The Metabolism of some Saturated Aliphatic Hydrocarbons, Alcohols and Fatty Acids by *Proactinomyces opacus* Jensen (*Nocardia opaca* Waksman & Hendrik)

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Erikson (1949), as a result of a study of about 300 strains of Proactinomycetes, mostly isolated from Rothamsted soils, came to the conclusion that the partially acid-fast strains in that locality were mainly accounted for by *Proactinomyces opacus*, *Pro. salmonicolor* and *Pro. paraffinae*. All three species grow well on liquid paraffin or paraffin wax as the sole energy source.

A few papers have been published on the oxidation of aliphatic hydrocarbons by washed suspensions of pure cultures of micro-organisms. Both Johnson, Goodale & Turkevitch (1942) and Imelik (1949) used species of *Pseudomonas*. It was shown by Johnson *et al.* (1942) that aliphatic hydrocarbons ranging from C_6 to C_{12} were oxidized. Saz (1949), using four strains of pathogenic mycobacteria, found that in particular the 14-, 16- and 18-membered hydrocarbons, both saturated and unsaturated, greatly increase the oxygen uptake. Cetyl (hexadecyl) and stearyl (octadecyl) alcohols had a similar effect. A useful review on the assimilation of hydrocarbons by micro-organisms has been published by ZoBell (1950).

In this paper we have made a study of the aerobic metabolism of a strain (T16) of the non-pathogenic *Pro. opacus* (Jensen) (*Nocardia opaca* Waksman & Hendrik, *Bergey's Manual*, 6th ed.), employing the Warburg technique.

METHODS

Growth of the organism. *Pro. opacus*, strain T16, isolated by Erikson from soil, was used throughout this work.

The organism was grown in liquid culture. Glucose proved to be the most suitable carbon source for maximum yield of cells. Although good growth took place in the presence of *n*-dodecane and *n*-tetradecane, it was found impossible to separate the cells from the hydrocarbons by centrifuging. The final medium employed was of the following composition: glucose, 20 g.; $NaNO_3$, 2 g.; KH_2PO_4 , 0.5 g.; $NaCl$, 0.2 g.; $MgSO_4$, 0.2 g.; $FeCl_3$, trace; 1 l. distilled water; pH adjusted to 7.2. The medium was distributed in 500 ml. media storage bottles (200 ml./bottle) and inoculated with 8 ml. of a 48 hr. culture of the organism. Growth was

carried out at room temperature on a rotary shaking machine. After 5–6 days, the cells were harvested by centrifuging, washed once with distilled water and finally suspended in distilled water. 1 ml. of suspension was usually made to contain about 6 mg. (dry wt.) of organism. The suspension could be kept for periods up to 2–3 weeks at 2° until needed. The experiments quoted in this paper have been performed with suspensions stored for 7–14 days at 2°.

Oxygen uptake. This was measured in a Warburg apparatus at 20°, the final total volume in each vessel being 2.5 ml., made up as follows: 1 ml. suspension; 0.5 ml. 0.066M phosphate buffer, pH 7.0; substrate and distilled water.

Owing to the high endogenous respiration, particularly when newly harvested, it was usually necessary to leave the organism and phosphate buffer shaking gently in the vessels overnight. This preliminary incubation, while markedly reducing the blank, had little effect on the oxidation of the subsequently added substrates. Next morning, 0.2 ml. of 5% (w/v) KOH and fluted filter paper, for the absorption of CO_2 , were introduced into the centre cup of each vessel. Distilled water was added directly to the vessel and substrates, if liquid, were tipped in from the side arm after equilibration. In the case of some hydrocarbons and alcohols, it was found advantageous to add 0.2 ml. distilled water to the side arm simultaneously to facilitate tipping, so ensuring that all the substrate passed into the main vessel. In the case of solid substances, these were added directly just before the equilibration period of 10–15 min. For the measurement of B.Q., three manometers were used for each determination (Umbreit, Burris & Stauffer, 1948).

RESULTS

Oxygen uptake of washed suspensions in the presence of liquid aliphatic hydrocarbons

Fig. 1 gives the result of an experiment showing the effect on the oxygen uptake of suspensions of *Pro. opacus* of the addition of the hydrocarbons decane, undecane, dodecane, tetradecane and hexadecane. It is evident that the higher paraffins (dodecane, tetradecane and hexadecane) most markedly affect the oxygen uptake. Saz (1949), working with tubercle bacilli, also found that with tetradecane, hexadecane and octadecane the oxygen uptake was

greatly increased. With *Pro. opacus* it was similarly observed (Saz, 1949) that the organism tended to migrate to the hydrocarbon-water interface.

As the organism is frequently cultured on liquid paraffin, it was thought of interest to find the effect of this substance on the rate of oxygen uptake by

Fig. 1. The oxygen uptake of *Proc. opacus* in the presence of *n*-decane, *n*-undecane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane. A washed suspension (1 ml.) of organism + 0.5 ml. phosphate buffer, pH 7.0, shaken gently overnight. Distilled water and substrates (0.2 ml. from side arm) added next morning. Temp. = 20°.

Pro. opacus. The results are given in Table 1. It appears from Table 1 that liquid paraffin is not as good a substrate as dodecane and tetradecane. Growth experiments also showed that better growth took place on dodecane and tetradecane than on liquid paraffin when these substances were used as sole carbon source in a mineral salt medium of the same composition as given under Methods (the hydrocarbons replacing the glucose).

Table 1. The oxygen uptake of *Proactinomyces opacus* in the presence of liquid paraffin, dodecane and tetradecane

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, incubated overnight. Next morning 0.2 ml. hydrocarbon and distilled water added to give a final volume of 2.5 ml.)

Addition	O ₂ Uptake in 120 min. (μl.)
None	36
Liquid paraffin	40
<i>n</i> -Dodecane	152
<i>n</i> -Tetradecane	92

Oxygen uptake in the presence of solid hydrocarbons

Solid paraffins increased the oxygen uptake of *Pro. opacus*. Included in Table 2 are dodecane, tetradecane and hexadecane (liquid at 20°) for comparison.

Table 2. Oxygen uptake of *Proactinomyces opacus* in the presence of some solid aliphatic hydrocarbons

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, incubated overnight at 20°. Next morning substrates and distilled water were added to give a final volume of 2.5 ml.)

Addition	O ₂ uptake in 120 min. (μl.)
None	23
Paraffin wax (0.2 g.)	56
<i>n</i> -Octadecane (0.2 g.)	95
<i>n</i> -Hexadecane (0.2 ml.)	173
<i>n</i> -Tetradecane (0.2 ml.)	82
<i>n</i> -Dodecane (0.2 ml.)	90

The effect of hydrocarbons on oxygen uptake in the presence of glucose

It will be seen from Table 3 that no stimulation of oxygen uptake occurs when glucose and hydrocarbon are present together. In fact, the sum of the separate rates is greater than when the two substrates are oxidized simultaneously. Johnson *et al.* (1942) report similar findings for *Pseudomonas* sp. when glucose and octane were added separately and together to their suspensions. These workers came to the conclusion that 'the fact that they are largely additive indicates that different dehydrogenase systems are involved'.

It is also of interest to note from Table 3 that the rate of oxygen uptake in the presence of glucose is substantially the same as for hexadecane.

Table 3. The effect of hydrocarbons on the oxygen uptake of *Proactinomyces opacus* in the presence of glucose

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, shaken overnight at 20°. Next morning substrates and distilled water added to give a final volume of 2.5 ml.)

Addition	O ₂ uptake in 120 min. (μl.)
None	27
Glucose (0.4 %, w/v)	196
<i>n</i> -Dodecane (0.2 ml.)	102
Glucose (0.4 %, w/v) + <i>n</i> -dodecane (0.2 ml.)	265
<i>n</i> -Hexadecane (0.2 ml.)	192
Glucose (0.4 %, w/v) + <i>n</i> -hexadecane (0.2 ml.)	322

Determination of R.Q. of dodecane

The theoretical R.Q. for the complete oxidation of a hydrocarbon is 0.65. For dodecane, the value obtained was 0.72 and that for the endogenous

respiration was 1.0. Saz (1949) found an identical value for one of his strains (H 37 Ra) using tetradecane. To account for the high value obtained he came to the conclusion that 'if the hydrocarbon is being oxidized, this would suggest that intracellular decarboxylation mechanisms are being stimulated as well'.

Reduction of methylene blue by extract from crushed cells

In order to obtain a sufficient weight of organism for this purpose, the organism was grown on the medium as previously given (see under Methods) but solidified with 2% agar. The poured plates were inoculated over the whole surface using a sterile glass spreader. The plates were incubated for 14 days at 23°, the surface growth was then harvested, washed once by centrifuging and then deposited in a tared tube by centrifuging. The cells were crushed by McIlwain's method (McIlwain, 1948), using Microid polishing alumina, Grade 3/50, slow cutting (Griffin and Tatlock Ltd.). After centrifuging, the supernatant was used for Thunberg experiments.

Table 4. *Reduction of methylene blue by the extract obtained from crushed cells of Proactinomyces opacus*

(The organisms (4.3 g. wet wt.) were crushed with 11 g. polishing alumina, taken up in 10 ml. phosphate buffer, pH 7.0, and centrifuged. The supernatant liquid was used in hooked Thunberg tubes, each of which contained 1 ml. of supernatant, 0.3 ml. 1/10 000 methylene blue and 0.2 ml. of substrate, etc., to give a total volume of 1.5 ml. Methylene blue and substrate were added from the 'hook' after evacuation and filling with O₂-free N₂.)

Addition	Time after which decoloration obtained (min.)
None	35
Dodecane	17
Hexadecane	17
Decane	40
Sodium lactate (0.2 ml. of 1%)	20

From Table 4 it will be seen that the rate of methylene-blue reduction is increased in the presence of dodecane and hexadecane. Decane was ineffective. It also had little effect on the oxygen uptake (Fig. 1). Sodium lactate has been included for purposes of comparison.

Metabolism of saturated aliphatic alcohols

Table 5 gives the results of an experiment with ethyl, butyl, amyl, *iso*amyl, *iso*hexyl, heptyl, octyl, decyl, lauryl, cetyl and octadecyl alcohols. It is clearly seen that the long-chain alcohols (decyl, lauryl (dodecyl) and cetyl (hexadecyl)) have a

marked effect on the oxygen uptake, while butyl, amyl, *iso*amyl, *iso*hexyl and heptyl alcohols are all toxic and no oxygen uptake is obtained in their presence. Saz (1949) found, too, that the oxygen uptake of the four strains of tubercle bacilli used by him were increased by long-chain (cetyl and stearyl (octadecyl)) alcohols. In the experiments

Table 5. *Oxygen uptake of Proactinomyces opacus in the presence of some saturated monohydric aliphatic alcohols*

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. 0.066 M-phosphate buffer, pH 7.0, incubated overnight at 20°. Next morning 0.2 ml. of liquid alcohols + 0.2 ml. distilled water added from side arm. In case of solid alcohols 0.2 ml. of the melted alcohol was added directly to suspension. Distilled water added to give final volume of 2.5 ml.)

Addition	O ₂ uptake in 120 min. (μl.)
None	15
Ethanol	104
Butanol	Nil
Amyl alcohol	Nil
<i>iso</i> Amyl alcohol	Nil
<i>iso</i> Hexyl alcohol	Nil
Heptyl alcohol	Nil
Octyl alcohol	16
Decyl alcohol	76
Dodecyl alcohol	98
Hexadecyl alcohol	101
Octadecyl alcohol	47

with the immiscible liquid and solid hydrocarbons and alcohols some variation was encountered in the amount of increase in oxygen uptake observed for different experiments. We believe this is connected with the difficulties in introducing the exact amounts (particularly in the case of solid substances) into the main vessel and also to the different degrees of dispersion obtained. The substances in the absence of the cells did not take up oxygen.

Metabolism of saturated aliphatic fatty acids

When used in low concentrations (0.0012 M) the longer chain acids give greater oxygen uptakes than acetate, propionate or butyrate (Fig. 2). This agrees with the findings of Randles (1950) for *Neisseria catarrhalis*. From Table 6 it will be seen that the very long-chain saturated fatty acids (laurate, myristate, palmitate, stearate and behenate) behaved in a similar way, although behenate is not so effective as the others.

In higher concentrations (1%, w/v) caprylate (octanoate), caprate (decanoate), undecanoate and laurate completely inhibited oxygen uptake. The toxic effect of high concentrations of caprylate, caprate, undecanoate and laurate may be connected with their detergent properties.

*Effect of fatty acids on oxygen consumption
in the presence of glucose*

The addition of the long-chain fatty acids (laurate and caprate) and glucose simultaneously to the washed suspension (Table 7) gave analogous results to those obtained for the hydrocarbons (see Table 3).

Fig. 2. The O_2 uptake of *Pro. opacus* in the presence of saturated aliphatic fatty acids. A washed suspension (1 ml.) of organism + 0.5 ml. phosphate buffer, pH 7.0, shaken gently overnight at 20°. Next morning substrates (0.5 ml. of 0.06M from side arm) and distilled water added to give a final concentration of 0.0012M-fatty acid as sodium salt on total volume of 2.5 ml.

DISCUSSION

Little work has been done on the metabolism of the partially acid-fast proactinomycetes. (For details regarding acid-fastness in this group see Erikson (1949). The capacity of these organisms to grow on liquid paraffin and paraffin wax has been known for a long time (Jensen, 1931). From work reported in this paper it is clear that a wide range of hydrocarbons and closely related compounds increase the rate of oxygen uptake by washed suspensions of

Pro. opacus. Of the hydrocarbons utilized, the higher members, dodecane, tetradecane and hexadecane were superior to the other members tried (Fig. 1). In this respect *Pro. opacus* differs from the Gram-negative, non acid-fast pseudomonas species as used by Johnson *et al.* (1942), who found that octane was the most actively used substance. Saz (1949), working with pathogenic strains of mycobacteria, obtained similar results to ourselves. He suggested two possibilities to account for the increased oxygen uptake: (a) that the hydrocarbons are used as energy source by the organism, and (b) that the hydrocarbons alter the respiration rate by changing the total free surface of the cells.

Table 6. The oxygen uptake of *Proactinomyces opacus* in the presence of long-chain fatty acids

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, shaken overnight at 20°. Next morning 0.5 ml. of 0.006M-sodium salt of acid tipped from side arm. Distilled water added to give final concentration of 0.0012M and final volume of 2.5 ml.)

Addition	O_2 uptake in 120 min. (μ l.)
None	17
Laurate (dodecanoate)	204
Myristate (tetradecanoate)	275
Palmitate	214
Stearate	193
Behenate (docosanoate)	37

Table 7. The effect of fatty acids on the oxygen consumption of *Proactinomyces opacus* in the presence of glucose

(A washed suspension (1 ml.) of *Pro. opacus* + 0.5 ml. of 0.066M-phosphate buffer, pH 7.0, shaken overnight at 20°. Next morning substrates and distilled water added to give final volume of 2.5 ml.)

Addition	O_2 uptake in 120 min. (μ l.)
None	47
Glucose (0.4%, w/v)	195
Sodium dodecanoate (0.0012M)	225
Glucose (0.4%, w/v) + sodium dodecanoate (0.0012M)	366
Sodium decanoate (0.0012M)	134
Sodium decanoate (0.0012M) + glucose (0.4%, w/v)	242

In the case of *Pro. opacus*, good growth is obtained using dodecane and tetradecane as sole carbon source in the medium. Saz (1949), on the other hand, only obtained stimulation of growth of one of his strains. The other strains tested were apparently unable to utilize the hydrocarbons as energy source.

As to the question of whether the oxygen uptake observed represents a true oxidation of the substrate added, our growth experiments would

support the view that the hydrocarbons are being actively metabolized. This is further borne out by the results obtained on the partially oxidized derivatives, such as long-chain monohydric alcohols and long-chain monocarboxylic acids. Methylene-blue reduction, moreover, points to the presence of a dehydrogenase enzyme present in the cells capable of oxidizing the long-chain hydrocarbons.

It seems that this organism offers very interesting possibilities for further studies on metabolism.

SUMMARY

1. The oxygen uptake of washed suspensions of *Proactinomyces opacus* is increased in the presence of *n*-dodecane, *n*-tetradecane, *n*-hexadecane, *n*-octadecane and paraffin wax.

2. Decyl, lauryl (dodecyl) and octadecyl alcohols give increased oxygen uptake, but amyl, *iso*amyl, *iso*hexyl, and heptyl alcohols are toxic.

3. The long-chain fatty acids (C_7-C_{18}) are all actively metabolized at very low concentrations (0.0012M). At 1%, octanoate, decanoate, undecanoate and laurate (dodecanoate) are toxic.

4. The rate of methylene-blue reduction by the supernatant liquid obtained from the crushed cells of *Proactinomyces opacus* is increased in the presence of dodecane and hexadecane.

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Reduction of Dehydroascorbic Acid by Bacteria

2. ROLE OF CYTOCHROME IN HYDROGEN TRANSPORT

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In a previous paper (Eddy, 1952) it was shown that the reduction of dehydroascorbic acid (DHA) to ascorbic acid (AA) by *Escherichia coli* can be effected with hydrogen supplied by various hydrogen donor/dehydrogenase systems in intact cells, but that corresponding cell-free systems are ineffective. It was therefore suggested that some additional factor, possibly a hydrogen carrier, is necessary.

Our attention was first directed to cytochrome because it was reported that some fifteen strains of streptococci were unable to reduce DHA (Gunsalus & Hand, 1941) and we found the same with species of *Lactobacillus* and *Clostridium*. These bacteria can produce reducing conditions of sufficiently high intensity for the reduction of DHA to be thermodynamically possible, but possess no cytochrome (Fujita & Kodama, 1934; Frei, Riedmüller &

Almasy, 1934). When a connexion between DHA reduction and cytochrome was in fact found in *Esch. coli*, comparison of the two properties was naturally extended over a range of bacterial types.

The present paper falls, therefore, into two parts, the first describing attempts to relate DHA reduction to the hydrogen carrier and the second being a survey of the types of bacteria able to effect the reduction.

METHODS

For spectroscopic work the organisms were grown in bulk on a solid medium containing 1% Protex, 1% peptone, 1% gelatin, 0.5% Yeastrel, 0.5% glucose, 1% $CaCO_3$ and 3% agar. After harvesting, the cells were washed twice with Ringer's solution (quarter strength) and suspended at the required concentration either in this solution or in phosphate buffer at pH 6.2.