) and bottle (4) and this result shows us whether a bacterial suspension possesses any heat-stable catalyst for the oxidation of catechol. Column 3, which records the values obtained for bottle (3), affords

a combined measure of any volatile basic substances present in the bacterial suspension and liberated under the conditions of the experiment, and of any ammonia produced by normal deamination of the alanine by the bacterial suspension.

Table II.

Mean values of ammonia content expressed in mg.

Organism used	System 1–system 4	System 2–system 4	System 3
Anthracoid	1·05	0·38	0·17
B. subtilis	0·81	0·05	0·25
Cholera	0·72	0·08	0·25
Gonococcus	0·66	−0·04	0·10
B. pyocyaneus	0·69	0·20	0·20
B. prodigiosus	0·86	0·26	0·37
V. tyrosinatica	0·89	0·22	0·25
Streptococcus	0·19	0·09	0·14
B. aertryck "Newport"	0·06	0·10	0·19
Staphylococcus	0·07	Nil	0·29

These results are in general the mean of three experiments. The difference in value between the results recorded in columns 1 and 3 for any one organism is a measure of the deamination caused by the enzymic oxidation of the catechol by the organism concerned and it is at once apparent that all those organisms which give the dimethyl-*p*-phenylenediamine reaction catalyse the oxidation of catechol. The results given in column 2 on the other hand indicate that with the possible exception of the anthracoid all other organisms lose this catalytic effect after subjection to a temperature of 100° for 30 minutes.

Various other phenols were now substituted for catechol *viz.* phenol, *p*-cresol and guaiacol and since these substances were not autoxidised the control bottle (4) could be withdrawn. The results can be briefly summarised as follows: of all the organisms tested none could cause deamination of amino-acids in the presence of the monohydric phenols, except the tyrosinase-containing *V. tyrosinatica* and though the two spore-bearing aerobes used catalysed an oxidative pigmentation of the guaiacol, neither they nor any of the other organisms tested could catalyse oxidative deamination of amino-acids in the presence of guaiacol. The following results indicate these facts. *B. subtilis* is taken as a type for the rest of the organisms which do not catalyse the oxidation of monohydric phenols and *p*-cresol as the representative of the two monohydric phenols used.

Table III.

Mean value of ammonia content expressed in mg.

Organism used	Suspension-*p*-cresol amino-acid	Heated suspension-*p*-cresol-amino acid	Suspension amino-acid
B. subtilis	0·09	0·09	Nil
V. tyrosinatica	1·05	0·40	0·51

The high ammonia value of the heated *V. tyrosinatica* suspension can be understood when one realises that the suspension is heated at a slightly acid reaction, so that there is not the same tendency to drive off ammonia and volatile bases by heating as there would be if the suspensions were made in

a neutral liquid. Since cultures of the organism are also of a mucilaginous consistency, satisfactory purification by washing is extremely difficult. The ammonia value in the absence of *p*-cresol is consequently high.

Action of washed bacterial suspensions on catechol in the presence of aniline. 2 g. of catechol, 4 cc. of aniline and the washed suspension prepared from 10 plate cultures of (1) the anthracoid and (2) *V. cholera* respectively were made up to 1 litre with Clark's buffer mixture (p_H 6·5) and the whole was aerated for 48 hours as recommended by Pugh and Raper [1927]. The reddish precipitate which separated in each of the two preparations was filtered off and washed alternately with 1 % HCl and distilled water so as to remove traces of aniline; it was then dried *in vacuo*. The dried filter papers were then extracted with ether in a Soxhlet apparatus and the ethereal solution was extracted with 0·02*N* NaOH; the aqueous layer was separated and acidified with 2 % HCl and the precipitate which formed was again filtered off and dried as before. The precipitate was finally extracted with acetone and was crystallised from this solvent. The crystals which were obtained in both experiments were bright reddish-coloured needles which gave all the reactions characteristic of the anilino-*o*-benzoquinone prepared by Pugh and Raper. The yield with the anthracoid was approximately 1·4 g. of the crystals having M.P. 193·5°, N, 9·61 %. The yield with cholera was approximately 1·3 g. of crystals, having M.P. 193·0°, N, 9·57 %. The highest M.P. found by Pugh and Raper was 193·5°, and $C_{18}H_{14}O_2N_2$ requires N, 9·66 %. Efforts to obtain anilino-quinones from control experiments using boiled bacterial suspensions were unsuccessful, though some pigmentation occurred.

The oxidation of catechol in the presence of aniline at alkaline reactions. The observation that catechol was oxidised at alkaline reactions without the intervention of any catalyst and that the oxidation proceeded by such channels that amino-acids if present were concurrently deaminated led the author to investigate whether or not *o*-benzoquinone was produced as an intermediate product. The investigation has been extremely difficult since there appeared to be a marked tendency to tar-formation. Aeration experiments were carried out on the same quantities of catechol and aniline as before, but the solutions were buffered at p_H 9·0 and no bacterial suspensions were added. The aeration was carried out for 48 hours. A first experiment gave reddish-coloured crystals whose M.P. was low and indeterminate; the amount was too small for further experimentation. A second experiment revealed two distinct types of crystals separating from an acetone solution of the products of the reaction, (1) typical reddish-coloured needles whose appearance was identical with that of anilino-*o*-benzoquinone and (2) orange-yellow coloured hexagonal plates. Both types of crystals came down together though the latter predominated. The yellow crystals have M.P. > 260° and differ from anilino-*o*-benzoquinone in that they are not readily soluble in *N*/50 sodium hydroxide.

SUMMARY.

The capacity of bacterial suspensions to catalyse the oxidation of catechol, guaiacol, orcinol, phenol, *p*-cresol and tyrosine has been studied and it has been shown that the oxidation of catechol with the formation of *ortho*-benzoquinone is only catalysed by those organisms which give the dimethyl-*p*-phenylenediamine reaction as applied by Gordon and McLeod. An apparent discrepancy exists with the strain of Staphylococcus used, since suspensions of the organism gave a typical though weak colour change with catechol and then failed to give evidence of deamination in the system bacterial suspension-alanine-catechol. Catechol which does not appear to be appreciably autoxidised at slightly acid reactions is markedly oxidised at p_H 9·0 and when the oxidation is carried out in the presence of aniline two distinct crystalline substances, one of which appears to be the anilino-*o*-quinone, can be isolated from the system.

The anthracoids studied differ from the other oxidase-containing bacteria in catalysing the oxidation of guaiacol to a maroon-coloured compound of unknown type, whilst *V. tyrosinatica* catalyses the oxidation of phenol and *p*-cresol, phenols which do not appear to suffer autoxidation.

The author is indebted to Prof. J. W. McLeod and Prof. H. S. Raper for encouragement during the course of this investigation and for useful suggestions made during proof reading. To the Medical Research Council whose grants made this work possible my grateful thanks are also due.

REFERENCES.

Batelli and Stern (1912). *Biochem. Z.* **46**, 324.
Chodat and Schweizer (1913). *Arch. Sci. Phys. Nat.* **35**, 140.
Ellingworth, McLeod and Gordon (1929). *J. Path. Bact.* **32**, 173.
Gordon and McLeod (1928). *J. Path. Bact.* **31**, 185.
Happold and Raper (1925). *Biochem. J.* **19**, 92.
Nishibe (1926). *Scientific reports, Govt. Institute for tropical diseases, Tokyo,* 185.
Pugh and Raper (1927). *Biochem. J.* **21**, 1370.
Schultze (1910). *Zentr. Bakt. Par. Orig.* **56**, 544.
Vernon (1911). *J. Physiol.* **42**, 402.