The Utilization of Substituted Acyl-Coenzyme A Derivatives in Fatty Acid Synthesis. II. Studies with Enzymes Obtained from Adipose Tissue

JOSEPH D. ROBINSON, ROY M. BRADLEY, AND ROSCOE O. BRADY

From the Laboratory of Neurochemistry, National Institute of Neurological Diseases and Blindness, National Institutes of Health, Bethesda, Maryland

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Partially purified enzyme preparations from rat epididymal adipose tissue which catalyze the synthesis of long-chain fatty acids from acetyl-coenzyme A (CoA) and malonyl-CoA also catalyze the reduction of acetoacetyl-CoA, D(−)-β-hydroxybutyryl-CoA, and crotonyl-CoA to the saturated derivative. This enzyme further catalyzes the incorporation of β-hydroxybutyryl-CoA and crotonyl-CoA into long-chain fatty acids, and evidence is presented which indicates that reduction to the saturated compound must occur prior to condensation with malonyl-CoA. These observations are offered in support of a reductive pathway for fatty acid synthesis passing through the β-keto, D(−)-β-hydroxy, and α,β-unsaturated derivatives.

We have recently presented spectrophotometric evidence for the reduction of acetoacetyl-CoA and crotonyl-CoA by a partially purified enzyme preparation obtained from rat adipose tissue (Robinson *et al.*, 1962b) which also catalyzes the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA (Martin *et al.*, 1961). Enzyme preparations obtained from pigeon and rat liver which catalyze the synthesis of long-chain fatty acids do not catalyze the reduction of β-hydroxybutyryl-CoA or crotonyl-CoA (Gibson *et al.*, 1958; Brady *et al.*, 1960). Enzymes obtained from yeast catalyze one-step reductions of certain thiolesters of acetoacetic acid and crotonic acid and the dehydration of thiolesters of D(−)-β-hydroxybutyric acid (Lynen, 1961). The incorporation of substituted acyl-CoA derivatives into long-chain fatty acids has not been demonstrated with these preparations. The situation is somewhat different with an enzyme preparation obtained from rat brain tissue which catalyzes the incorporation of acetoacetyl-CoA, D(−)-β-hydroxybutyryl-CoA, and crotonyl-CoA into saturated long-chain fatty acids in the presence of malonyl-CoA (Robinson *et al.*, 1962a).

It was of interest, therefore, to learn whether enzymes purified from adipose tissue could utilize substituted acyl-CoA derivatives for long-chain fatty acid synthesis. It is the purpose of the present communication to describe experiments demonstrating the reduction of labeled acetoacetyl-CoA, D(−)-β-hydroxybutyryl-CoA, and crotonyl-CoA to the saturated derivative, and the ability of the product of these reductive steps to serve as a primer for fatty acid synthesis in the presence of malonyl-CoA.

Experimental Procedure

Materials.—The acyl-CoA derivatives were prepared as described previously (Brady *et al.*, 1960; Robinson *et al.*, 1962a). The quinine salt of L(+)-β-hydroxybutyrate, prepared according to Clarke (1959), was a gift of Dr. Gilbert Ashwell; D(−)-β-hydroxybutyrate obtained from diabetic urine was a gift of Dr. Sidney Chernick. The labeled fatty acids, reduced triphosphopyridine nucleotide (TPNH), and CoA were obtained from commercial sources.

The fatty acid synthesizing enzyme preparations were obtained from epididymal fat pads of 150-g rats according to Martin *et al.* (1961). Fraction I is designated as the enzyme obtained from the high-speed supernatant fraction precipitating between 0 and 45% saturation with ammonium sulfate. Fraction II was obtained by adsorption and elution from calcium phosphate gel, and fraction III after adsorption and elution from alumina C_γ gel. Thiolase activity was measured according to Stern (1958).

Methods.—The short-chain fatty acids were recovered by adjusting the incubation mixtures to *p*H 10 with NaOH followed by lyophilization and finally suspension of the residue in 0.25 ml of 2 N H_2SO_4. The suspension was extracted with 0.1 ml of redistilled ethyl acetate, and the components in the organic phase were fractionated by vapor phase chromatography. A 5-foot column containing 30% substrate (composed of 10% stearic acid, 10% orthophosphoric acid, and 80% Dow-Corning Silicone 550-S) on acid-washed chromosorb was employed at a temperature of 126° and a carrier gas flow rate of 75 ml per minute. The short-chain fatty acids were collected in glass U-tubes immersed in a dry ice–acetone bath.

Methyl esters of the long-chain fatty acids were prepared from the petroleum ether extracts of the incubation mixtures (Brady *et al.*, 1960). The esters were dissolved in hexane and fractionated by gas chromatography; a 5-foot column containing 15% diethyleneglycol succinate polyester on acid-washed chromosorb was used at a temperature of 194° and a flow rate of 75 ml per minute. The effluent was collected in glass tubes fitted with Millipore filters (Hajra and Radin, 1962).

Butyric acid-C^{14} was decarboxylated as described previously (Robinson *et al.*, 1962b). The liberated $C^{14}O_2$ was collected in *p*-(diisobutylcresoxyethoxyethyl)-dibenzylammonium chloride (Hyamine) and the radioactivity counted.

Results

The reduction of acetoacetyl-CoA, D(−)-β-hydroxybutyryl-CoA, and crotonyl-CoA in the presence of TPNH was catalyzed by the adipose tissue enzyme fractions I to III (Figs. 1-3). The L(+) stereoisomer of β-hydroxybutyryl-CoA was not reduced by these preparations. Thiolase activity was present in all fractions. Attempts to purify enzyme Fraction III by adsorption and elution from a DEAE-cellulose column resulted in too great a dilution of enzymatic activity for satisfactory spectrophotometric demonstration of the reduction of these compounds. Since the enzyme obtained by this fractionation procedure was unstable to conventional concentrating techniques, these difficulties precluded the use of Fraction IV in the present studies.

The reduction of these acyl-CoA derivatives was confirmed by the use of the corresponding labeled compounds (Table I). Gas chromatography of the reaction products showed the formation of labeled butyrate

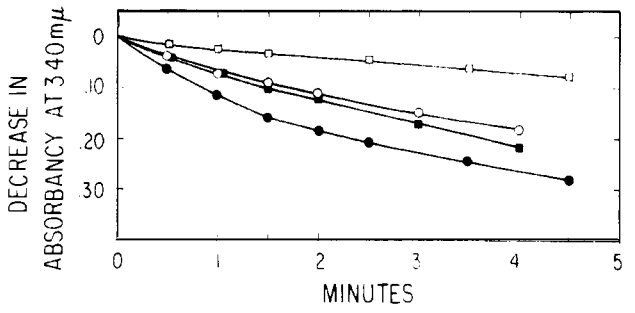

FIG. 1.—Spectrophotometric demonstration of the reduction of acyl-CoA compounds and of fatty acid synthesis with enzyme fraction I. Each cuvet contained 5 μmoles of triethanolamine hydrochloride buffer (*p*H 7.5), 7 μmoles of 2-mercaptoethanol, 30 mμmoles of TPNH, and the adipose enzyme fraction I (400 μg of protein) in a final volume of 0.2 ml. Decrease in absorbancy at 340 mμ was recorded after the addition of 65 mμmoles of malonyl-CoA plus 65 mμmoles of acetyl-CoA (closed circles), 65 mμmoles of acetoacetyl-CoA (open circles), 65 mμmoles of D(−)-β-hydroxybutyryl-CoA (open squares), or 65 mμmoles of crotonyl-CoA (closed squares). There were 7.3 units of thiolase activity present in each of the incubation mixtures. The figure was corrected for dilution and endogenous TPNH oxidation.

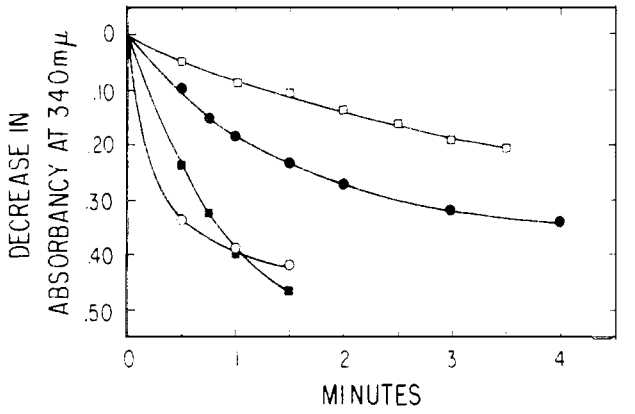

FIG. 2.—Spectrophotometric demonstration of the reduction of acyl-CoA compounds and of fatty acid synthesis with enzyme fraction II. Each cuvet contained 10 μmoles of potassium phosphate buffer (*p*H 7.8), 1 μmole of 2-mercaptoethanol, 30 mμmoles of TPNH, and the adipose enzyme fraction II (250 μg of protein) in a final volume of 0.2 ml. Decrease in absorbancy at 340 mμ was recorded after the addition of 65 mμmoles of malonyl-CoA plus 65 mμmoles of acetyl-CoA (closed circles), 65 mμmoles of acetoacetyl CoA (open circles), 65 mμmoles of D(−)-β-hydroxybutyryl-CoA (open squares), or 65 mμmoles of crotonyl-CoA (closed squares). There were 7.8 units of thiolase activity present in this preparation. The figure was corrected for dilution and endogenous oxidation of TPNH.

in all cases. Because of the presence of thiolase in the enzyme preparation employed, an aliquot of the C^{14}-labeled butyrate collected after gas chromatography from the incubation with acetoacetyl-1-C^{14} CoA was degraded. Of 445 cpm in the butyric acid sample examined, 430 cpm were recovered in the $C^{14}O_2$ by decarboxylation.

When C^{14}-labeled D,L-β-hydroxybutyryl-CoA, crotonyl-CoA, and butyryl-CoA were incubated with enzyme fraction III in the presence of TPNH and unlabeled malonyl-CoA, radioactive long-chain fatty acids were formed (Table II). Extracts of the corresponding nonincubated control samples contained negligible radioactivity. Gas chromatography of the methyl esters of the recovered fatty acids demonstrated that the major product was palmitate in all cases.

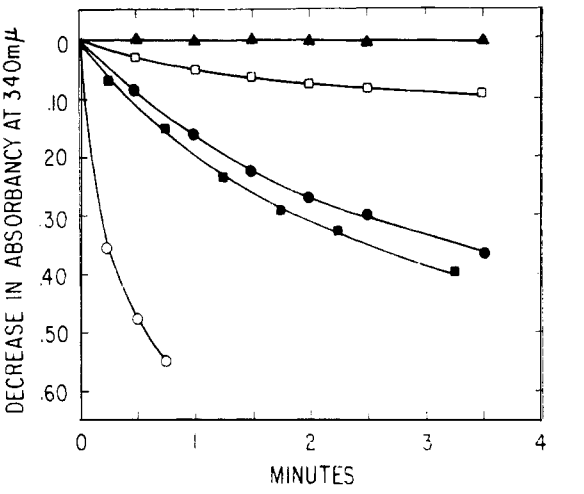

FIG. 3.—Spectrophotometric demonstration of the reduction of acyl-CoA compounds and of fatty acid synthesis with enzyme fraction III. Each cuvet contained 10 μmoles of potassium phosphate buffer (*p*H 7.8), 10 μmoles of KCl, 53 μmoles of $(NH_4)_2SO_4$, 1 μmole of 2-mercaptoethanol, 30 mμmoles of TPNH, and the adipose enzyme fraction III (140 μg of protein) in a final volume of 0.2 ml. Decrease in absorbancy at 340 mμ was recorded after the addition of 65 mμmoles of malonyl-CoA plus 65 mμmoles of acetyl-CoA (closed circles), 65 mμmoles of acetoacetyl-CoA (open circles), 65 mμmoles of D(−)-β-hydroxybutyryl-CoA (open squares), 65 mμmoles of L(+)-β-hydroxybutyryl-CoA (closed triangles), or 65 mμmoles of crotonyl-CoA (closed squares). There were 5 units of thiolase activity present in this preparation. The figure was corrected for dilution and endogenous TPNH oxidation.

TABLE I

REDUCTION OF SUBSTITUTED C^{14}-ACYL-CoA COMPOUNDS TO C^{14}-BUTYRATE

In experiments 1 and 3 each tube contained 50 μmoles of potassium phosphate buffer (*p*H 7.8), 50 μmoles of KCl, 265 μmoles of $(NH_4)_2SO_4$, 5 μmoles of 2-mercaptoethanol, 1.5 μmoles of TPNH, the adipose enzyme fraction III (580 μg of protein), plus the labeled acyl-CoA compounds listed below in a final volume of 0.7 ml. In experiment 2 the incubation mixture contained 120 μmoles of potassium phosphate buffer (*p*H 7.8), 12 μmoles of 2-mercaptoethanol, 1.5 μmoles of TPNH, the adipose enzyme fraction II (2.3 mg of protein), plus the labeled acyl-CoA compound listed in a final volume of 1.6 ml. The incubation time was 60 minutes at 37°. The reaction was stopped by the addition of 0.2 ml 2 N NaOH. The samples were prepared for gas chromatography as described in the text.

Experiment No.	Additions	Radioactivity Recovered in the Butyric Acid Fractions After Gas Chromatography (cpm)
1	Acetoacetyl-1-C^{14}-CoA, 500 mμmoles (930 cpm per mμmole)	1,650
	Same, nonincubated control	104
2	D,L-β-hydroxybutyryl-3-C^{14}-CoA, 300 mμmoles (233 cpm per mμmole)	8,216
	Same, nonincubated control	580
3	Crotonyl-1-C^{14}-CoA, 500 mμmoles (2,675 cpm per mμmole)	73,556
	Same, nonincubated control	4,021

TABLE II

INCORPORATION OF LABELED ACYL-CoA COMPOUNDS INTO LONG-CHAIN FATTY ACIDS

The reaction tubes contained 50 μmoles of potassium phosphate buffer (*p*H 7.8), 50 μmoles of KCl, 265 μmoles of $(NH_4)_2SO_4$, 5 μmoles of 2-mercaptoethanol, 1.5 μmoles of TPNH, 500 mμmoles of malonyl-CoA, and the adipose enzyme fraction III (580 μg of protein), plus the respective labeled acyl-CoA compounds, in a final volume of 0.75 ml. After incubation for 60 minutes at 37° the reaction was stopped by the addition of 0.2 ml 2 N H_2SO_4. The fatty acids were extracted into petroleum ether and this extract was washed twice with 0.05 M butyric acid and three times with water. The preparation of the methyl esters and the conditions of the gas chromatography are described in the text.

Additions	Radioactivity Recovered in Long-Chain Fatty Acids (cpm)	Radioactivity in the Methyl Palmitate Fraction After Gas Chromatography (%)
D,L-β-Hydroxybutyryl-3-C^{14}-CoA, 500 mμmoles (870 cpm per mμmole)	1,709	56
Crotonyl-1-C^{14}-CoA, 500 mμmoles (2675 cpm per mμmole)	32,810	67
Butyryl-1-C^{14}-CoA, 500 mμmoles (502 cpm per mμmole)	21,412	63

TABLE III

THE EFFECT OF ACYL-CoA DERIVATIVES ON THE ENZYMATIC DECARBOXYLATION OF MALONYL-1,3-C^{14}-CoA

The decarboxylation flasks (Brady *et al.*, 1960) contained 10 μmoles of potassium phosphate buffer (*p*H 7.8), 10 μmoles of KCl, 1 μmole of $KHCO_3$, 53 μmoles of $(NH_4)_2SO_4$, 1 μmole of 2-mercaptoethanol, 65 mμmoles of malonyl-1,3-C^{14}-CoA (670 cpm per mμmole), and the adipose enzyme fraction III (140 μg of protein) in a final volume of 0.2 ml. The reaction was stopped after 10 minutes at 37° by the addition of 0.2 ml of 1 N H_2SO_4, and the $C^{14}O_2$ was recovered as described previously (Brady, 1960).

Additions	Radioactivity in CO_2 (cpm)
None	563
Acetyl-CoA, 65 mμmoles	2,854
Acetoacetyl-CoA, 65 mμmoles	2,043
D(−)-β-hydroxybutyryl-CoA, 65 mμmoles	598
L(+)-β-hydroxybutyryl-CoA, 65 mμmoles	307
Crotonyl-CoA, 65 mμmoles	386
Butyryl-CoA, 65 mμmoles	1,554

Of the substituted 4-carbon acyl-CoA compounds examined, only acetoacetyl-CoA stimulated the enzymatic decarboxylation of malonyl-1,3-C^{14}-CoA above control levels (Table III). However, the significance of this observation is lessened by the fact that the enzyme fraction III employed in these determinations contained 5 units of thiolase activity in each incubation vessel. Butyryl-CoA was roughly half as effective as acetyl-CoA in stimulating the decarboxylation of malonyl-CoA.

DISCUSSION

The relative rates of the reduction of D(−)-β-hydroxybutyryl-CoA and crotonyl-CoA compared to the synthesis of long-chain fatty acids from acetyl-CoA and malonyl-CoA, measured spectrophotometrically, either remained roughly parallel or increased through the purification steps examined in these studies. Exact comparisons cannot be made because of different compositions of the suspending media and some variation between equivalent enzyme preparations. The disproportionate increase in the relative rate of reduction of acetoacetyl-CoA is most likely a reflection of the decrease in the amount of thiolase activity relative to fatty acid synthesizing activity in the course of the purification procedures.

The reduction of labeled acetoacetyl-CoA, D,L-β-hydroxybutyryl-CoA, and crotonyl-CoA to labeled butyrate has been demonstrated in the present experiments. Since only the D(−) stereoisomer of β-hydroxybutyryl-CoA was observed spectrophotometrically to be reduced, it is likely that only the D(−) stereoisomer gave rise to butyrate. It is also likely that only the D(−) stereoisomer gave rise to butyrate. It is also likely that only the D(−) stereoisomer was incorporated into long-chain fatty acids, since the L(+) isomer did not appear to condense with malonyl-1,3-C^{14}-CoA.

No studies were undertaken to try to show the incorporation of labeled acetoacetyl-CoA into long-chain fatty acids, since the presence of thiolase would give rise to labeled acetyl-CoA which could then serve as a primer for the formation of labeled long-chain fatty acids in the presence of unlabeled malonyl-CoA.

The stimulation of the decarboxylation of malonyl-CoA by acyl-CoA compounds has been interpreted as indicating a concerted condensation-decarboxylation between the acyl-CoA compound and malonyl-CoA (Brady, 1960). Of the substituted acyl-CoA compounds examined, only acetoacetyl-CoA caused significant decarboxylation above control levels. This result may most likely also be attributed to the formation of acetyl-CoA due to thiolase. Since both labeled β-hydroxybutyryl-CoA and crotonyl-CoA served as precursors for long-chain fatty acids, it is proposed that reduction to the saturated derivative occurs in all cases prior to the condensation with malonyl-CoA.

The presence of enzyme-bound acetoacetyl-CoA formed from malonyl-CoA and acetyl-CoA by enzyme preparations from yeast (Lynen *et al.*, 1960) and bacteria (Alberts and Vagelos, 1961) have been reported, and recently the incorporation of enzyme-bound acetoacetate into long-chain fatty acids has been demonstrated (Goldman and Vagelos, 1962). It therefore seems likely that the reductive steps in fatty acid synthesis proceed from the acetoacetyl-*S*-enzyme generated by the condensation of acetyl-CoA and malonyl CoA through the enzyme-bound derivative of D(−)-β-hydroxybutyrate and crotonate, resulting in the saturated 4-carbon compound. The greater facility with which long-chain fatty acids are synthesized from acetyl-CoA and malonyl-CoA compared with these substituted compounds probably reflects the relative difficulty for these compounds to attain the enzyme site when supplied exogenously.

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The Occurrence of Cytochrome and Coenzyme Q in *Thiobacillus thiooxidans**

THOMAS M. COOK AND WAYNE W. UMBREIT

From the Department of Bacteriology, Rutgers—The State University, New Brunswick, New Jersey

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The existence of a cytochrome system and coenzyme Q_8 has been demonstrated in *Thiobacillus thiooxidans* and *Thiobacillus thioparus*. Cytochrome 550 of *T. thiooxidans* has an absorption spectrum resembling that of cytochrome *c*. The coenzyme Q_8 content of *T. thiooxidans* was estimated to be 4.3 μmoles (3.2 mg) per gram of dry weight.

Thiobacillus thiooxidans is a chemosynthetic autotrophic bacterium which derives its energy from the oxidation of elemental sulfur. This oxidation, and consequently the growth of the organism, is aerobic and proceeds only in the presence of molecular oxygen. Earlier studies on the effects of inhibitors (Vogler *et al.*, 1942) showed sulfur oxidation to be blocked by low concentrations of sodium cyanide, sodium azide, and carbon monoxide. All of these were known to be potent inhibitors of the cytochrome system. The aerobic nature of the process and its sensitivity to these inhibitors naturally led to the assumption that the cytochrome system was involved in sulfur oxidation by *T. thiooxidans*.

Subsequently, the presence of cytochromes was demonstrated in various species of the genus *Thiobacillus*. Klimek *et al.* (1956) showed the presence of a respiratory pigment resembling cytochrome *c*, which they termed cytochrome *s*, in *Thiobacillus thioparus*.

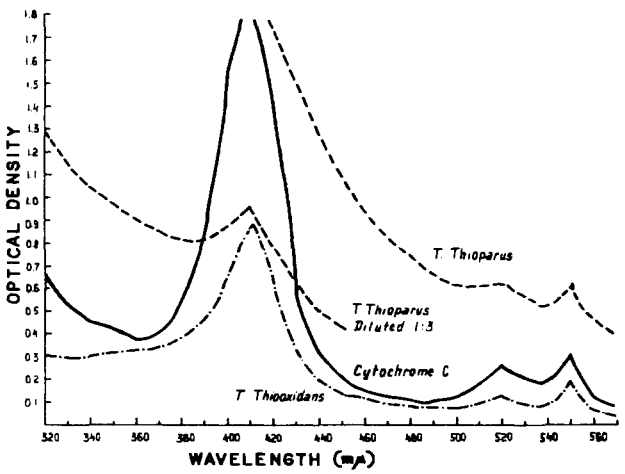

FIG. 1.—Absorption spectrum of reduced bacterial cytochromes compared with reduced mammalian cytochrome *c*. The *T. thioparus* preparation was a crude sonic extract containing 10 mg protein per ml. The *T. thiooxidans* material was a partially purified preparation (calcium phosphate gel-treated acid extract) containing 1.5 mg protein per ml.

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A cytochrome of the *c* type was also shown in *Thiobacillus denitrificans* by Aubert *et al.* (1958). In the thiosulfate-oxidizing organism, *Thiobacillus* X, Trudinger (1961a) found at least three separate cytochrome *c* type pigments.

On the other hand, Szczepkowski and Skarzynski (1952) were unable to demonstrate typical cytochrome absorption spectra in extracts of *T. thiooxidans*, although cyanide and azide were inhibitory to growth. As pointed out in a recent review (Peck, 1962), this finding casts some doubt on a role for cytochrome and oxidative phosphorylation in sulfur oxidation. We would therefore like to report the demonstration of a cytochrome *c* type pigment and a coenzyme Q compound in *T. thiooxidans*, as well as *T. thioparus*.

EXPERIMENTAL PROCEDURES

Cultures.—The strain of *T. thiooxidans* used in these studies was originally obtained from Dr. R. L. Starkey. A culture of *T. thioparus* was generously supplied by Dr. H. D. Peck, Jr., Oak Ridge National Laboratory, Tenn.

Growth Media.—*T. thiooxidans* was grown on a mineral medium described by Vogler and Umbreit (1941) plus elemental sulfur. Growth of *T. thioparus* was made on the thiosulfate medium of Starkey (1935).

Extraction of Cytochromes.—Washed cell suspensions, freed of sulfur, were prepared in *p*H 8 Tris buffer (0.05 M) and disrupted for 15 minutes in the Raytheon 10KC sonic oscillator. Unbroken cells and debris were removed by centrifugation at 10,000 × *g* for 15 minutes. Acid extracts of such sonicates were made by adjusting to *p*H 3 and centrifuging down the precipitated protein. After neutralization of the acid extract to *p*H 7.5, the cytochromes could be absorbed on calcium phosphate gel prepared by the method of Dixon and Webb (1958), washed with distilled water, and then eluted with *p*H 5 ammonium acetate (1.0 M). The absorption spectra of these fractions (adjusted to *p*H 7.5) were determined with and without reduction by sodium hydrosulfite with the Beckman DU spectrophotometer.

Cytochrome Oxidase Assay.—The cytochrome oxidase activity of the sonic extracts was determined in the colorimetric assay using the nadi reagent as described by Straus (1954). The development of color was followed with a Beckman DU spectrophotometer equipped with a standard Beckman recorder.