Adaptation of *Bacillus subtilis* to Fatty Acids

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It has previously been shown (Laser, 1951) that respiration and growth of *Bacillus subtilis* are inhibited by *cis*-vaccenic acid, the degree of inhibition being dependent on the ratio of acid to cells. The inhibition is transient at suitable ratios, respiration and growth ultimately proceeding at rates equal to or even greater than those of untreated controls. Bacterial cells, which have recovered from the initial inhibition by vaccenic acid, were found to be resistant to further addition of the acid, a result which resembles acquired drug resistance by bacteria. These studies have now been extended to other fatty acids and the behaviour of such treated cells after subculture in absence of fatty acids has been examined.

MATERIAL AND METHODS

The strain of *B. subtilis* used was no. 2586 of the National Collection of Type Cultures. Vegetative forms were obtained and prepared for manometric experiments as previously described (Laser, 1951).

The medium in which washed cells were suspended was 0.15M-phosphate buffer, pH 8.0, containing 2% glucose $\pm$ 0.5 ml. nutrient broth/3.0 ml.

The fatty acids tested were stearic, linoleic, oleic and synthetic *cis*-vaccenic acid (Morton & Todd, 1950). They were dissolved with heating in 0.3M- K_3PO_4 (usually 2.0 mg./ml.), diluted with water to 0.15M, and pipetted into the reaction vessel immediately before adding the organisms. The acids and their solutions in K_3PO_4 were kept under N_2 in the cold. The solutions were warmed before use and in the case of stearic acid, all the components of the medium were heated to 40° and quickly pipetted into warmed vessels. In order to maintain the pH of the phosphate buffer at pH 8.0, 0.12 ml. 0.15M- KH_2PO_4 were added per 0.1 ml. 0.15M- K_3PO_4 containing the dissolved fatty acid.

Respiration was measured manometrically in Barcroft-Warburg manometers at 37°. The gas phase was O_2 . As a rule all components of the medium were filled into the main compartment of the flasks before the beginning of the experiment, the fatty acid being added immediately before the organisms. In those experiments in which the fatty acid was added at $t=0$ or at a convenient time during the course of the experiment, it was tipped into the main compartment of the vessel from a side bulb, the required amount of acid being dissolved in 0.05 ml. 0.15M- K_3PO_4 . The same amount of 0.15M- K_3PO_4 was tipped at the same time into the control vessel.

The initial (dry) weight of cells per vessel varied between 0.5 mg. (growing cells) and 1.0–2.0 mg. (washed cells), contained in 0.1–0.4 ml. of a suitably diluted washed

suspension. Its turbidity was determined by means of the Spekker absorptiometer (1 cm. cell, Ilford filters H 503 and 608) and the cell content (on a dry weight basis) obtained by reference to a calibration curve.

To subculture cells after treatment with a fatty acid at the end of a manometric experiment, the following procedure was observed. One small drop of the cell suspension was plated on nutrient agar in a Petri dish and incubated overnight at 37°. In order to exclude accidental contamination during the manometric procedure, the culture was examined microscopically and cells from a single colony were then incubated in 10.0 ml. nutrient broth + 2% glucose for 8 hr. and transferred into Roux flasks upon peptone-agar for about 16 hr., as previously described (Laser, 1951).

Symbols and abbreviations. R = 'acid ratio' = ratio of wt. of fatty acid in the medium : dry wt. of cells. Recovered cells = cells whose respiratory and growth rates, after initial inhibition by a fatty acid, had equalled those of the untreated control. Q_{O_2} = μ l. O_2 uptake/mg. dry wt./hr.

RESULTS

The present experiments deal mainly with the effect of fatty acids on the respiratory rate of washed cell suspensions in phosphate buffer solution + glucose and on the respiratory and growth rates of cells in the same medium to which nutrient broth had been added. No growth occurs in washed suspensions in absence of broth, so that the rate of O_2 uptake, in absence or presence of fatty acid, is a measure of the ability to oxidize glucose. In growing cells the rate of O_2 uptake is, in addition, an index of growth, i.e. the logarithmic increase in cell population is reflected in the logarithmic increase of the respiratory rate.

The effect of stearic acid. Fig. 1 shows the effect of different concentrations of acid on the O_2 uptake of 2.0 mg. washed cells. It will be seen that (1) at the concentrations used, the respiration is initially inhibited; (2) the inhibition increases with increasing amounts of acid; (3) the cells gradually recover from the inhibitory effect of the acid and eventually attain a rate of O_2 uptake which approaches or equals that of the control. Similar curves are obtained if the same concentration of acid is allowed to act on decreasing amounts of cells, i.e. the inhibition becomes greater as the cell concentration falls. This confirms earlier results with vaccenic acid (Laser, 1951) and demonstrates that the inhibition depends much less on the concentration of acid in the medium than on the ratio of acid to cells. This ratio (R) has

therefore been used throughout the descriptive part of the paper while the corresponding molar concentrations are given in the figures.

Stearic acid, which was the only saturated acid tested, has been found to inhibit respiration less than the unsaturated acids. This became especially apparent at higher concentrations, the cells tolerating and recovering from acid ratios as high as 1, which was not the case with the other acids. Such a result could be ascribed to the low solubility of stearic acid which might prevent a uniform distribution of acid among the cells. Preliminary experiments, however, in which the precaution of warming

Fig. 1. Effect of varying ratios of stearic acid: cells (R) on O_2 uptake of washed suspensions (2.0 mg. dry wt./4.7 ml.), plotted as % of Q_{O_2} (μ l. O_2 /mg. dry wt./hr.) of control in absence of acid; 0.15 M-phosphate buffer, pH 8.0, + 2% glucose; gas phase, O_2 . The figures on the curves denote R . Concentration of acid in the medium (from top to bottom): $0.94, 1.89, 3.75, 7.57$ and 15.1×10^{-4} M.

all components of the medium prior to setting up the vessels was not taken (see Methods), allowed this possibility to be excluded. Under these conditions the paradoxical effect was obtained that the inhibition of O_2 uptake increased with decreasing concentrations of acid. This can be explained by the readiness of dissolved stearic acid to precipitate out of solution more quickly at high than at low concentrations, thereby diminishing the effective R . This effect was not encountered when the proper precautions were taken. The relatively low inhibitory effect of stearic as compared with the unsaturated acids therefore appears to be real. A similar observation, namely the relatively low toxicity of stearic acid as compared with unsaturated acids, has been made by Kodicek &

Fig. 2. Effect of different acids on O_2 uptake of washed cell suspensions (2.0 mg. dry wt./3.0 ml.), plotted as percentage of Q_{O_2} of control in absence of acid; 0.15 M-phosphate buffer, pH 8.0, + 2% glucose; gas phase, O_2 ; ratio of acid: cells = 0.05; concentration of acids in medium = 1.18×10^{-4} M; A, stearic; B, oleic; C, vaccenic; D, linoleic acid.

Fig. 3. Effect of different acids on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.); 0.15 M-phosphate buffer, pH 8.0, + 2% glucose + 0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 ; ratio of acid: cells = 0.05; concentration of acid in medium = 1.18×10^{-4} M; A, control without acid; B, stearic; C, oleic; D, vaccenic; E, linoleic acid.

Worden (1945) in their work on growth and lactic acid formation by *Lactobacillus casei*.

The relative effect of different fatty acids. Figs. 2 and 3, which demonstrate the effect of different

acids on the O_2 uptake of the micro-organisms at equal ratios of acid : cells, show that the magnitude of the initial inhibition increases in the following order:

stearic < oleic < vaccenic < linoleic.

Thus washed cells treated with linoleic acid, $R = 0.1$, did not recover from the respiratory inhibition during an experiment of 6 hr. duration, while with oleic acid at the same ratio recovery set in after 3 hr., leading after 6 hr. to a 70 % increase of the

being superimposable on that representing washed cells at $R = 0.1$.

Repeated addition of fatty acids. Growing *B. subtilis*, which have been treated with and have recovered from the initial inhibition by vaccenic acid, are resistant to a further addition of this acid (Laser, 1951). It was therefore determined whether this is also the case with other fatty acids and whether the developed resistance is specific for the acid with which the cells have been treated. Fig. 5 shows the effect of a second addition of different amounts of linoleic acid on the respiration of washed cells, which are recovering from an initial inhibition

Fig. 4. Effect of varying ratios of linoleic acid : cells (R) on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.) and of washed suspensions (2.0 mg. dry wt./3.0 ml.); 0.15M-phosphate buffer, pH 8.0, + 2 % glucose $\pm$ 0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 ; growing cells, $\bullet$ — $\bullet$; washed suspensions, $\circ$ — $\circ$.

oxidation rate above that of the control. The inhibition of O_2 uptake of growing cells was always accompanied by an inhibition of growth, as in the case of vaccenic acid (Laser, 1951). When the cells recover from the respiratory inhibition, however, the rate of growth approaches or even exceeds the normal rate.

Relative tolerance for linoleic acid of washed and growing cells. The question of the necessity of growth for the development of resistance is an important one. The behaviour of washed and growing cells of the same batch was therefore examined on addition of two different concentrations of linoleic acid ($R = 0.05$ and 0.1) the higher of which inhibited the respiration of washed cells completely and without recovery (Fig. 4). It will be noted that growing cells are able to overcome the inhibition. The increased tolerance, however, conferred by the conditions of growth is small, only twofold, as at a ratio $R = 0.2$ no recovery took place even with growing cells, the corresponding curve (not shown in Fig. 4)

Fig. 5. Effect of second addition of linoleic acid on O_2 uptake of washed cell suspensions (2.0 mg./3.0 ml.) plotted as percentage of Q_{O_2} of control in absence of acid; 0.15M-phosphate buffer, pH 8.0, + 2 % glucose; gas phase, O_2 . Initial ratio of acid : cells (R_0) and concentration of acid in the medium are shown on the heavy line (A-D), which indicates four initially identical vessels, to three of which (B, C, D) a second addition of acid (0.05, 0.1 and 0.2 mg.) has been made at $t = 1.5$ hr. (indicated by arrow), leading to $R_1 = 0.05$ for B, 0.075 for C and 0.125 for D.

by linoleic acid ($R = 0.025$). It will be seen that the cells not only tolerate amounts of acid in excess of the initial concentration, but show an increase of O_2 uptake with increasing amounts of added acid. This cannot have been caused by the oxidation of the acid itself, because it was many times greater than could be accounted for by the oxidation of the added acid. This increased rate of metabolism may be likened to, or be one of the underlying reasons for, the increased growth rate of certain bacteria, e.g. *Lb. casei*, in presence of oleic acid, after recovery from the primary growth inhibition (Williams & Fieger, 1946). Fig. 6 illustrates the effect of a second addition of acid to growing cells. Here too the treated cells, during the process of recovery, have become resistant to approximately six times the

initial acid ratio, as expressed by the steady logarithmic increase of respiration, which is a measure of the increase in cell population. In a number of experiments with oleic acid this has also been directly confirmed by density determinations. Similarly, treated recovered cells grew at a normal rate when transferred into a new medium in presence of fatty acid at a concentration several times higher than the initially inhibiting dose. Increasing the amount of acid by a second addition, however, did not produce an increase in the growth rate above that of the controls. This is probably due to the fact that, under the experimental conditions, growth proceeded at the maximal rate.

Fig. 6. Effect of second addition of linoleic acid on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.); 0.15 M-phosphate buffer, pH 8.0, + 2 % glucose + 0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 . Initial ratio of acid to cells (R_0) and concentration of acid in the medium are indicated on the heavy line (B-F). This represents five initially identical vessels, to four of which (C-F) a second addition of acid (0.025, 0.05, 0.1 and 0.2 mg.) has been made at $t=1.5$ hr. (indicated by arrow), leading to approximate $R_t=0.066$ for C, 0.1 for D, 0.166 for E and 0.3 for F, on taking the increase in cell population at $t=1.5$ hr. into account.

The question whether the resistance is specific for the acid with which the cells have initially been treated was examined by cross-reaction of growing cells with all the acids used in these experiments. The results were uniform and unambiguous: treatment with any one acid produced resistance against any of the other acids. Even cells treated with and recovered from the inhibition by a saturated acid (stearic acid) showed unaffected rates of O_2 uptake and growth on addition of linoleic acid.

Development of resistance. In previous work (Laser, 1951) a second addition of acid to treated cells had been made at a later stage of the experiments, i.e. after complete recovery had taken place.

Enzyme adaptation by bacteria, however, is believed to occur fairly rapidly under suitable conditions. Thus Pollock (1950) has demonstrated that, after a brief treatment of bacteria with penicillin, an adaptive enzyme is produced within about 1 hr. In the experiments illustrated in Figs. 5 and 6 of this paper, a second addition of acid had therefore been made at a time when the cells were only beginning to recover from the respiratory inhibition. It will be seen that at that time resistance had already developed. It was then determined whether under the conditions of these experiments, which differ from those maintained by Pollock, a relatively

Fig. 7. Effect of second addition of oleic acid, at different times of the experiment, on O_2 uptake of growing cells (0.5 mg. initial dry wt./3.0 ml.); 0.15 M-phosphate buffer, pH 8.0, + 2 % glucose + 0.5 ml. nutrient broth/3.0 ml.; gas phase, O_2 . Initial ratio of acid : cells (R_0) and concentration of acid in the medium are indicated in the figures on the curves (for curve D, see text). The heavy line (B, C) represents two initially identical vessels. Second additions (0.05 mg.) were made to C and E at $t=1.5$ hr., to D at $t=0$, leading to R_t (approximate R_t for C and E) = 0.0835 for C, 0.1 for D and 0.07 for E, on taking the increase of cell population at $t=1.5$ hr. into account.

short contact of the bacteria with a fatty acid would also be sufficient to produce resistance. Accordingly, at $t=0$ a second addition of acid was made to cells, which had been in contact with acid for the time involved in setting up the manometer vessels, gassing with O_2 and establishing temperature equilibrium, which amounted to about 25–30 min. (Fig. 7). It will be seen that the second addition of acid at $t=0$ decreased the O_2 uptake by about 50 % (curve D, Fig. 7), compared with control cells (curves B, C, Fig. 7) and that recovery was delayed for about 30 min., i.e. resistance had not developed during the preliminary incubation period. On the

other hand, 90 min. contact of the cells with a small amount of acid ($R = 0.00625$; $3.7 \times 10^{-6}M$), which by itself did not produce respiratory inhibition (curve *E*, Fig. 7), caused the cells to become resistant. That very small concentrations of substrate, as, for example, $10^{-4}M$ -nitrate or $8 \times 10^{-9}M$ -penicillin, produce marked adaptation in *Escherichia coli* and *B. cereus* respectively, has previously been reported by Wainwright (1950) and by Pollock (1950). Since in the present experiments resistance developed only after a lag period, it seems to depend on some metabolic activity of the cell during that period, which in these experiments, as in those of Pollock, lasted about 1 hr.

Subculture of treated recovered cells. Treated cells have so far only been shown to be resistant to a second treatment with a fatty acid immediately after recovery from the first addition, i.e. without interposition of a period of growth in absence of acid. Experiments were therefore carried out to determine the effect of fatty acids after subculturing recovered cells in absence of fatty acid. In the present arrangement the shortest interval between the removal of the bacteria from the acid-containing medium and re-examination of subcultured cells was 40 hr. The following results were obtained: (1) cells which had developed resistance either as washed suspensions or in a nutrient medium had completely lost their resistance; (2) their O_2 uptake and growth on renewed exposure to any of the fatty acids were inhibited to the same extent and recovery took place at the same rate as that of untreated controls; (3) the process can be repeated many times, apparently indefinitely, without any alteration in the behaviour of the organisms. Whether the complete loss of resistance occurred immediately on subculturing in absence of acid or only gradually could not be decided from the present experiments.

DISCUSSION

The experiments dealing with the inhibition of growth and respiration of *B. subtilis* and the subsequent recovery after treatment with stearic, oleic and linoleic acids differ but little from previous experiments with vaccenic acid (Laser, 1951). The conclusion had then been drawn that the inhibition results from a temporary blocking by penetrated acid of the oxidative system inside the cell and that recovery is due to the ability of the cell to metabolize penetrated acid. It appeared unlikely that physico-chemical surface phenomena could account for the observed facts, especially the recovery from the inhibition and the subsequent resistance of treated cells. The results of the present work make this even more improbable because (1) a second addition of fatty acids still further increases the inhibition, if the acid is added before a certain

degree of recovery by the cells has been attained (curve *D*, Fig. 7); (2) resistance develops also in cells treated with a small dose of acid, which by itself does not cause respiratory inhibition; (3) the addition of fatty acid to treated recovering cells stimulates the O_2 uptake of washed suspensions; (4) treated recovered cells respire and grow at an undiminished rate if transferred into a new medium in presence of fatty acid; (5) the resistance of treated recovered cells is lost after the organisms have been subcultured in absence of added fatty acid. The joint significance of these facts, however, makes it reasonably certain that the phenomenon of resistance is due to the development of an adaptive enzyme. Whether the complete loss of resistance occurred immediately on subculturing in absence of acid or only gradually could not be decided by the present arrangement. However, as resistance had been completely lost, the rate of its loss has no significant bearing on the above conclusion.

Since washed cell suspensions are able to develop resistance, they must be capable of doing so by rearrangement of their protein constituents. That such rearrangements do occur has been clearly demonstrated by Spiegelman & Dunn (1947-8) and Spiegelman & Reiner (1947-8) in their work on the interrelationship of enzymes in bacteria during adaptation, and by Chin (1950), who has described changes of the different components of cytochrome in washed, non-growing yeast cells following upon aeration. The increase in tolerance afforded by a nutrient medium, however, is small, only twofold if, as in these experiments, the substrate concentration is at the tolerance level for washed cells. It is therefore considered likely that the development of resistance in washed cell suspensions and in growing cells, at least in these experiments, differs only in its quantitative aspect, i.e. growth by itself does not confer on the cell a new property. However, where the substrate is either indifferent, as, for example, galactose with *Streptococcus lactis* grown in presence of, and adapted to, glucose, or where it only partly inhibits growth, the process of multiplication can obviously greatly potentiate tolerance and/or enzyme formation. These views are in agreement with those expressed by Spiegelman & Dunn (1947-8) and Spiegelman & Reiner (1947-8), although in the past contradictory conclusions have been drawn by a number of workers (see Yudkin, 1938).

Evidence put forward in this paper, especially the coincidence of the period of inhibition of growth and respiration with the time lag before resistance develops, is believed to support the conclusion that the substrate, towards which the cell ultimately develops resistance, has first to be metabolized by the cell and that the acquired resistance is dependent on this metabolic process.

SUMMARY

1. Stearic, oleic, vaccenic and linoleic acids, within the range of concentrations used, produce an inhibition of respiration of washed suspensions of *Bacillus subtilis* and a concomitant inhibition of respiration and growth of growing cells.

2. The inhibition is transient, its degree and duration being dependent on the ratio acid : cells.

3. Washed suspensions and growing cells which have recovered from the inhibition are resistant to further additions of acid at concentrations higher than the initially inhibiting dose.

4. Treatment with any one acid produces resistance against any of the other acids.

5. A time lag ensues on treatment with a fatty acid before resistance develops.

6. Low concentrations of acid, which do not inhibit growth or oxygen uptake, nevertheless produce resistance.

7. The tolerance of growing cells with subsequent development of resistance is slightly greater than that of washed suspensions.

8. The resistance is lost on subculturing the cells in absence of fatty acid.

9. The evidence suggests that the resistance of acid-treated cells is due to the development of an adaptive enzyme, which is formed subsequently to the incorporation into the cell and the metabolic degradation of the fatty acid.

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The Inhibition of Erythrocyte Cholinesterase by Tri-Esters of Phosphoric Acid

1. DIETHYL *p*-NITROPHENYL PHOSPHATE (E 600) AND ANALOGUES

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The ester diethyl *p*-nitrophenyl phosphate (E 600) is a powerful and irreversible inhibitor of cholinesterase. The kinetics of its reaction with cholinesterase have been studied (Aldridge, 1950).

The work to be reported here began as a study of the inhibitory powers of analogues of E 600 containing different substituents in various positions on the phenyl ring. Comparisons of the activities of other groups of irreversible inhibitors of cholinesterase have been reported. Mackworth & Webb (1948) compared the activity of dialkyl fluorophosphonates prepared by McCombie & Saunders (1946) against a purified preparation of horse-serum cholinesterase. Metcalf & Marsh (1949) compared the activity of substituted diethyl phenyl phosphates and thiophosphates against bee-brain cholinesterase. In these reports a direct comparison was made between

the concentrations of inhibitor that produced 50 % inhibition of the cholinesterase preparation.

Aldridge (1950) showed that the inhibition of erythrocyte cholinesterase by E 600 followed first-order kinetics and was bimolecular. The bimolecular rate constants were considered to be a much better index of inhibitory power than the usual 50 % inhibition concentrations. When these constants were determined for this series of compounds it was found that the kinetics of the inhibition were not like those obtained for E 600. It was then realized that these differences could be accounted for by the presence of a very active, but unstable, inhibitor of cholinesterase. When this impurity was removed the compounds reacted with cholinesterase in the same way as E 600. This aspect of the work seemed to be important, and the work carried out with one of