LV. AN ENZYME OF THE TUBERCLE BACILLUS¹

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THE possible importance of bacterial enzymes in the diseases of man has not received the attention it deserves. Particularly in a chronic disease such as tuberculosis the bacterial enzymes, disseminated slowly but with cumulative effect, may well play a decisive role. The study which follows deals with an oxidizing enzyme produced by the tubercle bacillus.

The findings of four groups of workers are in direct conflict in regard to the presence of an indophenol oxidase in the tubercle bacillus. Gordon & McLeod [1928], using *p*-aminodimethylaniline (*p*-dimethylphenylenediamine) alone, reported a 1 plus result for the tubercle bacillus on a scale where the *meningococcus* was 4 plus, confirming the 1 plus result of Schultze [1910] and Kramer [1912] who used the diamine in combination with α -naphthol. But Terada & Nozaki [1937], in a study concerning human and rat lepra bacilli and the other acid-fast organisms, using the diamine alone as did Gordon & McLeod, found no indophenol oxidase in either the pathogenic or non-pathogenic acid-fast bacilli, while their controls of *B. subtilis* and *meningococcus* gave the proper positive results. Kawabata [1934] also reported negative results for indophenol oxidase in human, bovine and frog tubercle bacilli.

No reference has been found in the literature to the presence in the tubercle bacillus of a catechol or phenol oxidase, but a catechol oxidase frequently occurs in organisms producing an indophenol oxidase as shown by Happold [1930]. The intensities of the enzymic reactions were not parallel for the two, and one organism (*Staph. albus*) possessed the catechol but not the indophenol oxidase.

A search for the enzyme which is the subject of this paper was undertaken because it appeared that some of the properties of a phenolase or catechol oxidase might explain certain observations made in the laboratory regarding pigmentation in tuberculous tissue, and the reddening and darkening of masses of tubercle bacilli which had been left for some weeks in a phenol solution. The work described is preliminary to a more extended study now under way.

EXPERIMENTAL

Testing of bacillary masses with phenolic substances

The original observation regarding the darkening of masses of bacilli was verified by placing equivalent masses of tubercle bacilli of human type in duplicate bottles of 5% phenol, sterile physiological saline and sterile distilled water; one trio was kept in the dark and the other was exposed to the usual laboratory lighting. The bacillary masses changed colour only in phenol, and the reddening occurred sooner and darkened more in those exposed to the light. In all subsequent tests the materials were accordingly exposed as evenly as possible to light.

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Variation of phenol concentration. Aqueous solutions were used. Ten concentrations, from 0.5 down to 0.1 %, and from 0.05 to 0.01 % respectively, were tested in 5 ml. portions. The human type bacillus (Gary strain) was used; one culture had been recently planted and the other was an old one, suspected of being dead. Comparable crumbs of bacilli (20–40 mg.) were removed from the solid medium and placed in the solutions with a nichrome wire loop.

Reddening of both organisms was observed in 1 day in the 0.5, 0.4 and 0.3 % tubes. Both reddened in 2 days in the 0.2 % phenol. The freshly recovered strain reddened in the 0.1 % tube in 4 days, but not the old culture. None of the 0.05–0.01 % tubes gave any coloration within 2 weeks. For practical purposes, therefore, it would be advisable not to use a lower concentration of phenol for testing than 0.2 %.

An upper limit for the phenol concentration cannot be determined. Homogeneous mixtures of phenol and water cannot be obtained between approximately 10 and 70 % phenol. Human type bacilli (Gary), taken off solid medium, were tested in 90, 80 and 75 % phenol. Swelling and some dispersion of the crumbs occurred, but no darkening within 12 days. Five months later complete solution of the bacillary masses was the only change to be observed.

Phenol in phosphate buffer. KH_2PO_4 -NaOH mixtures, made up according to Clark [1925] at pH 5.8, 6.0, 6.2, 6.4, 6.6, 7.0 and 7.4, containing 5 % phenol, were used in 5 ml. portions with equal-sized crumbs of human bacilli (Gary) freshly grown on 5 % glycerol broth. In 1 day a faint, shrimp-pink tint was seen on the edges of the crumbs in all the tubes. After 3 days the colour had spread throughout the bacillary masses, that in the pH 6.0 tube being the darkest; next day there was a suggestion of dark speckling in this tube. Two months later only a uniformly deep rose colour appeared in all the tubes, somewhat greyer in tinge than the original.

Preliminary trials with substances other than phenol

Orcinol, *thymol* and *p*-benzoquinone were tested in saturated aqueous solution; *catechol* and *resorcinol* were used in 1 % aqueous solution. Crumbs of the same human type bacilli (Gary), used above for the phenol-phosphate tests, were used here, in 5 ml. of the solutions of which another 5 ml. were kept as a control in which colours developed by autoxidation could be seen.

There was no immediate change in any tube except that containing the quinone, where a deep red-brown developed in the solution around the bacillary masses; in 22 hr. the flakes were deep brown and the solution itself darker than the control. At 22 hr. the catechol solution containing bacilli was pink, the control colourless; the orcinol test mixture was slightly pinker than its control. After 48 hr. the quinone-coloured flakes were very dark, those in the catechol were reddish grey (with the autoxidation control still colourless), and those in orcinol had faint pink edges. After 7 weeks the quinone mixture was dark and very turbid, preventing further description; the bacilli had become brownish black in catechol (while the control was only a weak tea-colour); in orcinol the masses were a rosy, greyish brown, the control a light brownish red. Resorcinol and thymol solutions remained negative throughout, no colour developing with or without bacilli.

In connexion with the colour developed in the *p*-benzoquinone, curiosity prompted the filtration of some of the culture fluid from this same lot of bacilli through a Berkefeld grade N candle, and the testing of the filtrate with quinone. A deep brownish red colour developed instantaneously and became more intense overnight. A portion of sterile glycerol broth of the same batch used for the culture was also tested with quinone and gave qualitatively similar results but

very much more slowly. Presumably substances capable of interaction with quinone had been added to the medium during bacillary growth. It should be noted that the bacilli themselves became darkened in the presence of quinone, and while this might mean further oxidation of quinone by enzymic means, it might mean a localized combination of quinone with bacillary substance, which was dark in colour.

Phenol, catechol, resorcinol, quinol, pyrogallol, hydroxy-quinol
(1:2:4-trihydroxybenzene), *phloroglucinol, tyrosine, adrenaline*

Phosphate buffer at pH 6.6 was used to prepare 1% solutions of all the phenol family, and a saturated solution of tyrosine; a 5 ml. portion of each solution was used for the test, and 5 ml. used as a control for observation of colour developing with autoxidation; the adrenaline used was the commercial 1:1000 solution of the hydrochloride, containing preservatives. Crumbs of equal size of human type bacilli (strain Gary), grown on solid medium, were placed in the test solutions.

At this pH (6.6), during 44 hr. observation, the phloroglucinol, resorcinol, phenol and tyrosine tests were all negative. Results with the other compounds follow. 1:2:4-Trihydroxybenzene: the control itself darkened too readily to permit observation of the bacilli, but they were already very dark at 8 hr. Pyrogallol: the bacillary mass was orange at 8, very brown at 31 and black at 44 hr. Quinol: a questionable coloration of the mass was noted at 8 hr., which had deepened to a definite rose at 31, and to a dark reddish brown at 44 hr. Catechol: the bacillary mass was brownish at 8 hr., and the brown colour became progressively more intense at 31 and 44 hr. Adrenaline: no colour change had been observed at 44 hr., but in view of the preservatives present (chloretone $2\frac{1}{2}$ grains per ounce, and sodium bisulphite not over 0.1%) the tube was kept for further observation; after 27 days the bacillary mass had become a brownish grey, which was still darker after 6 more days. In the absence of preservatives the adrenaline would be expected to show an activity similar to that of catechol; the concentration of the adrenaline was also only one-tenth that of the catechol.

From the respective colorations of the bacillary masses with passage of time, one would derive the following order of diminishing oxidizability (diminishing reducing power) at pH 6.6: 1:2:4-trihydroxybenzene, pyrogallol, catechol, hydroquinone, adrenaline (with preservatives present), and phenol (other long-term experiments with Gary cultures).

PRODUCTION OF DIANILINO-O-BENZOQUINONE

Because of the several chemical manipulations necessary, the non-virulent type of tubercle bacillus (BCG) was chosen for the first experiment, which followed substantially the preparation of dianilino-o-quinones by Pugh & Raper [1927] and by Happold [1930] using enzymes from various sources. Sterile apparatus was used throughout.

A suspension of BCG was prepared by grinding crumbs of bacilli with physiological saline in an agate mortar; the 35 ml. of suspension used for the experiment contained 105 mg. of bacilli. The suspension was added to 100 ml. of Clark's K_2HPO_4 -NaOH buffer at pH 6.5, containing 1 g. catechol and 2 ml. aniline. No immediate colour developed after mixing, and the preparation was allowed to stand overnight, free access of air being provided through the sterile cotton plug in the flask. After 16 hr. a red precipitate with a somewhat brownish tinge appeared, consisting of just-visible tufts of fine short needles; the liquid

was coloured red also. The flask was aerated for 2 hr. with gentle bubbling, first passing the air through chloroform-water, and a marked increase in the amount of precipitate resulted.

A few drops of the mixture, containing some precipitate, were examined microscopically. Beautiful garnet red bundles of needles were seen among considerable débris; feather and burr forms and single needles were all present. The red crystals in the precipitate were extremely friable; a portion of the mixture was filtered, but the crystals could not be removed from the filter paper without admixture with paper fibres; the filter paper was dried over sulphuric acid. The m.p. of the crude material, still containing bacilli and débris, was 186.5° (uncorr.).

Of all preparations of dianilino-*o*-benzoquinone made subsequently, the one to be described next was the most successful.

Culture fluid with catechol and aniline

The culture fluid (75 ml.) from a 3 months' growth of human type bacillus (Gary) upon 5% glycerol broth was decanted from bacillary sediment after centrifuging. To it was added an equal volume of phosphate buffer at pH 6.5, containing 1 g. catechol and 2 ml. aniline.

A faint pink colour developed immediately, rapidly darkening in tint; the reaction mixture was red and a few red needles had already formed in 15 min. In 5 hr. a heavy precipitate had settled, which was increased in bulk on standing overnight; the liquid still contained much red material in suspension. This preparation was not aerated at any time, but absorbed the necessary oxygen at its surface. (Owing to the virulence of this strain, whose bacilli might be present in the precipitate, no chemical manipulation of the product was attempted.)

The control flask, made up of 75 ml. (sterile) of the same lot of glycerol broth on which the culture had been grown, with the same buffer, catechol and aniline, produced a small amount of dirty light brown flocculent material after an hour; this settled out overnight, leaving a clear light brown liquid; no red crystals were seen at any time during the 18 hr. the mixture was under observation. It is therefore clear that the growth of tubercle bacilli in this broth had produced an agent capable of very marked catalysis of the oxidation of catechol, as manifested by the rapid and voluminous formation of the dianilino-derivative of its oxidation product, *o*-benzoquinone.

Since the result of using decanted culture fluid instead of a bacillary suspension was so satisfactory, the next step was to try a fluid which could not be suspected of containing any bacilli whatever.

Berkefeld filtrate from human type bacilli

The culture fluid from a growth of the virulent strain H 37 upon 5% glycerol broth was passed through a grade N Berkefeld candle and tested with the usual proportions of catechol and aniline in phosphate buffer at pH 6.5. A very marked catalysis of the oxidation of catechol was observed.

Further possible avenues of approach to a study of bacillus-free preparations of the enzyme were thus opened.

Note. Another preparation of the dianilino-benzoquinone was made, using a sterile Berkefeld filtrate from a culture of the non-virulent BCG. The red crystals, after decanting the reaction mixture, were washed once, centrifuged and spread on a clay plate. The m.p. of this still relatively crude product (air-dried for 3 days) was 190°, with decomposition, which compares favourably with 193°, the value reported by Happold for his most highly purified product.

*Effect of bacillary suspension on phenol, guaiacol, orcinol and quinol
in presence of aniline*

A suspension of washed human type bacilli (Gary) was used for all these experiments. Approximately 50 mg. of bacilli were used for each 50 ml. of phosphate buffer at pH 6.5, containing the usual proportions of the phenol in question, and aniline.

None of these phenols formed the red dianilino-*o*-benzoquinone. No visible product appeared in the guaiacol mixture; brownish yellow substances were observed in the phenol, orcinol and hydroquinone mixtures.

The failure to obtain evidence of the formation of an *o*-quinone in the case of orcinol agrees with the conclusion of Happold [1930] concerning the oxidation of that substance, and in the cases of phenol and guaiacol it may perhaps be considered additional evidence that the mode of action of the enzyme under discussion is not that of tyrosinase [Pugh & Raper, 1927].

Old tuberculin, long-dried bacilli

It seemed of interest to investigate the retention of this particular enzymic activity in old tuberculin (which is essentially a concentrated sterile filtrate of culture broth from the human type bacillus), and in a stock of dried human type bacilli (H 37) which had been kept over sulphuric acid for approximately 10 years.

10 ml. of old tuberculin (representing 100 ml. of fresh culture fluid) were mixed with 90 ml. of distilled water and 100 ml. of phosphate buffer at pH 6.5, containing the usual 1 g. catechol and 2 ml. aniline. The weight of the dried bacilli used, 0.54 g., was approximately five times that used in the fresh bacillary suspensions previously tested; the bacilli were suspended in 80 ml. buffer at pH 6.5, containing 0.75 g. catechol and 1.5 ml. aniline. In this case an autoxidation control of the reagents was kept, since it was expected that observations would continue for a long period. (The control of sterile broth used with the decanted culture fluid-catechol experiment was also available for comparison with the old tuberculin.) After 2 months so much brownish deposit had formed that it was useless to try to weigh the yields of red crystals of the dianilino-ortho-benzoquinone, and only visual comparison was possible.

There appeared to be little difference between control and dried bacilli. Apparently the enzymic activity had been lost during the ageing and drying of the bacilli.

The old tuberculin mixture, however, showed quite definitely a heavier yield than the controls. This particular preparation thus had seemingly retained some of its oxidase activity, but whether this represented the total activity of the old tuberculin after manufacture could only be decided by tests at repeated intervals of storage.

ATTEMPT TO USE METHYLENE BLUE AS HYDROGEN ACCEPTOR IN THE
OXIDATION OF CATECHOL

It was thought that if methylene blue could be utilized as hydrogen acceptor in place of molecular oxygen, a quantitative method for estimation of the enzyme could be worked out, using catechol as the substrate and depending on the rate of decoloration of the dye. An attempt was accordingly made to form the dianilino-*o*-benzoquinone from catechol (as evidence of oxidation to the quinone) in the presence of methylene blue, with air excluded from the reaction by a deep layer of paraffin oil.

The test mixture contained 2 ml. of a thin suspension of human type bacilli (H 37) ground in physiological saline, 3 ml. of 1 : 5000 methylene blue solution, 5 ml. of phosphate buffer at pH 6.5, 0.05 g. catechol, 1 drop of aniline; after thorough mixing the liquid was covered with 2 in. of sterile paraffin oil. A control with 2 ml. distilled water in place of the bacillary suspension, and a control containing only buffer, methylene blue and aniline, properly diluted, were also prepared and covered with oil.

No reduction of methylene blue occurred during the 7 days the tubes were under observation. After 3 days there was evidently some leakage of air through the oil layer, for in the tube containing bacilli a few red crystals formed very slowly at the oil-water interface. At pH 6.5 and 23° methylene blue did not serve as hydrogen acceptor in this reaction.

THERMOSTABILITY OF THE ENZYME

The supernatant fluid from a centrifuged culture of human type bacilli (H 37), grown on 5% glycerol broth, was used for the tests in place of a Berkefeld filtrate, in order to avoid dilution from a steam-sterilized candle and any possible physico-chemical effects of the material making up the candle. Sterile graduated centrifuge tubes, selected for uniform shape and size of tip, were used to heat the fluid, of which 2 ml. were used for each test. The tube was clamped over a water bath so that the fluid was well immersed, heated for $\frac{1}{2}$ hr. at the selected temperature, and then stored immediately in a mixture of ice and water. An unheated tube was stored in the ice as control. The temperatures used were 45, 55, 65, 75, 85 and 98° .

In this experiment the formation of dianilino-quinone was used as a test for any enzymic activity remaining. To each of the chilled tubes 2 ml. of phosphate buffer at pH 6.6, containing 1% catechol and 2% aniline, were added, and a chemical control tube with no culture fluid was also made up. The tubes were allowed to stand with sterile cotton plugs to admit air. The only observable difference 4 hr. after mixing was the slightly darker colour of the heated tubes compared with the unheated control; 17 hr. after mixing there were a few amorphous reddish bits to be seen in the culture fluids, but none in the control. In another 24 hr. there were clumps of definite crystals of red dianilino-o-benzoquinone in all the culture fluids, while there was only the merest suspicion of red material in the chemical control. There was no differentiation between tubes heated to different temperatures, or between heated and unheated controls, at this stage. (Although this was not a potent fluid compared with most of those used in the foregoing experiments, it still caused the appearance of an effect in 17 hr. which required 41 hr. to manifest itself unaided.) After another 48 hr. (89 all told) there were still very few crystals in the chemical control, but a moderately heavy red precipitate in all the culture fluids. The tubes were centrifuged to pack the sediment evenly in the graduated tips. There was still no obvious difference to be seen between the test temperatures, or between heated and unheated fluids. It was concluded that the enzyme is thermostable, contrary to expectations. Various plant polyphenolases have been reported as thermostable, according to Haldane [1930].

SEPARATION FROM CULTURE FLUID OF A PROTEIN POSSESSING ENZYMIC ACTIVITY

The amount of red dianilino-quinone formed, again served as a rough measure of the enzymic activity, using catechol as substrate. The fluid used was that decanted after centrifuging a 5% glycerol broth culture of human type bacilli

(H 37). Freshly prepared 50% trichloroacetic acid was used as protein precipitant, in the proportion of 1 vol. acid to 4 vol. fluid, as used by Seibert [1934] at a certain stage in the preparation of the tuberculo-protein known as P.P.D. The protein in four 5 ml. portions of fluid was thrown down and allowed to settle overnight. After being centrifuged the tubes were allowed to stand another 24 hr.; a light secondary coagulum formed, which was adherent to the walls of the tubes and was not affected by centrifuging. From two of the tubes the supernatant fluid was decanted, and the precipitated protein stirred up well with 5 ml. of freshly prepared 10% trichloroacetic acid, centrifuged, the wash-liquid discarded and the washing with 10% acid repeated. Then the protein was dissolved carefully in the least possible amount of $N/10$ NaOH, 1.5 ml. being required for this lot, and made up to 5 ml. with distilled water. The originally decanted supernatant fluids were carefully neutralized with 10% NaOH (to dilute them as little as possible, instead of using $N/10$ NaOH). The other two tubes, still containing protein and original reaction mixture, were likewise neutralized approximately with the strong alkali, and finally with the weak; these were to serve as controls of the possibly destructive effect of trichloroacetic acid upon the total enzyme content of the fluid. A control of neutralized trichloroacetic acid itself was also prepared. To each of these (redissolved protein, neutralized supernatant fluid, neutralized whole reaction mixture and acid control) and also to portions of the original culture fluid, an equal volume of phosphate buffer at pH 6.6 was added. The buffer had 1% of catechol and 2% of aniline already dissolved in it. Sterile cotton plugs permitted absorption of oxygen as usual.

After 21 hr. it was apparent from the relative volumes of precipitated dianilino-quinone that part of the enzymic activity had been carried down with the protein precipitate, and part had remained in the supernatant fluid. After another 24 hr. the dark red crystals were packed down by centrifuging and the amounts in the tubes compared. The precipitated, twice washed, redissolved protein contained about three-fifths of the original activity, while two-fifths remained unprecipitated and still active in the supernatant reaction mixture. The enzymic activity had apparently not been damaged by trichloroacetic acid, since the neutralized whole reaction mixture had as much dianilino-quinone formation as the untreated original culture fluid. As usual, the few crystals formed in the autoxidation control were negligible in amount.

SUMMARY

Various experiments have been described wherein the human type of tubercle bacillus or a product of its growth was found to catalyse the oxidation of various phenolic substances. The properties of the active agent concerned in this catalysis, as observed under the conditions employed, suggest the formation of a diffusible, thermostable enzyme of the polyphenol oxidase type, whose behaviour is more consistent with catechol- than with phenol-oxidase, and which is not a tyrosinase. Part of the activity could be removed from a culture fluid of human type tubercle bacillus by use of a protein precipitant, and recovered from a solution of the washed protein. Methylene blue did not serve as a hydrogen acceptor for the oxidation of catechol catalysed by this enzyme.

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