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 were extracted overnight at room temperature with 1 liter of 2:1 $CHCl_3$:CH_3OH. The suspension was filtered, and the extracts were evaporated to dryness under reduced pressure below 40°C The residue was taken up in a small quantity of 2:1 $CHCl_3$:CH_3OH, filtered, and concentrated under a stream of N_2 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 H_2SO_4 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 silver nitrate (2). Glass plates were coated with a slurry of 60 g silica gel G in 120 ml of water containing 15 g of dissolved silver nitrate. Three 200 × 200 × 0.1 mm thin layers could be produced from the slurry. 40 separate 0.5 µl aliquots were applied to each plate, and chromatography was performed with 15% ether in petroleum ether (v/v). The plates were sprayed with 0.2% 2,7-dichlorofluorescein in 95% ethanol and examined under ultraviolet light. Methyl vaccenate (R_f .45) was cleanly separated from methyl palmitoleate (R_f .35) and the esters of the saturated fatty acids (R_f .65). The unsaturated esters were eluted with methanol, concentrated in vacuo, dissolved in heptane, and analyzed by gas-liquid chromatography. Cis-vaccenate was 97% pure

with 3% palmitoleate present, and the palmitoleate was 89% pure with 11% cis-vaccenate present. Recovery of applied ester was 94% for cis-vaccenate and 87% for palmitoleate. The free fatty acids were released by saponification in alcoholic sodium hydroxide and extracted in petroleum ether as described in the preceding section.

Preparation of Solutions of Fatty Acids

0.1 M fatty acid in 0.1 M KOH was sonicated 15 sec with a Branson sonifier (Model S75, Branson Instruments Co., Stamford, Conn.) 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 then 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 M fatty acid in 0.005 M albumin.

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 GS1 (glucose supernatant 1 [1]). The oxidative activities of GS1 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 (GS1), substrate, HCN, 0.01 M histidine, 0.002 M $MgSO_4$, 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, Monrovia, Calif.) connected to a circulating 30°C water bath.

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 H$_2$O. Rates of NAD reduction were followed for an additional 10 min[1]. 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 MgSO$_4$ 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 MgSO$_4$ 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 nanoatoms/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

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[1] The unsaturated fatty acids tended to cause an increase in the turbidity of the solution. This increase was most pronounced directly after addition of the fatty acid; after several minutes either no further increase or a slow steady increase resulted. When this phenomenon interfered with the assay, it was found that a reversal of the order of adding the GS1 E. coli extract and fatty acid circumvented the problem.

TABLE I

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

Time after addition	Albumin	Myristate		Palmitate		Stearate		Oleate		Palmitoleate		Cis-vaccenate		Mixed fatty acids	
		alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin
sec															
30	200§	50	160	40		75	105	11	105	30	130	30	135	10	170
60	280		150			50(130)‖	105		145	15	150	15	190	5	220
90		40(80)‖		25(170)‖					200		175		260		
120										0				0	
150								0							
180		11(117)‖													
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.
‡ Mg⁺⁺ 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		Palmitate		Stearate		Oleate		Palmitoleate		Cis-vaccenate		Mixed fatty acids	
		alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ albumin
NADH Oxidase															
Oxygen uptake	110	60	115	97	100	75	105	0	6	0	18	0	10	0	9
Spectrophotometric	107	38	105	97	115	54	110	3	5	0	2	0	4	0	4
Succinate															
Oxidase	0	75	0	100	0	80	0	0	0	0	0	0	0	0	0
Dehydrogenase	10	93	28	88	16	81	30	54	45	45	70	17	58	23	81

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 GSl 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 $MgCl_2$ 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 $MgCl_2$ (2 mm) to the NaCl solution did not enhance the degree of inhibition caused by fatty acid.

(*c*) GSl (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 palmitic 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		Palmitate		Stearate	
	alone	+ albumin	alone	+ albumin	alone	+ 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		Palmitoleate		Cis-vaccenate		Mixed Fatty Acids	
	alone	+ albumin	alone	+ albumin	alone	+ albumin	alone	+ 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	-0.01 ± 0.06
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 μmole/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 μmole/10 min per 10 mg protein).
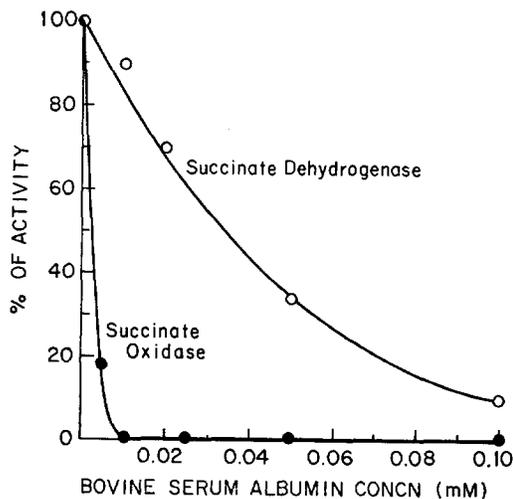
EFFECT OF ALBUMIN ON SUCCINATE OXIDATION

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.

oxygen electrode to determine directly whether any measurable extent of oxidation could be found. 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.[2]

DISCUSSION

The ability of an *E. coli* accellular 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

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[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.[3] 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-methyl-

[3] R. W. Hendler and N. Nanninga, manuscript in preparation.

ene 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. Mg^{++} 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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