RESPIRATION AND PROTEIN SYNTHESIS
IN ESCHERICHIA COLI
MEMBRANE-ENVELOPE FRAGMENTS

II. Effects of Fatty Acids
and Albumin on Respiration

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ABSTRACT
Fatty acids inhibited the ability of Escherichia coli membrane-envelope fragments to catalyze
the oxidation of succinate and nicotinamide adenine dinucleotide, reduced form (NADH)
and also inhibited the response of the Clark oxygen electrode to nonenzymatic oxygen
uptake. In all cases, unsaturated fatty acids were much more inhibitory than saturated fatty
acids. Albumin afforded complete protection from inhibition in the nonenzymatic oxygen-
uptake experiments but only partial protection for the respiratory activities of the mem-
brane fragments. The succinoxidase activity was totally inhibited by bovine serum albumin
at concentrations that inhibited succinate dehydrogenase only slightly and NADH oxidase
not at all. The E. coli acellular preparation showed no dehydrogenase or oxidase activity
for any of the fatty acids under a variety of conditions. These conditions included variations
of pH, concentration of fatty acids, and the presence or absence of albumin, CoA, ATP,
NAD, cysteine, succinate, and carnitine. It thus appears that E. coli grown in the absence
of fatty acid can not use fatty acids as an energy source.

INTRODUCTION
This paper is the second in a series that describes experiments designed to study the respiratory and
protein-synthesizing capabilities of membranes isolated by gentle techniques from Escherichia coli.
In addition to learning more about these membrane-oriented activities, our ultimate goal is to
determine whether it is possible to supply energy for protein synthesis directly from high energy
intermediates formed during respiration (without the required participation of ATP). The first paper
described techniques for preparing membrane-envelope fragments from E. coli, and presented
data for their dehydrogenase and oxidase activities with use of the following substrates: NADH,
NADPH, succinate, malate, isocitrate, glutamate, pyruvate, and α-ketoglutarate (1). In the current
work we have tried to determine whether these preparations are capable of using fatty acids as an
energy source. We have also determined the effect of fatty acids on respiration, as well as on the
Clark oxygen electrode under nonenzymatic conditions.
MATERIALS AND METHODS

Extraction of Fatty Acids from E. coli

E. coli W-6 was grown on Difco Antibiotic medium #3 (Difco Laboratories, Inc., Detroit, Mich.) to early log phase, harvested by centrifugation, and stored frozen at -13°C. 52 g of cells was extracted overnight at room temperature with 1 liter of 2:1 CHCl₃/CH₃OH. The suspension was filtered, and the extract was evaporated to dryness under reduced pressure below 40°C. The residue was taken up in a small quantity of 2:1 CHCl₃/CH₃OH, filtered, and concentrated under a stream of N₂ below 40°C to yield 1.34 g of lipid. The lipid was hydrolyzed for 3 hr at 80°C in 134 ml of 1 N NaOH in 50% ethanol in a glass-stoppered flask. The hydrolysate was extracted three times with equal volumes of petroleum ether, and the ether extract was discarded. The aqueous solution was acidified with 10 N H₂SO₄ and extracted three times with equal volumes of petroleum ether. The ether was removed by evaporation under nitrogen to yield a residue of 478 mg of fatty acids.

Fractionation of E. coli Fatty Acids

The fatty acids were esterified by heating with 48 ml of 14% boron trifluoride in methanol (Applied Science Laboratories, Inc., State College, Pa.) at 80°C for 2 min. The mixture was cooled, and an equal volume of water was added followed by 2 volumes of heptane. The mixture was vigorously shaken and left overnight in the cold. The heptane layer was separated, and the aqueous layer was reextracted with an equal volume of heptane. The combined heptane extracts were evaporated to dryness below 40°C to yield a residue of 463 mg of esters. Gas-liquid chromatography revealed a mixture consisting of 37% (by weight) cis-vaccenate, 30% palmitoleate, 26% palmitate, and traces of other fatty acids including myristate and stearate.

The fatty acid ester mixture was resolved by chromatography on thin-layer plates of silica gel G and consisting of 37% (by weight) cis-vaccenate, 30% palmitoleate, 26% palmitate, and traces of other fatty acids including myristate and stearate.

E. coli Extract

E. coli W-6, a proline auxotroph (4), was grown on proline-supplemented Difco Antibiotic medium #3, and spheroplasts were produced by growth in the presence of penicillin (1). The spheroplasts were recovered by centrifugation, washed, stored overnight in liquid nitrogen, and thawed as previously described (1). The suspension was then subjected to two 20-sec bursts of ultrasonic irradiation with the Branson sonifier and centrifuged for 10 min at 3500 g (1). The resulting preparation is referred to as GSl (glucose supernatant 1 [1]). The oxidative activities of GSl prepared at many different times during the course of 1½ yr have been highly reproducible for the substrates listed in the introduction to this paper. The experiments reported here were carried out with two different preparations used interchangeably. One had a protein concentration of 7.1 mg/ml and the other a concentration of 8.2 mg/ml. Protein concentration was determined by the method of Lowry et al. (5).

Fatty Acid Dehydrogenase Assay

Reaction mixtures contained E. coli extract (GSl), substrate, HCN, 0.01 M histidine, 0.002 M MgSO₄, and cofactors (when indicated) in a final volume of 1 ml at pH 6.8. Final concentrations (unless otherwise noted) were 1 mM fatty acid, 0.1 mM albumin, 12 mM HCN, "FAM" which consisted of 2 mM ATP, 2 mM NAD, 0.13 mM CoA, and 3 mM cysteine; and 0.3 mM carnitine. Spectrophotometric measurements were made with an automatic recording Cary 16K spectrophotometer (Cary Instruments Corp., Midland, Mich.) at a power setting of 6. Dow Corning silicone antifoam AF (Dow Corning Corp., Midland, Mich.) was used to break the resulting foam. Fatty acid in albumin solution was prepared by first neutralizing the fatty acid with KOH and than diluting with a neutral solution of fatty acid-depleted (3) bovine serum albumin (Armour Pharmaceutical Co., Chicago, Ill. Fraction V) to produce a solution of 0.05 mM fatty acid in 0.005 mM albumin.
This is a split beam instrument that consecutively monitors each of five cuvettes against a blank.

The general procedure was based on determining the rates of NAD reduction in control samples, which sometimes contained cofactors but which did not contain fatty acid, and comparing these rates with those obtained with corresponding experimental samples containing the same cofactor additions plus fatty acid. The difference between these two rates was attributed to oxidation of fatty acid. In order to compensate for subtle variations from assay to assay, one of the five samples for both control and experimental determinations was always used as a reference. The five samples comprising each determination were as follows: (a) "reference" containing 25 µl GS1 E. coli extract, 2 mm NAD, and 12 mm HCN; (b) same as (a); (c) same as (a) but "FAM" (defined above) replaced the NAD; (d) same as (a) + carnitine; (e) same as (a) (- NAD) + FAM and carnitine.

The rate of change of optical density at 340 mµ was recorded. After 5 min no additions were made to the reference cuvette; 0.1 ml of 0.01 m fatty acid was added to each of the other four cuvettes in the experimental run, and 0.1 ml of water was added to each of the other four cuvettes in the control run. When assays were performed with fatty acid in albumin solution, 0.1 ml of albumin solution was added to the control in place of 0.1 ml H2O. Rates of NAD reduction were followed for an additional 10 min.

Slopes were determined from the rates measured in the interval from 5 to 10 min.

Calculations were made as follows:

(a) The net rate was determined for all conditions by subtracting the reference sample rate.

(b) The rate of fatty acid oxidation under each of the four conditions was determined by subtracting the net control rate (absence of fatty acid) from the net experimental rate (presence of fatty acid).

(c) The rate of change of optical density was converted to µmoles of fatty acid oxidized by use of 6.22 as the millimolar extinction coefficient of NADH (6).

Oxygen Uptake Experiments

Polarographic measurements were made with a model #4004 Clark oxygen electrode fitted with a 0.001 in. Teflon membrane (Yellow Springs Instrument Co., Yellow Springs, Ohio) at 30°C under conditions previously described (1). Endogenous oxygen uptake was recorded with the E. coli extract in 0.01 M histidine, 0.002 M MgSO4 pH 6.8. Additions were made sequentially and spaced sufficiently far apart to enable the establishment of a new rate of oxygen uptake. The order of additions was the following: (a) fatty acid or fatty acid in albumin solution; in control experiments either no addition was made or just albumin solution was added; (b) FAM; (c) succinate; (d) carnitine; (e) NADH.

The change in rate of oxygen uptake after each addition in an experimental case employing fatty acid minus the change in rate of oxygen uptake after the corresponding addition in a control experiment (minus fatty acid) was taken to represent the rate of fatty acid oxidation.

Nonenzymatic Oxygen Uptake

Nitrogen gas was passed through a Pasteur pipette immersed in 1 ml buffer (0.01 M histidine and 0.002 M MgSO4 at pH 6.6) is an open reaction vessel and bubbled at the rate of 90 bubbles/min. The tip of the Clark electrode was also immersed in the vessel and the solution was continuously stirred with a magnetic flea and maintained at 30°C.

An alternative method was based on the fact that methylene blue can be nonenzymatically reduced by NADH and oxidized by oxygen. With 2 mm NADH and 0.14 mm methylene blue in the closed 1 ml vessel used for polarographic measurements, the rate of oxygen uptake was 75 nacnatom/s/min.

RESULTS

Effect of Fatty Acids on Response of Clark Electrode to Nonenzymatic Oxygen Uptake

Table I shows that fatty acids inhibited electrode response to the loss of oxygen caused by bubbling nitrogen through the buffer solution. The inhibition tended to increase with time so that after 2-3 min electrode response frequently was abolished. When fatty acids were added with albumin no inhibition resulted. In fact, a stimulation was usually observed. The addition of albumin to vessels already containing fatty acids completely reversed the inhibition. Albumin added in the absence of fatty acid caused an enhanced response of the electrode, indicating that small quantities of fatty acid or other inhibitor may be present on the electrode membrane.

E. coli extract GS1 was tested for the presence of substances that might inhibit electrode response (fatty acids?) or enhance the response (albumin?). The addition of 50 µl GS1 did not effect the electrode response in the absence or presence of fatty acid.
## Table I

Effect of Fatty Acids on Response of Clark Electrode (N₂-Bubbling)**

| Time after addition | Albumin alone | Myristate + albumin | Palmitate + albumin | Stearate + albumin | Oleate + albumin | Palmitoleate + albumin | Gir-vaccenate + albumin | Mixed fatty acids + albumin |
|---------------------|---------------|---------------------|---------------------|-------------------|-----------------|------------------------|--------------------------|--------------------------|
| sec                 |               |                     |                     |                   |                 |                        |                          |                          |
| 30                  | 200           | 50                  | 160                 | 40                | 30              | 130                    | 30                       | 135                      |
| 60                  | 280           | 150                 | 50                  | 105               | 105             | 150                    | 150                      | 220                      |
| 90                  | 40 (80)       | 150                 | 50 (130)            | 105               | 145             | 150                    | 150                      | 190                      |
| 120                 | 25 (170)      | 200                 | 15                  | 175               | 15              | 260                    | 15                       | 0                        |
| 150                 |               | 0                   |                     |                   |                 |                        |                          |                          |
| 180                 | 11 (117)      | 0                   |                     |                   |                 |                        |                          |                          |
| 260                 | 0 (5)         | 0 (80)              |                     |                   | 0 (75)          | 0 (25)                 |                          |                          |

* Oxygen was removed from a standard vessel at the rate of 0.17 μatoms/min by bubbling N₂ gas through the vessel at a rate of 90 bubbles/min.

** Mg²⁺ is required for inhibitions in the presence of fatty acids.

§ All numbers represent the rate of oxygen removal as a percentage compared to the corresponding rate obtained when no additions were made.

|| Numbers in parenthesis represent rates (as percentages) obtained by adding 0.1 μmole albumin to the "inhibited vessel" at the indicated time.

## Table II

Effect of Fatty Acids on Oxidative Enzymes

| Albumin | Myristate + albumin | Palmitate + albumin | Stearate + albumin | Oleate + albumin | Palmitoleate + albumin | Gir-vaccenate + albumin | Mixed fatty acids + albumin |
|---------|---------------------|---------------------|-------------------|-----------------|------------------------|-------------------------|-----------------------------|
| NADH Oxidase | Oxygen uptake | Spectrophotometric | Succinate Oxidase | Dehydrogenase |
| 110 | 60 | 97 | 100 | 75 | 105 | 0 | 0 | 18 | 0 | 10 | 0 | 9 |
| 107 | 38 | 97 | 115 | 54 | 110 | 3 | 5 | 0 | 2 | 0 | 4 | 0 | 4 |

NADH oxidase was measured both by oxygen uptake with use of the Clark oxygen electrode and by the change of optical density at 340 mμ (in the absence of cyanide) with the Cary 16K automatic recording spectrophotometer. Succinate oxidase was measured by oxygen uptake and succinate dehydrogenase by change of optical density at 600 mμ with the dye 2,6-dichlorophenol indophenol as electron acceptor (1). All numbers represent per cent of activity observed compared to controls where neither fatty acid nor albumin was added. Before addition of fatty acid or albumin, the level of uninhibited enzyme activity was in the range of previously reported values (1) (i.e., average for NADH oxidase by oxygen electrode or spectrophotometer, 15.5 units; succinoxidase, 2.0 units; succinate dehydrogenase, 1.3 units.) A unit represents the oxidation of 1 μmole substrate/10 min per mg protein.
acid. When the electrode was already poisoned by 1 mm palmitoleate and the addition of 50 µl GSI caused no response, the subsequent addition of albumin completely restored normal electrode response. These observations show that E. coli extract exerted no detectable influence on the response of the electrode under conditions of oxygen removal by nitrogen bubbling.

The inhibition of electrode response (to nitrogen bubbling) caused by fatty acid required the presence of Mg++. This was shown by replacing the histidine-Mg++ buffer with 0.73% NaCl, in which case 1 mm oleate caused no inhibition until 2 mM MgCl₂ was provided.

When nonenzymatic oxygen uptake was caused by NADH and methylene blue, it was observed that:

(a) Either palmitoleate (1 mm) or mixed fatty acids (Total = 1 mm composition described in Methods section) caused 70-75% inhibition of electrode response. The addition of albumin (0.1 mM) completely restored normal electrode response.

(b) Replacing the histidine-Mg++ buffer by 0.73% NaCl did not lessen the degree of inhibition caused by palmitoleate or mixed fatty acids. The addition of MgCl₂ (2 mM) to the NaCl solution did not enhance the degree of inhibition caused by fatty acid.

(c) GSI (50 µl) did not reverse the inhibition caused by palmitoleate, nor did it interfere with the removal of the inhibition caused by the subsequent addition of albumin.

(d) Inhibition caused by addition of fatty acid was immediate in contrast to the situation described in the experiments where oxygen was removed by bubbling with nitrogen.

Effect of Fatty Acids on Oxidase Enzymes

NADH Oxidase: Table II shows that the saturated fatty acids, myristate and stearate, partially inhibited NADH oxidase, with myristate being somewhat more inhibitory than stearate. The differences in extent of inhibition shown by the two assay methods might reflect the fact that different enzyme and substrate concentrations are used in each assay. Albumin afforded complete protection from these inhibitions and palmitoleic acid caused no inhibition. The unsaturated fatty acids caused 100% inhibition and albumin afforded only partial protection from these inhibitions.

The inhibition caused in the oxygen-uptake assay for NADH oxidase differed from the inhibition noted above in the nonenzymatic nitrogen bubbling experiments with the Clark electrode in that the inhibition observed in the oxidase assay was immediate upon addition of fatty acid, and albumin could not protect the oxidase from poisoning by the unsaturated fatty acids.

Succinoxidase: The response of the succinoxidase enzymes to saturated fatty acids was similar to that noted for NADH oxidase. Myristate was more inhibitory than stearate, and palmitate was not inhibitory at all. A major difference between succinoxidase and NADH oxidase activities was revealed by the response to added albumin. Succinoxidase was completely inhibited by 0.1 mM albumin whereas NADH oxidase was slightly stimulated. Unsaturated fatty acids also completely eliminated succinoxidase activity.

Succinate Dehydrogenase: Succinate dehydrogenase was inhibited about 10-20% by the saturated fatty acids and about 50-80% by the unsaturated fatty acids. Albumin was inhibitory but not as much as toward succinate oxidase activity. It is also shown that the presence of albumin and unsaturated fatty acid together was less inhibitory than when either was present alone. This is consistent with the known ability of albumin to bind (and be bound by) these fatty acids quite strongly.

Fatty Acid Dehydrogenase Activity: Fatty acid-NAD dehydrogenase activity for each of the fatty acids was measured and the results are shown in Table III. Changes in the rate of NAD reduction due to the addition of fatty acid were uniformly negligible. No improvement of this rate was obtained by the addition of FAM, which provided ATP, CoA, and cysteine, or by the addition of carnitine or albumin, or by various combinations of these factors.

In addition to the experiments shown in Table III, dehydrogenase assays were carried out for each of the four sets of additions with each fatty acid, at 1 mM concentration plus or minus albumin at pH 8 and with 0.1 mM fatty acid concentration at pH 6.8 and pH 8.0. In no case was any appreciable difference in the rate of reduction of NAD caused by the addition of fatty acid.

Fatty Acid Oxidase Activity: Although the absence of demonstrable fatty acid dehydrogenase activity would indicate the concurrent absence of fatty acid oxidase activity, we used the
TABLE III

Fatty Acid Dehydrogenase Activity

| Additions               | Myristate (alone) | Myristate (+ albumin) | Palmitate (alone) | Palmitate (+ albumin) | Stearate (alone) | Stearate (+ albumin) |
|-------------------------|-------------------|-----------------------|-------------------|-----------------------|-----------------|----------------------|
| 1 Fatty Acid + NAD      | 0.30 ± 0.06       | 0.00 ± 0.02           | 0.04 ± 0.09       | -0.02 ± 0.02          | 0.08 ± 0.24     | 0.01 ± 0.06          |
| 2 Fatty Acid + FAM      | -0.02 ± 0.25      | -0.11 ± 0.05          | 0.09 ± 0.12       | 0.00 ± 0.08           | 0.23 ± 0.23     | 0.01 ± 0.06          |
| 3 Fatty Acid + carnitine + NAD | 0.14 ± 0.04     | -0.03 ± 0.08          | -0.01 ± 0.07      | -0.03 ± 0.03          | 0.29 ± 0.20     | 0.06 ± 0.04          |
| 4 Fatty Acid + FAM + carnitine | 0.09 ± 0.21     | -0.02 ± 0.04          | -0.08 ± 0.22      | -0.15 ± 0.10          | 0.00 ± 0.17     | -0.06 ± 0.04         |
| No. of experiments      | 4                 | 3                     | 6                 | 3                     | 5               | 3                    |

| Additions               | Oleate (alone) | Oleate (+ albumin) | Palmitoleate (alone) | Palmitoleate (+ albumin) | Glu-vaccenate (alone) | Glu-vaccenate (+ albumin) | Mixed Fatty Acids (alone) | Mixed Fatty Acids (+ albumin) |
|-------------------------|----------------|-------------------|----------------------|--------------------------|------------------------|---------------------------|-----------------------------|-------------------------------|
| 1 Fatty Acid + NAD      | 0.09 ± 0.01    | 0.25 ± 0.17       | -0.32 ± 0.04         | 0.12 ± 0.04              | -0.21 ± 0.05          | 0.02 ± 0.02               | -0.23 ± 0.34                | -0.01 ± 0.06                  |
| 2 Fatty Acid + FAM      | -0.06 ± 0.09   | 0.03 ± 0.08       | -0.64 ± 0.18         | -0.06 ± 0.12             | -0.43 ± 0.13          | 0.07 ± 0.08               | 0.16 ± 0.18                 | -0.02 ± 0.05                  |
| 3 Fatty Acid + carnitine + NAD | 0.06 ± 0.03   | 0.14 ± 0.13       | -0.26 ± 0.04         | 0.16 ± 0.05              | -0.34 ± 0.09          | 0.00 ± 0.02               | -0.07 ± 0.07                | -0.07 ± 0.01                  |
| 4 Fatty Acid + FAM + carnitine | -0.28 ± 0.27  | 0.03 ± 0.03       | -0.66 ± 0.18         | 0.14 ± 0.03              | -0.51 ± 0.12          | 0.03 ± 0.03               | 0.23 ± 0.31                  | -0.03 ± 0.05                  |
| No. of experiments      | 3               | 4                 | 3                    | 3                        | 3                      | 3                         | 3                           | 4                             |

The numbers represent changes in rate of reduction of NAD (or oxidation of fatty acid) in units of \( \mu \text{mole/10 min per 10 mg} \), due to the addition of fatty acid or fatty acid plus albumin. Standard errors of the mean are also shown. Complete details are presented in the experimental section. Final concentrations were: 1 mM fatty acid; 0.1 mM albumin, FAM = 2 mM ATP, 2 mM NAD, 0.13 mM coenzyme A, 3 mM cysteine; and 0.3 mM carnitine. Final volume was 1 ml and the buffer was 0.01 M histidine containing 0.002 M MgSO\(_4\) at pH 6.8. 12 mM HCN was present in all cases. The base level for NAD reduction in the absence of fatty acid was near zero (±0.02 \( \mu \text{mole/10 min per 10 mg protein} \)).
Figure 1 Succinate dehydrogenase activity was determined spectrophotometrically by measuring the reduction of 2,6-dichlorophenol indophenol with a Cary 16 K spectrophotometer. Succinate oxidase activity was measured polarographically with the Clark oxygen electrode (1). Reaction volumes were 1 ml and the protein concentration of E. coli extract (GS1) was 0.41 mg/ml for oxidase assays and 0.16 mg/ml for dehydrogenase assays.

In a series of 63 experiments (data not shown) it was found that no significant oxygen uptake occurred with any fatty acid in the presence or absence of albumin, succinate, carnitine, or FAM (cofactor mixture).

**Effect of Albumin on NADH Oxidase, Succinoxidase, and Succinate Dehydrogenase Activities**

In Table II it was shown that 0.1 mm fatty acid-free albumin (0.69%) completely blocked succinoxidase activity, inhibited succinate dehydrogenase activity by 90%, and stimulated NADH oxidase about 10%. When succinoxidase activity had been reduced to zero by the addition of albumin, the subsequent addition of NADH to the same reaction vessel caused the immediate uptake of oxygen at the stimulated rate usually observed for NADH and albumin. Fig. 1 shows the remarkable sensitivity of succinoxidase activity to albumin at concentrations that have minimal effects on succinate dehydrogenase activity.  

**DISCUSSION**

The ability of an E. coli acellular preparation to oxidase fatty acids has been studied in order to determine whether fatty acids could serve as a source of energy for metabolic reactions occurring in E. coli membranes. A search of the literature has revealed little direct information on the ability of E. coli acellular preparations to oxidize fatty acids. We have looked for fatty acid oxidase and dehydrogenase activities in a homogenate of E. coli that has been shown to contain oxidative enzymes for NADH, NADPH, succinate, malate, isocitrate, pyruvate, and α-ketoglutarate (1). Three saturated fatty acids, myristate, palmitate, and stearate, and three unsaturated fatty acids, oleate, palmitoleate, and cis-vaccenate, were tested as well as a mixture of fatty acids isolated from our strain of E. coli.

Attempts to stimulate fatty acid oxidation included the addition of various combinations of the following components: bovine serum albumin, ATP, NAD, coenzyme A, cysteine, succinate, and carnitine. Dehydrogenase assays were performed at 1 mm and at 0.1 mm concentrations of fatty acids at pH 6.8 and at 8.0. The combined results indicate that E. coli (W-6 grown on Difco Antibiotic Medium 3) is deficient in enzymes capable of fatty acid oxidation. It is not possible, however, from these negative data, to conclude that such enzymes are not present.

Overath et al. (7) have recently shown that, in E. coli K 12Y mel, individual enzymes involved in fatty acid oxidation are present in minute amounts when the cells are grown in the absence of fatty acids. When the cells are adapted to growth on fatty acid, a very substantial increase in concentration is noted for the individual enzymes involved.

2 Albumin inhibition of succinoxidase has been found for both malate-grown cells and glucose-grown cells. Subsequent work has shown, however, that although oxidative activities of GS1 are highly reproducible, there is a variability in the quantitative aspects of the albumin sensitivity. For example, complete inhibition of succinoxidase activity in a different preparation of GS1 required 0.2 mm albumin instead of 0.01 mm. Under conditions of complete inhibition of succinoxidase activity, NADH oxidase was still 70% active. Other preparations were insensitive to albumin.

The identity of the variable that determines sensitivity is currently under investigation.
in fatty acid oxidation (7). From these observations, as well as from our own findings we conclude that, unless *E. coli* is induced to grow in the presence of fatty acids, fatty acid oxidation plays a very minor role in the energy metabolism of this organism.

Unsaturated fatty acids at 1 mM completely blocked NADH oxidase and succinoxidase activities. Succinate dehydrogenase, which is also membrane bound, was only partially inhibited. Although myristate was more inhibitory than palmitate or stearate, the saturated fatty acids were much less inhibitory than the unsaturated fatty acids. Albumin completely protected NADH oxidase from inhibition by saturated fatty acids but only partially protected the oxidase from inhibition by the unsaturated fatty acids. The surprising finding was made that succinate oxidase activity was completely inhibited by 0.01 mM albumin (0.069%)², whereas succinate dehydrogenase was only 10% inhibited and NADH oxidase was slightly stimulated. The effect of albumin on succinate oxidase is in marked contrast to the situation in mammalian adipose tissue mitochondria, where the addition of bovine serum albumin (0.2 mM) caused a 190% stimulation of succinoxidase activity (8). The striking difference in sensitivities for succinate and NADH oxidases to albumin and other inhibitors (1) suggests that in *E. coli* these two activities may be separate. However, we find that either NADH or succinate can reduce nearly all of the cytochromes present.² The locus of the albumin sensitivity appears to be at a point prior to the participation of the cytochromes. The observation that succinate dehydrogenase activity was not particularly sensitive to albumin indicates that the situation is somewhat complex. The phenomenon is currently under investigation.

Fatty acids were able to interfere with the response of the Clark oxygen electrode under conditions of nonenzymatic oxygen uptake. In the experiments where oxygen was replaced with nitrogen, the inhibition progressively increased over a 3 min period at which time electrode response was completely abolished. This situation is in distinction to the inhibitions by fatty acids of NADH oxidase and of the nonenzymatic NADH-methylene blue reaction, which effects were immediate. The inhibition of the response of the Clark electrode under both nonenzymatic conditions was completely prevented or reversed by albumin. This was not the case for NADH oxidase where albumin afforded minimal protection against inhibition by unsaturated fatty acids. Mg²⁺ was required for fatty acid to interfere with the response of the Clark electrode when oxygen removal was accomplished by nitrogen replacement but not when the methylene blue-NADH system was used. It seems likely that an insoluble magnesium soap, which can be solubilized or degraded by albumin, rather than the fatty acid is the inhibitory agent in the nitrogen bubbling experiments. In the methylene blue-NADH experiments, either the mechanism of inhibition is different or some other inhibitory substance may be formed.

It is important to note that in our studies nonenzymatic oxygen uptake was caused by two different procedures, one physical and the other chemical. Although fatty acids interfered with electrode response in both cases, it has not been definitely established that the effects were exerted directly on the electrode. It is possible that gaseous diffusion rates were affected in the nitrogen bubbling experiments and that the chemistry of oxidation and/or reduction of methylene blue was affected in the chemical method.

Nevertheless, these observations signal a note of caution in the use of the Clark electrode for measuring oxygen uptake in the presence of fatty acids. In our own work we have noted the difference in time of onset of the inhibition in the NADH oxidase and nitrogen bubbling experiments, the difference in protection afforded by albumin against unsaturated fatty acids in the enzymatic and nonenzymatic situations, and we have confirmed the effects of fatty acid on NADH oxidase by both polarographic and spectrophotometric techniques.

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