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# Metabolism and fine structure of Acetobacter suboxydans

George William Claus
*Iowa State University*

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METABOLISM AND FINE STRUCTURE

OF *ACETOBACTER SUBOXYDANS*

by

George William Claus

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Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

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# GENERAL INTRODUCTION

"It is apparent that the field of physiological bacteriology is wide, and that for an understanding of its phenomena we must draw upon a great variety of sources for our information" (Buchanan and Fulmer, 1928, p. 1).

The quantity and quality of these "sources" have grown almost exponentially in the past, and, for a while, the tendency was to create distinct disciplines of study. More recently, this growth has been coupled with greater interaction between these disciplines. The present day interlocking of morphology, physiology, and biochemistry is evident, and the results of this continuum have contributed much to our understanding of the biological system as a whole. At present one must take care, since too much generalization "...could in fact divert our appreciation of the hard problems of complex and specialized systems; just as the intensive study of specialized systems, when successful, can lead us to forget that they are embedded in diverse organisms and situations" (Mazia and Tyler, 1963, p. xi).

The present investigation with the bacterium *Acetobacter suboxydans* deals with two specialized problems; one of metabolism, and the other of structure. The results of these studies can be of interest in themselves, but, if any value is to be found, it will be only when related to the activities of the microorganism as a whole.

# PART I

# METABOLISM OF *ACETOBACTER SUBOXYDANS*

# INTRODUCTION

Acetobacter suboxydans (Kluyver and de Leeuw, 1924) has long been of industrial importance in vinegar fermentation and one- or two-step oxidations of polyols (Fulmer and Underkofler, 1947; Suhadolnik, 1953).

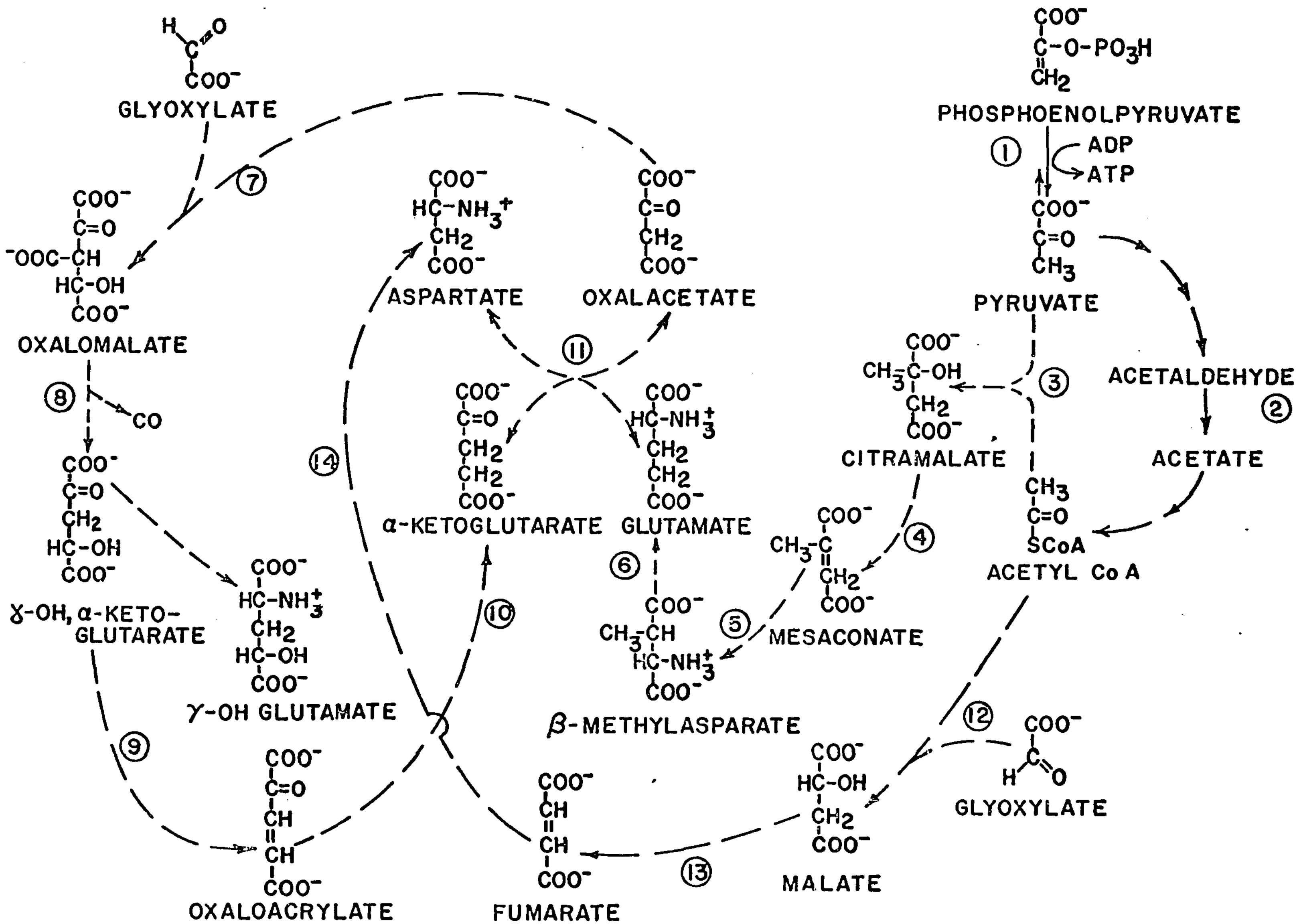
The metabolism of the acetic acid bacteria has been comprehensively reviewed by Rao (1955), Cheldelin (1961) and rainbow (1961). Hauge et al. (1955) have shown that cell-free extracts of A. suboxydans contain the enzymes of the pentose cycle, and Kitos et al. (1958) have presented radiorespirometric evidence which indicates that the pentose cycle is the only significant pathway for complete oxidation of glucose in this obligate aerobe.

Neither whole cells nor cell-free extracts of three strains of A. suboxydans (including ATCC 621) were able to oxidize acetate or intermediates of the tricarboxylic acid (TCA) cycle; this indicates that the TCA cycle is not operative in this organism (King and Cheldelin, 1952; Fewster, 1958). This concluded absence of the TCA cycle, unique among Acetobacter species, is supported by other manometric, chemical, and radiotracer studies (King and Cheldelin, 1954a; Kitos et al., 1957). Even though this bacterium can incorporate acetate and ethanol into cellular material (Rao and

Stokes, 1953; Rao, 1955; Kitos *et al.*, 1956), only aconitase and fumarase of the TCA cycle enzymes are reportedly present, and even these are present in much smaller concentrations than other species of *Acetobacter* (Rao, 1955). These unusual characteristics have prompted Rao (1955) and Cheldelin (1961) to question the mechanism of amino acid formation, such as aspartate and glutamate, which normally arise from the TCA cycle intermediates. This organism seems to require only serine, glutamate, histidine or proline for "healthy growth" when glycerol is used as a substrate (Cheldelin, 1961).

Cheldelin and his associates (Sekizawa *et al.*, 1962) have postulated two complex pathways for the formation of glutamate; the first is initiated with the condensation of glyoxylate and oxalacetate (Fig. 1, reactions 7 through 11) and the second with a reaction between pyruvate and acetyl coenzyme A (Fig. 1, reactions 3 through 6). The ability of cell-free extracts to form acetate by means of a yeast-type pyruvate decarboxylase and subsequent oxidation has been demonstrated (King and Cheldelin, 1954 a and b; King and Cheldelin, 1956); acetyl Co A is then thought to be formed by an ATP-dependent mechanism similar to acetate thiokinase (Kitos *et al.*, 1957) (Fig. 1, reaction sequence 2). Very recently, Cheldelin *et al.* (1963) have stated that the intermediates formed upon condensation of pyruvate and acetyl Co

Fig. 1. Postulated pathways for the formation of glutamate and aspartate in undialyzed cell-free extracts of *A. suboxydans*. Reactions 7 through 11 and 3 through 6 postulated by Sekizawa *et al.* (King and Cheldelin's group), Biochem. Biophys. Res. Comm., *9*, 361, 1962. Compounds indicated as formed by reactions 3 through 6 were reportedly identified and reactions 12 through 14 postulated by Cheldelin *et al.*, Fed. Proceedings, *22*, 652, 1963



GLYOXYLATE
PHOSPHOENOLPYRUVATE
ADP
ATP
OXALOMALATE
CO
ASPARTATE
OXALACETATE
PYRUVATE
ACETALDEHYDE
ACETATE
CITRAMALATE
α-KETOGLUTARATE
GLUTAMATE
ACETYL CoA
MESACONATE
γ-OH, α-KETO-GLUTARATE
γ-OH GLUTAMATE
β-METHYLASPARTATE
GLYOXYLATE
MALATE
OXALOACRYLATE
FUMARATE

A, leading to the synthesis of glutamate, have been isolated and identified; they have also preliminarily reported that isotopically-labeled acetate and glyoxylate condense to form malate, then fumarate, and finally aspartate in the presence of a nitrogen donor (Fig. 1, reactions 12 through 14).

The present study was initiated to determine whether an enzymatic mechanism for the carboxylation of phosphoenolpyruvate or pyruvate existed in cell-free extracts of *Acetobacter suboxydans*, and, if such a mechanism(s) did exist, which of the five known mechanisms (Wood and Stjernholm, 1962) were responsible. If one or more of the phosphoenolpyruvate or pyruvate carboxylating enzymes did catalyze the formation of oxalacetate, it was originally thought that such a mechanism might be important in the subsequent biosynthesis of amino acids.[1]

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[1]A preliminary report of the author's $CO_2$ assimilation studies have been previously presented: T. Mÿoda, G. W. Claus and C. H. Werkman, Certain Aspects of the Fixation of $CO_2$ by *Mycobacterium phlei* and *Acetobacter suboxydans*, Unpublished paper presented before The Society of American Bacteriologist's North Central Branch Meeting, Milwaukee, Wisconsin, October, 1959.

# MATERIALS AND METHODS

## Organism and Medium

The bacterium used in this investigation was Acetobacter suboxydans obtained from the American Type Culture Collection as number 621 and maintained on Acetobacter agar (Weiss, 1957) for over eight years in this laboratory. Cells were grown on a medium described by King and Cheldelin (1954a) containing 5 per cent (w/v) glycerol, 1 per cent yeast extract, 0.5 per cent $KH_2PO_4$ and adjusted to pH 6.0. Eighty parts per million of General Electric-60 antifoam were substituted for the Dow Corning AF used by King and Cheldelin.

Subcultures were prepared by inoculating 100 ml. of this medium placed in 1,000 ml. Erlenmeyer flasks and subjecting them to vigorous agitation (Burrell Wrist-Action Shaker, Model DD, Pittsburgh) for 24 hours at 30$^{\circ}$C. This subculture served as an inoculum for 10 liters of the same medium contained within a Model FS614, New Brunswick Fermentor (New Brunswick, N. J.). Since previous results (Claus, unpublished; Razumouskya, 1955) indicated that the rate of growth of this obligate aerobe was greatly influenced by the rate of aeration, care was exercised in standardizing the rate of oxygen adsorption to 84 µmoles per liter per hour, thus somewhat stabilizing the environmental conditions for growth and the subsequent cell yield. The cells were harvested at 40,000 r.p.m. with a refrigerated, Sharpels Super-Centrifuge (Type TIF; Philadelphia, Pa.) at the end of the logarithmic phase of growth. The cells were then suspended in distilled water and centrifuged three times prior to being stored at -20°C.

## Preparation and Purification of Cell-free Extracts

### Preparation

Cells were resuspended in 0.1 M tris (hydroxymethyl) aminomethane (Tris) buffer, pH 7.4, at a ratio of 2:1 (w/v). Fifty mililiter aliquots were treated in the 10 Kc Raytheon Sonic Oscillator (Model DF101; Waltham, Mass.) for 3 minutes at a current flow of ca. 1.3 amps. Unbroken cells and cellular debris were removed by centrifugation at 24,000 x g for 20 minutes in a Model PR-1 International Refrigerated Centrifuge (Boston, Mass.).

The cell-free extract was 1) used without further treatment, 2) dialyzed from 8 to 16 hours at 10°C. against ca. 200 volumes of 0.001 M Tris-HCl, pH 7.4, buffer, or 3) dialyzed from 1 to 4 hours in an Electroconvection Dialysis Apparatus (Model EC-25, Swathmore, Pa.) against ca. 150 volumes of the same buffer. Treatment of extracts with ion exchange resin for removal of nucleotides was carried out according to Stadtman et al. (1951) on samples that were electrodialyzed for 1.5 hours. After resin treatment, the samples were electrodialyzed for an additional 1.5 hours.

Partial purification

Undialyzed cell-free extracts were adjusted to pH 6.5 and a temperature of 0°C. Cold protamine sulfate (20 mg./ml., pH 5.0) was slowly added to the cell-free extract until a concentration of ca. 0.12 mg. protamine sulfate per mg. of protein was obtained[1]. This treatment usually decreased the 260/280 mμ absorption ratio from 1.6 to 1.3 and ca. 60 per cent of the protein was precipitated. The precipitate was removed by centrifugation for 15 minutes at ca. 20,000 x g. Ammonium sulfate solutions previously saturated at 100°C. were filtered and cooled to 0°C. for further protein precipitation. An appropriate volume of saturated ammonium sulfate (pH 4.0) was slowly added to the buffered extract so that the desired level of saturation was obtained; the extract was immediately adjusted to ca. pH 7 and left for 10 minutes at this

[1]This concentration was based on the original concentration of protamine sulfate at room temperature (20 mg./ml.); however, this solution became saturated when cooled to 0°C. Thus, the actual ratio of protamine sulfate to protein used was somewhat less than indicated.

degree of saturation. The ammonium sulfate precipitate was removed and the supernate brought to the next degree of saturation in a similar manner. Ammonium sulfate precipitates were dissolved in 0.1 Tris, pH 7.5, buffer and dialyzed in a Model B Oxford Multiple Dialyzer (San Francisco, Calif.) against 0.001 M solutions of the same buffer for 8 to 16 hours at 10$^{o}$C.

## Chemicals

$NaHC^{14}O_3$ or $KHC^{14}O_3$ was prepared from $BaC^{14}O_3$ obtained from Oak Ridge National Laboratory according to the procedure described by Hug (1956).

All other chemicals were commercial preparations: malic dehydrogenase was obtained from C. F. Boehringer & Soehne; pyridoxal phosphate, the sodium salt of nicotinamide-adenine dinucleotide phosphate (reduced), and the tricyclohexylamine salt of phosphoenolpyruvic acid from the California Foundation for Biochemical Research; 2,4-dinitrophenylhydrazine and L-glutamic acid from Eastman Kodak Company; L-aspartic decarboxylase from General Biochemicals; oxalacetic acid from Mann Research Laboratories; aspartic acid, α-ketoglutaric acid and reduced glutathione from Nutritional Biochemicals Corporation; disodium nicotinamide adenine dinucleotide (reduced), adenosine di- and triphosphate, cytidine di- and triphosphate, guanosine di- and triphosphate, inosine di- and triphosphate, and uridine di- and triphosphate from Pabst Laboratories; and sodium pyruvate was obtained from Schwarz Laboratories.

## Analytical Procedures

### Conditions for detection of $C^{14}O_2$ assimilation

The $CO_2$ fixation reactions were carried out in Warburg flasks under an atmosphere of 100 per cent nitrogen. A solution of Na- or $KHC^{14}O_3$ was added to one side arm and cell-free extract was added to the other. If oxalacetate was used as a substrate, it was placed in a sidearm to reduce nonenzymatic decarboxylation due to the presence of $Mn^{++}$.

The rate of spontaneous decarboxylation at 30°C. under normal atmospheric conditions was twice that under 100 per cent nitrogen when simulated assay conditions were used. The presence of 5 μmoles of $MnCl_2$ stimulated 4 times more non-enzymatic decarboxylation than solutions were $Mn^{++}$ was absent. Since a divalent cation was essential for $C^{14}O_2$ assimilation, this could not be avoided; however, the presence of a nitrogen atmosphere did increase the detectable quantities of organically-fixed $C^{14}$ in the assay system.

Temperature equilibration and a change of atmosphere were obtained by flushing the flasks with oxygen-free nitrogen for 15 minutes at 31°C. The reaction was started by tipping the contents of the side arms into the main chamber. After incubation for 30 minutes, the reactions were stopped by the addition of acid in one of several ways.

Preparations of radioactive samples for counting

Aliquots of acidified reaction mixtures Early in this investigation, the reactions were stopped by the addition of trichloracetic acid to a final concentration of 5 per cent. The precipitated protein was removed by centrifugation and an aliquot of the reaction mixture was pipetted onto a ground-glass-planchet with an Adams suction apparatus. Aliquots of the deproteinized mixtures were applied to the planchets, according to the procedure described by Baugh (1961), and counted immediately upon drying.

Later in this study, it was determined that when 3 ml. solutions containing 5 µmoles each of oxalacetate and $MnCl_2$ were incubated at 31°C. for 30 minutes then acidified, 98 per cent nonenzymatic decarboxylation to pyruvate occurred. Other similar observations have been reported (Richter, 1962). Since the $Mn^{++}$ containing reaction mixtures were routinely acidified prior to determination of the amount of $C^{14}O_2$ fixed, it can be seen that radioactive examination of such aliquots probably detected only a small portion of the formed radioactive oxalacetate.

2,4-Dinitrophenylhydrazone of oxalacetate   Most aldehydes and ketones when treated with 2,4-dinitrophenylhydrazine yield dinitrophenylhydrazones that are very insoluble (Shriner *et al*., 1956). Prior to determining the extreme lability of oxalacetate, aliquots of acidified reaction mixtures were routinely treated with 2,4-dinitrophenylhydrazine, according to the method of Suzuki (1958), to determine the quantity of radioactive oxalacetate formed. This method was time consuming, involved many variable factors (e.g. self adsorption correction of the packed crystals), and still involved prior acidification of the reaction mixtures.

The method of Maruyama and Lane (1962b) was later adopted, especially where fractionated extracts of relatively low protein concentrations were used. The reactions were stopped by directly adding 2,4-dinitrophenylhydrazine saturated in 2 N HCL. After 30 minutes of incubation at 30°C., the radioactive dinitrophenylhydrazones formed were extracted with an equal volume of ethyl acetate to which carrier (nonradioactive) oxalacetate (at a concentration of 1 mg. per ml.) had been added. A 1 ml. aliquot of the ethylacetate phase was dried on a ringed nickel-plated-steel planchet then counted under a thin-window Geiger-Müeller counter.

Assay of radioactivity

End-window counting Radioactivity was measured with a lead-shielded, mica end-window Geiger-Müeller tube (Type TGC2, Tracerlab, Waltham, Mass.). The window thickness was 1.4 mg. per $cm.^2$. A Nuclear Instrument and Chemical Corp. scaling unit (Model 163) was used. In most instances, the method of counting was selected to produce a standard deviation within 5 per cent of the net count. Correction for self adsorption was necessary when 2,4-dinitrophenylhydrazones were prepared according to Suzuki (1958); however, such corrections were not necessary when deproteinized aliquots or ethylacetate-extracted dinitrophenylhydrazones (Maruyama and Lane, 1962b) were used.

Radioactive analysis The location of radioactive compounds, separated by paper chromatography, was determined by radioautography (Fink and Fink, 1948). Exposure time was estimated by counting the most active region after chromatographic development. Radioactivity of 200 counts per minute in a 4 $cm.^2$ area insured a well defined darkening of the film after 10 days of exposure. The exposed film was developed with D-16 Eastman Kodak Developer and cleared with a solution of sodium thiosulfate (294 grams per liter).

Chromatography

The 2,4-dinitrophenylhydrazones were spotted on Whatman No. 3 paper and ascendingly developed in one gallon wide-mouthed jars. The solvent used for chromatography was either n-Butanol, ethanol, 0.5 N $NH_4OH$ (7:1:2) (El Hawary and Thompson, 1953) or n-Butanol saturated with 3 per cent (w/w) $NH_4OH$ (Suzuki, 1958).

Separation of amino acids was accomplished on Whatman No. 3 paper with descending solvent flow in a 12"x12"x24" Pyrex Chromatography jar containing a CALAB Pyrex glass rack (Calif. Lab. Equipment Co., Berkeley, Calif). The solvent used was pyridine, acetic acid, water (50:35:15) (Decker and Riffart, 1950). The separated amino acids were detected by spraying with a solution of 0.25 per cent ninhydrin in acetone.

Spectrophotometric analysis of oxalacetate

Nonenzymatic decarboxylation of oxalacetate was determined in 3 ml. solutions containing 100 μmoles Tris, pH 7.5, buffer, 0.5 μmoles oxalacetate, 0.15 international enzyme units (Cooper *et al.*, 1958) of malate dehydrogenase, and 0.8 μ moles reduced nicotinamide adenine dinucleotide (NADH). These reaction mixtures were subjected to changes in pH, inorganic ions, and atmosphere prior to the addition of NADH and malate dehydrogenase to initiate the reactions. Change in absorption at 340 mμ was determined with a Beckman Model DB recording spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.).

The quantity of oxalacetate formed due to the transamination of aspartate with α-ketoglutarate was determined in a manner similar to that described above (Bergmeyer and Bernt, 1963). Unfractionated cell-free extracts contained other enzymes and substrates which would allow the NADH to be oxidized in the absence of added malate dehydrogenase or substrates for the transaminase. However, an adsorption increase due to reduction of the formed oxalacetate could easily be detected if the following procedure were used: a) 0.8 μmoles of NADH were added to the reaction mixture containing cell-free extract, pyridoxal phosphate and Tris buffer, and the oxidation of NADH was allowed to go the completion; b) α-ketoglutarate, malate dehydrogenase and an additional 0.8 μmoles of NADH were then added to the reaction mixture; and c) the transamination reaction was initiated by adding aspartate and following the oxidation of NADH upon formation of oxalacetate.

# EXPERIMENTAL

## Fixation of Carbon Dioxide by Cell-free Extracts

Initial experiments were carried out with a variety of microorganisms in order to test the ubiquity of enzymic phosphoenolpyruvate carboxylation. It was surprising to find that the specific activity of A. suboxydans extracts was equal to or exceeded that of other heterotrophs known to use the tricarboxylic acid cycle. Since all of the heterotrophic bacteria studied to date primarily exhibit a nucleotide dependent mechanism for phosphoenolpyruvate carboxylation (Kurahashi et al., 1957; Baugh et al., 1959), early assay conditions included inosine diphosphate in an attempt to stimulate carboxylation. A typical experiment demonstrating the stimulation of $C^{14}O_2$ fixation in the presence of phosphoenolpyruvate is illustrated in Table 1.

## Identification of Carboxylation Product

The most likely product formed by the carboxylation of phosphoenolpyruvate would be oxalacetate or malate. Early in this investigation, it was noticed that aliquots of deproteinized reaction mixtures lost as much as 20 per cent of the initial radioactivity after remaining at room temperature.

Table 1. Phosphenolpyruvate stimulated $C^{14}O_2$ fixation by cell-free extracts[a]

| Number | Deletions | Specific activity fixed (counts/min./mg. protein) |
|---|---|---|
| 1 | none | 50,000 |
| 2 | PEP | 720 |
| 3 | cell-free extract | 0 |

[a]The complete system contained; undialyzed cell-free extract (2.5 mg.), 3 μmoles phosphoenolpyruvate, 3 μmoles inosine diphosphate, 10 μmoles $MnCl_2$, 5 μmoles $NaHC^{14}O_2$ ($1.8 \times 10^5$ counts/min.), 100 μmoles tris (hydroxymethyl) aminomethane buffer pH 7.4, adjusted to a total volume of 2.0 ml. with distilled water. The reaction was incubated at 31°C. under a nitrogen atmosphere for 30 min. and stopped by the addition of 5 per cent trichloroacetic acid. A deproteinized aliquot was dried on a glass planchet and counted under an end-window Geiger Mueller tube.

Since it is known that oxalacetate can easily lose its carboxyl group attached to the β-carbon forming pyruvate and $CO_2$ (Richter, 1962), and since enzymatic carboxylation of phosphoenolpyruvate occurs at the β-carbon (Wood and Stjernholm, 1962), thèse data suggest that oxalacetate was being formed in the above experiments.

Malate and oxalacetate can be distinguished from one another as aldehydes form very insoluble and stable dinitrophenylhydrazones when treated with dinitrophenylhydrazine (Shriner *et al.*, 1956).

Aliquots of deproteinized reaction mixtures were added to carrier oxalacetate and placed with a solution of 2,4-dinitrophenylhydrazine saturated in 2 N HCL. The washed dinitrophenylhydrazone crystals were found to be radioactive and corresponded to known oxalacetate-2,4-dinitrophenylhydrazone when subjected to chromatography and radioautography. The results of a typical analysis are shown in Table 2.

## Substrate Specificity

In well dialyzed cell-free extracts, oxalacetate might be formed from phosphoenolpyruvate (PEP) and $CO_2$ in several ways (Wood and Stjernholm, 1962):

a) by the combined action of pyruvate kinase, malic enzyme, and malate dehydrogenase;

$$\text{PEP} \xrightarrow[\text{pyruvate kinase}]{\text{ADP} \quad \text{ATP}} \text{pyruvate} \xleftrightarrow[\text{malic enzyme}]{CO_2 \quad \text{NADPH} \quad \text{NADP}} \text{malate} \xleftrightarrow{\text{NADH} \quad \text{NAD}} \text{oxalacetate} \qquad (15)$$

b) or by the presence of pyruvate kinase and pyruvate carboxylase;

$$\text{PEP} \xrightarrow[\text{pryuvate kinase}]{\text{ADP} \quad \text{ATP}} \text{pyruvate} \xleftrightarrow[\text{pyruvate carboxylase}]{CO_2 \quad \text{ATP} \quad \text{acetyl Co A} \quad \text{ADP+Pi}} \text{oxalacetate} \qquad (16)$$

Table 2. $C^{14}$-Phenylhydrazone identification

| Solvent[a] | Conc. | $R_f$ of knowns[b] | | $R_f$ of unknown |
|---|---|---|---|---|
| | | Oxalacetate | Pyruvate | $C^{14}$-Phenylhydrazone[c] |
| butanol: ethanol: $NH_4OH$ (7:1:2) | primary spot | .23 | .37 | .23 |
| | secondary spots | .49, .65 | .60 | |
| n-butanol saturated in 3 per cent $NH_4OH$ | primary spot | .08 | .28 | .09 |
| | secondary spots | .32, .50 | .56 | |

[a]Ascending chromatography was employed at room temperature.

[b]Oxalacetic acid (Mann Research Laboratories) and Na-Pyruvate (Schwarz Laboratories) were used.

[c]The developed chromatogram was exposed to Kodak ''No-Screen'' x-ray film prior to development in Kodak D-16 developer.

c) or by direct carboxylation of PEP with PEP carboxylase (17), PEP-carboxykinase (18) or PEP-carboxytransphosphorylase (19);

$$\text{PEP} \xrightarrow{CO_2} \text{Oxalacetate} + \text{Pi} \qquad (17)$$

$$\text{PEP} + \text{IDP} \xleftrightarrow{CO_2} \text{Oxalacetate} + \text{ITP} \qquad (18)$$

$$\text{PEP} + \text{Pi} \xleftrightarrow{CO_2} \text{Oxalacetate} + \text{PPi} \qquad (19)$$

In cases a) and b) above (reactions 15 and 16) the substance being directly carboxylated is pyruvate; in these situations, one would expect pyruvate to stimulate the fixation as well as, if not better than, phosphoenolpyruvate. If oxalacetate were being formed by carboxylation of phosphoenolpyruvate, as in c) (reactions 17 through 19), one would expect pyruvate to react only to the extent that pyruvate kinase could be reversed.

The effect of substituting pyruvate for phosphoenolpyruvate as a substrate for the fixation of $C^{14}O_2$ is shown in Table 3. Phosphoenolpyruvate is <u>ca</u>. 20 times more reactive than pyruvate. The inhibitory effect of adenosine diphosphate on the phosphoenolpyruvate carboxylation reaction is consistent with the slight stimulatory effect of adenosine triphosphate on fixation observed in the presence of pyru-

Table 3. Substrate specificity for cell-free extract carboxylation[a]

| Number | Additions[b] | Activity fixed (cpm/mg. protein) |
|---|---|---|
| 1 | PEP | 5,850 |
| 2 | PEP, ADP | 3,223 |
| 3 | pyruvate | 177 |
| 4 | pyruvate, ATP | 306 |
| 5 | pyruvate, NADPH | 32 |
| 6 | none | 5 |

[a]The complete system contained; 2.7 mg. (protein) cell-free extract treated for removal of nucleotides (Stadtman *et al*., 1951) and dialyzed (see Materials and Methods), 5 μmoles $MnCl_2$, 5 μmoles $KHC^{14}O_3$ ($1.8 \times 10^5$ counts/minute), 100 μmoles tris (hydroxmethyl) aminomethane buffer pH 7.4, 5 μmoles reduced glutathione, and adjusted to a total volume of 2.0 ml. with distilled water. The reaction conditions and radioactive assay procedures are the same as in Table 1.

[b]Additions were; 3 μmoles each of phosphoenolpyruvate (PEP), adenosine diphosphate (ADP), adenosine triphosphate (ATP), pyruvate, and 1.5 μmoles of reduced nicotinamide adenine dinucleotide (NADPH).

vate. The presence of pyruvate kinase in extracts of *A. suboxydans* has been infered (King and Cheldelin, 1954a and b; King and Cheldelin, 1956); and demonstrated by Claus (unpublished results). Since both pyruvate kinase and the known phosphoenolpyruvate carboxylating enzymes (reactions

17 through 19) require the same substrate, one would expect them to compete for this substrate and show an inhibition of $C^{14}O_2$ fixation in the presence of ADP, as shown in Table 3. The slight increase in activity indicated when pyruvate and ATP are substituted for phosphoenolpyruvate might be explained upon the assumption of slight reversibility of the pyruvic kinase reaction or slight activity of a pyruvate carboxylase-like reaction. The absence of an active malic enzyme is shown (Table 3) by the failure of NADPH to stimulate fixation of $C^{14}O_2$.

Thus, under these conditions, phosphoenolpyruvate is the primary substrate for the carboxylation reaction in _A. suboxydans_.

Formation of $C^{14}$-Aspartate from Phosphoenolpyruvate and $C^{14}O_2$

The culture of _A. suboxydans_ ATCC 621 used in this study has been maintained for over 8 years in this laboratory. It seemed advisable to compare this working culture with a newly acquired culture of ATCC 621 with regard to ability to form radioactive aspartate from PEP and radioactive $CO_2$.

Presence of glutamate-oxalacetate transaminase

Since _A. suboxydans_ is reported to have only aconitase and fumarase of the tricarboxylic acid cycle enzymes (Rao, 1955), it was postulated that the oxalacetate formed by the enzymic phosphoenolpyruvate carboxylation might serve as a substrate for transamination with glutamate to form aspartate in the presence of pyridoxal phosphate. Such a reaction has been indirectly implicated by the work of Cheldelin and associates (Sekizawa et al., 1962; Cheldelin et al., 1963).

The presence of such a transaminase was investigated by adding aspartate, α-ketoglutarate, pyridoxal phosphate, commercially prepared malate dehydrogenase, and reduced nicotinamide adenine dinucleotide (NADH) to the cell-free extract. If oxalacetate was formed by transamination, it would be enzymatically reduced to malate as shown by reaction 20.

$$\text{oxalacetate} \xrightarrow[\text{malate dehydrogenase}]{\text{NADH} \quad \text{NAD}} \text{malate} \qquad (20)$$

The formation of oxalacetate by such a transaminase was investigated by measuring the rate of decrease in absorption due to the presence of cell-free extracts as shown in Table 4. If the extract or aspartate and α-ketoglutarate are not added to the assay system, oxalacetate formation does not occur. The oxidation of NADH in the presence of extract and only α-ketoglutarate may be due to the presence of glu-

Table 4. Presence of glutamate-oxalacetate transaminase in cell-free extracts

| Extract | Number | Deletions[a] | NADH oxidized[b] (μmoles/3 min.) |
|---|---|---|---|
| 1) Laboratory culture of ATCC 621 | 1 | none | 0.60 |
| | 2 | aspartate, α-ketoglutarate | 0 |
| | 3 | aspartate | 0.20 |
| | 4 | extract | 0 |
| 2) Newly acquired ATCC 621 | 5 | none | 0.62 |
| | 6 | aspartate, α-ketoglutarate | 0 |
| | 7 | aspartate | 0.18 |

[a]The complete system contained 10 μmoles Na-aspartate 4 μmoles α-ketoglutarate, 0.5 μmoles pyridoxal phosphate, 0.25 enzyme units (Cooper *et al.*, 1958) malate dehydrogenase, 2.1 μmoles nicotinamide adenine dinucleotide (NADH), 28.4 and 30.4 mg. of 10 hour-dialyzed extracts (1 and 2 respectively), and distilled water to 3.0 ml. 1.6 μmoles NADH were added to the system to reduce the substrates for other dehydrogenases in the extract prior to initiating the transamination reaction.

[b]Oxidation of NADH was followed at 340 mμ on a Model DB Beckman recording spectrophotometer.

tamate dehydrogenase. The increased NADH oxidation in the presence of aspartate and α-ketoglutarate indicates the formation of oxalacetate by transamination in cell-free extracts of *A. suboxydans*.

Both the laboratory culture of ATCC 621 and the recently acquired culture of ATCC 621 demonstrated glutamate-oxalacetate transaminase activity; however, the newly acquired culture contained about two times more units of activity (International Units; see Cooper _et al_., 1958) than the culture being used in the laboratory.

## Formation and identification of $C^{14}$-aspartate

Since it had been shown that cell-free extracts of _A. suboxydans_ could catalyze the carboxylation of phosphoenolpyruvate and the transamination of aspartate with α-ketoglutarate, it was of interest to determine whether this bacterium would form aspartate from phosphoenolpyruvate, $CO_2$ and an amino acid.

The assay conditions were the same as those required to show phosphoenolpyruvate carboxylation except that, in the complete system, 5 μmoles of Na-glutamate, 1.5 μmoles of pyridoxal phosphate, and an appropriate volume of undialyzed cell-free extract were added to a total volume of 2.7 ml. The reactions were stopped after 45 minutes by the addition of 5 per cent trichloroacetic acid, and deproteinized aliquots were assayed for radioactivity and chromatographed.

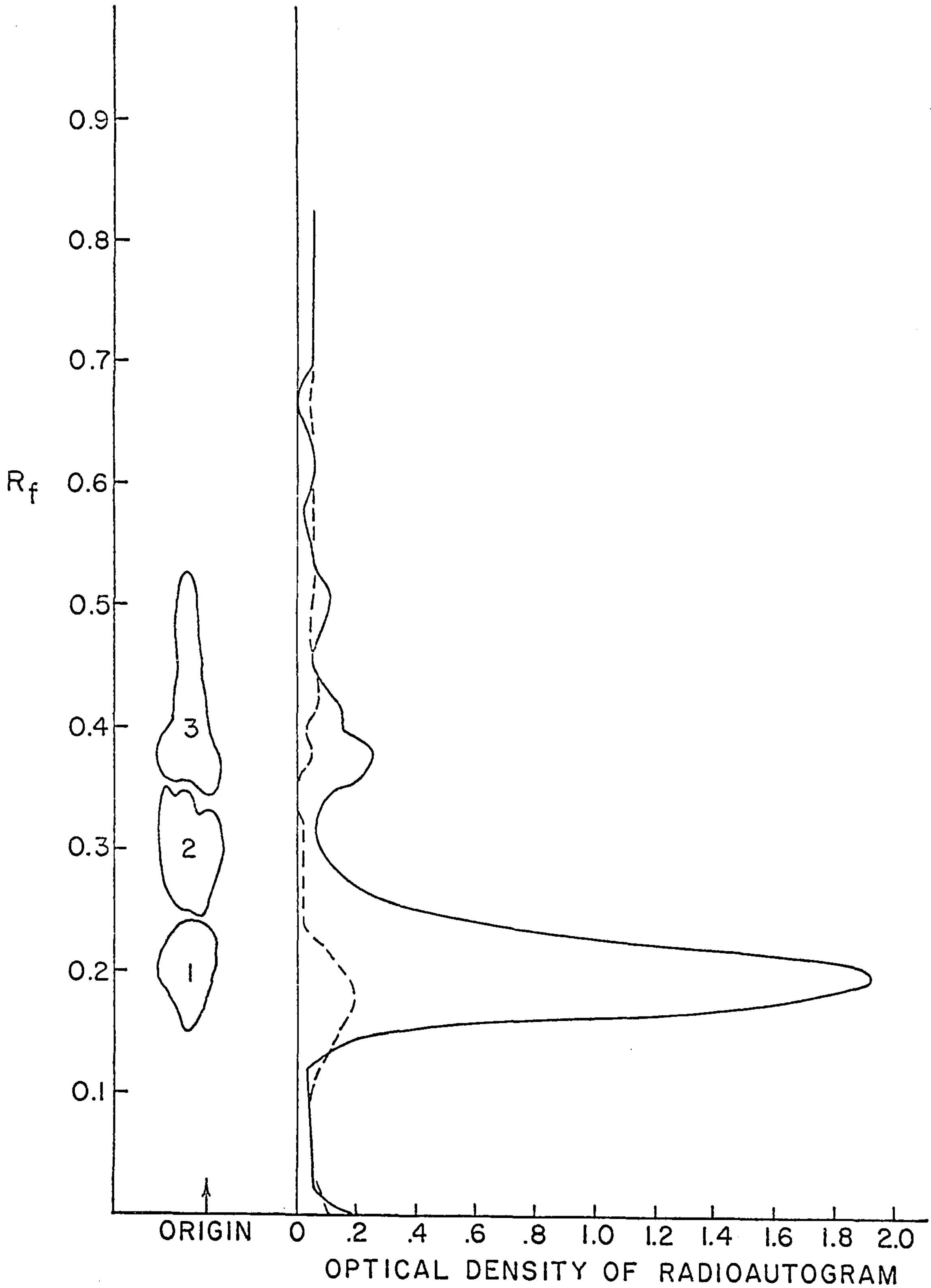
In undialyzed cell-free extracts of the laboratory culture of ATCC 621 and that recently acquired from the

American Type Culture Collection, the total amount of $C^{14}O_2$ assimilation was reduced from 50 to 90 per cent in the absence of phosphoenolpyruvate.

Aliquots of the reaction mixtures were cochromatographed with known aspartate. The solvent-developed chromatograms were sprayed with ninhydrin then placed on x-ray film for sufficient exposure. The exposed-developed x-ray was cut into strips and the intensity of exposure measured with a Photovolt densitometer (Model 425; G. T. Waker and Co., Minneapolis, Minn.). Figure 2 compares the results obtained from reaction mixtures with and without phosphoenolpyruvate. Both the ATCC and the laboratory culture extracts show large increases in radioactive aspartate formed in the presence of added phosphoenolpyruvate.

Since undialyzed cell-free extracts were used, the small amount of endogenous activity in the absence of phosphoenolpyruvate is understandable. More radioactive aspartate was demonstrated in reaction mixtures not containing added glutamate than those where glutamate was added; however, a relatively high concentration of glutamate existed in the undialyzed extracts, and the glutamate added may have been in excess of the optimum concentration. When well dialyzed extracts were used, radioactive aspartate, formed in the absence of phosphoenolpyruvate, was eliminated.

Fig. 2. Comparison of radioactivity fixed in aspartate (spot 1) when phosphoenolpyruvate is present (solid line) or absent (broken line) in reaction mixtures containing undialyzed cell-free extracts (laboratory culture of ATCC 621). Spots 1 and 2 cochromatographed with known aspartate and glutamate respectively. Spot 3 (unidentified) formed a yellow color upon reaction with ninhydrin. The chromatogram was developed at 25°C. with pyridine, acetic acid, and water (50:35:15); the solvent front descended 40 cm. from the origin. Kodak No-screen x-ray film was exposed for 70 days to assure adequate detection of all radioactivity.



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OPTICAL DENSITY OF RADIOAUTOGRAM

## Characteristics of Enzymatic Phosphoenol-pyruvate Carboxylation

The results show that pyruvate and NADPH or pyruvate and ATP will not replace phosphoenolpyruvate as a substrate for the assimilation of $CO_2$ into oxalacetate. Of the three $CO_2$ assimilation reactions known to be catalyzed by phosphoenolpyruvate (reactions 17 through 19), two of them are readily reversible and require either a nucleotide or inorganic phosphate as the enol-phosphate acceptor (reactions 18 and 19); the third reaction is essentially irreversible and requires no acceptor for the enol-phosphate (reaction 17). A study of the possible reaction requirements and reversibility was initiated to determine the characteristics of the enzyme(s) responsible for such carboxylation in *A. suboxydans*.

### Requirement for nucleotide, cation, and pH

Early in this study, it was noted that the addition of nucleotides somewhat inhibited the assimilation of $C^{14}O_2$. The extent of this inhibition depended upon the type of diphosphonucleotide introduced. To eliminate the possibility that the optimum nucleotide concentration was being exceeded, cell-free extracts were dialyzed by electroconvection, treated with ion-exchange resin according to Stadtman *et al.* (1951), and dialyzed by electroconvection a second time.

Table 5. Effect of nucleotides on carboxylation of phosphoenolpyruvate[a]

| Reaction | Additions[b] | Activity fixed (cpm/mg. protein) |
|---|---|---|
| 1 | PEP | 2,034 |
| 2 | PEP, ADP | 953 |
| 3 | PEP, IDP | 714 |
| 4 | PEP, GDP | 728 |
| 5 | PEP, CDP | 1,584 |
| 6 | PEP, UDP[c] | 1,800 |
| 7 | none | 0 |

[a]The complete system was the same as in Table 3. 2.7 mg. (protein) of the same cell-free extract treated for removal of nucleotides was used.

[b]Additions were; 3 µmoles each of phosphoenolpyruvate (PEP), adenosine diphosphate (ADP), inosine diphosphate (IDP), guanosine diphosphate (GDP), cytodine diphosphate (CDP), and uridine diphosphate (UDP).

[c]In many experiments, UDP had even less or no inhibitory effect on $C^{14}O_2$ assimilation.

Table 5 shows the effect of various nucleotides upon $C^{14}O_2$ assimilation catalyzed by these extracts. It is interesting to note that purine nucleotides have a greater inhibitory effect than the pryimidine nucleotides. The fact that $C^{14}O_2$ assimilation can take place without addition of diphosphonucleotides strongly suggests that a nucleotide independent enzyme is active. The inhibition of $CO_2$ assimilation in the presence of nucleotides and the greater inhibition observed with purine nucleotides suggests that pyruvate kinase may be competing with the carboxylating enzyme(s) for phosphoenolpyruvate.

Experiments with well-dialyzed cell-free extracts indicate that $Mn^{++}$ stimulates 4 times more activity than $Mg^{++}$. The presence of ethylendiaminetetraacetate (EDTA) to the assay system completely inhibits $HC^{14}O_2^-$ fixation; whereas, the addition of excess $MnCl_2$ to EDTA-containing reaction mixtures completely restores the original activity. The effect of pH on oxalacetate formation (dinitrophenylhydrazone isolated) has been briefly studied. In the absence of a diphosphonucleotide, phosphoenolpyruvate carboxylation is 4 to 5 times greater at pH 7.4 than at 7.0.

Search for a reversible carboxylating enzyme

If *A. suboxydans* extracts contain a reversible enzymatic mechanism for phosphoenolpyruvate carboxylation (as reactions 18 and 19), one would expect release of the nonradioactive $CO_2$ and the subsequent incorporation of radioactive $C^{14}O_2$ when oxalacetate and $HC^{14}O_3$- are used as substrates (Krampitz *et al.*, 1943). The presence of such an "exchange reaction" was checked repeatedly in various extracts made from 5 different cell harvests.

Slight indications of activity fixed by such an exchange reaction occurred in only 60 per cent of the experiments, from which the following generalizations have been made: 1) in undialyzed extracts, a triphosphonucleotide appears necessary and adenosine triphosphate stimulates from 2 to 4 times more activity than either guanosine or inosine triphosphate, 2) $MnCl_2$ stimulates ca. 3 times more $C^{14}O_2$ to be fixed than $MgCl_2$, 3) the amount of $C^{14}O_2$ fixed is ca. 30 per cent greater at pH 7.0 than at pH 7.4, and 4) when found, the amount of activity fixed using oxalacetate and a triphosphonucleotide is only from 2 to 6 per cent of the activity fixed using phosphoenolpyruvate and no diphosphonucleotide.

It was noted that pyruvate and ATP will replace ca. 5 per cent of the activity produced with phosphoenolpyruvate and no nucleotide (Table 3), and oxalacetate and ATP will replace ca. 4 per cent of this activity. It was also noted, in subsequent studies, that a 10 per cent loss of oxalacetate occurred within 30 minutes when 3 ml. solutions containing 5 µmoles of oxalacetate were buffered at pH 7.5 under an atmosphere of nitrogen. Solutions of oxalacetate are easily β-decarboxylated to pyruvate and $CO_2$ (see review by Richter, 1962); therefore, it seems possible that this small amount of $C^{14}O_2$ fixation can be accounted for by the spontaneous decarboxylation of oxalacetate and the slight reversibility of the pyruvate kinase reaction, thereby forming very small quantities of phosphoenolpyruvate which can in turn be carboxylated.

Purification

In order to clearly demonstrate the enzyme(s) responsible for this nucleotide independent, irreversible carboxylation of phosphoenolpyruvate, it is necessary to determine the stoichiometric relationship between the substrate(s) and products of the reaction. The stoichiometry can be determined only upon partial purification, since 1) the lability of oxalacetate (decarboxylation of the radioactive carboxyl-carbon) does not permit accurate radioactive analysis, 2) the presence of contaminating dehydrogenases prevents spectrophotometric analysis of oxalacetate formation, and 3) the change in phosphoenolpyruvate concentration is greatly effected by contaminating pyruvic kinase.

It was found that the protein precipitating at an ammonium sulfate concentration from 30 to 40 per cent saturation had the highest specific activity (Table 6); up to a 4.6 fold increase in specific activity was obtained in

Table 6. Efficiency of ammonium sulfate in fractionating the carboxylating enzyme

| Stage of purification | Volume (ml.) | Activity[a] (c.p.m./ml.) | Total activity (c.p.m.) | Total protein[b] (mg.) | Specific activity (c.p.m./mg.) |
|---|---|---|---|---|---|
| 1. Initial cell-free extract | 85.0 | 24,550 | 2,082,500 | 1181.5 | 1,766 |
| 2. After precipitation with protamine sulfate (0.13 mg. per mg. protein) | 49.5 | 5,020 | 248,490 | 267.3 | 930 |
| 3. 0-30% $(NH_4)_2SO_4$ precipitate | 4.3 | 1,680 | 7,224 | 23.2 | 368 |
| 4. 30-40% $(NH_4)_2SO_4$ precipitate | 2.4 | 6,070 | 14,568 | 5.0 | 2,890 |
| 5. 40-80% $(NH_4)_2SO_4$ precipitate | 10.0 | 16,764 | 167,640 | 145.0 | 1,156 |

[a] Radioactivity was measured after ethylacetate extraction of oxalacetate-2,4-dinitrophenylhydrazones according to Maryuma and Lane (1962b). The assay conditions used were the same as shown in Table 1, except no triphosphonucleotide was added.

[b] Protein was determined by the method of Warburg and Christian (Layne, 1957).

this fraction, but this was rapidly lost with the simultaneous formation of an insoluble material. Protamine sulfate precipitation prior to precipitation with ammonium sulfate inhibits this early loss of activity but also decreases the specific activity by *ca*. 50 per cent; this precipitate exhibits a specific activity *ca*. 3 times greater than that of the protamine sulfate-treated extract or *ca*. 1.5 times greater than the original extract. Although *ca*. 96 per cent of the original protein has been removed from the extract, this latter enzyme preparation is still not sufficiently pure to accomplish accurate stoichiometric analysis. The inorganic orthophosphate concentration has been observed to be routinely higher upon termination of phosphoenolpyruvate carboxylation reactions. Further purification on DEAE-SEPHADEX is presently being carried out in an attempt to better elucidate the mechanism of phosphoenolpyruvate carboxylation in *A. suboxydans*.

# DISCUSSION

Wood and Stjernholm (1962) have recently presented a comprehensive review in which they list 12 enzymatic mechanisms known to assimilate $CO_2$ into a new carbon-carbon bond in heterotrophic organisms. Four of these mechanisms are recent modifications of the Wood-Werkman reaction orginally proposed in 1935 (reactions 16 through 19); one of which uses pyruvate (reaction 16) and the others phosphoenolpyruvate as the substrate for carboxylation. Only the reaction catalyzed by phosphoenolpyruvate carboxylase (reaction 17) is irreversible and requires no acceptor for the phosphate liberated from the phosphoenolpyruvate upon carboxylation.

Since pyruvate and NADH or pyruvate and ATP cannot be substituted for phosphoenolpyruvate in the $C^{14}O_2$ assimilation studies in _A_. _suboxydans_ extracts, it is believed that an active mechanism for carboxylation of pyruvate does not exist in this bacterium grown under these experimental conditions (see Materials and Methods). These conclusions are not surprising since it appears that the distribution of a bacterial malic enzyme (Kaufman _et al_., 1951) and a bacterial pyruvate carboxylase (Seubert and Remberger, 1961) is very limited.

If the mechanism(s) for phosphoenolpyruvate carboxylation in _A_. _suboxydans_ is reversible, one should be able to demonstrate this by the $CO_2$-exchange reaction demonstrated by

Krampitz *et al*. (1943). Very slight evidence for such $C^{14}O_2$-exchange was found in only 60 per cent of the experiments, and this amount can be explained by spontaneous decarboxylation of the oxalacetate to pyruvate and subsequent formation of very small quantities of phosphoenolpyruvate in the presence of ATP and pyruvate kinase. Since both phosphoenolpyruvate carboxykinase (reaction 18) and phosphoenolpyruvate carboxytransphosphorylase (reaction 19) in other organisms are readily reversible (Utter and Wood, 1946; Siu and Wood, 1962), it appears that the primary phosphoenolpyruvate carboxylating reaction in *A. suboxydans* extracts is irreversible.

Both reversible phosphoenolpyruvate carboxylation reactions require an acceptor for the liberated inorganic orthophosphate. Heterotrophic bacterial extracts containing appreciable amounts of phosphoenolpyruvate carboxykinase catalyze increased amounts of $C^{14}O_2$ incorporation upon addition of a diphosphonucleotide (Baugh *et al*., 1960; Bates and Werkman, 1960; Myoda and Werkman, 1960). The autotroph *Thiobacillus thiooxydans* exhibits only slight evidence of a reversible carboxylase, and the amount of $C^{14}O_2$ fixed into phosphoenolpyruvate is inhibited by diphosphonucleotides (Suzuki and Werkman, 1958a). Such an inhibition with purine diphosphonucleotides in extracts of *A. suboxydans* is probably due to the conversion of phosphoenolpyruvate to pyruvate by pyruvate kinase. The possible stimulation of carboxylation by inorganic orthophosphate has not been fully investigated, but the apparent lack of reversibility in crude extracts, the formation of inorganic phosphate in reaction mixtures, and the inability of a diphosphonucleotide to stimulate fixation all indicate that neither phosphoenolpyruvate carboxykinase nor phosphoenolpyruvic carboxytransphosphorylase are active in significant amounts in extracts of _A_. _suboxydans_. It should be noted, however, that extracts containing both carboxytransphosphorylase and an active pyrophosphatase could give results where the reaction appeared irreversible and inorganic orthophosphate was produced.

Phosphoenolpyruvate carboxylase is an enzyme catalyzing the apparently irreversible carboxylation of phosphoenolpyruvate and was first described by Banderski and Greiner (1953) in spinach-leaf extracts. This enzyme catalyzes the formation of oxalacetate and inorganic orthophosphate from phosphoenolpyruvate and $CO_2$ (more probably $HCO_3^-$; see Maruyama and Lane, 1962a). The enzyme occurs in plants (Tchen _et al_., 1955; Rutman and George, 1961; Banderski, 1955; Walker, 1962; Tchen and Vennesland, 1955; Maruyama and Lane, 1962b) autotrophic and heterotrophic bacteria (Suzuki and Werkman, 1958b; Baugh _et al_., 1960; Myoda and Werkman, 1960), but as yet has not been demonstrated in animal tissue (Walker, 1962). In some cases the enzyme seems to be stimulated by small quantities of inorganic phosphate (Tchen and Vennesland, 1955), but this is not an absolute requirement for activity (Siu, 1962). The carboxylase has a high affinity (low Km value) for both $CO_2$ and phosphoenolpyruvate (Wood and Stjernholm, 1962) and, therefore, thought to be very effective in forming oxalacetate.

Oxalacetate and pyruvate (acetyl coenzyme A) are the only intermediates common to the oxidation and interconversion of carbohydrates, proteins and fats. Special significance in intermediary metabolism is given then to those reactions in which carbon dioxide is added to pyruvate or phosphoenolpyruvate. These enzymatic reactions constitute an important mechanism governing the passage of metabolites into and out of the tricarboxylic cycle. If an organism such as A. suboxydans has been shown incapable of using this cycle yet forms oxalacetate from phosphoenolpyruvate when grown in an excess of carbohydrate (energy source), one might presume that this demonstrated formation of aspartate by oxalacetate-amino acid transamination would be the primary purpose of such a carboxylating mechanism. Since the primary phosphoenolpyruvate-carboxylating mechanism of A. suboxydans (grown under these conditions) appears very similar to the reaction catalyzed by phosphoenolpyruvate carboxylase, it is of interest to compare the equilibrium constants and functions proposed for the reactions catalyzed by the various carboxylases in plant and animal tissue.

Walker (1962) discussed the literature available on the three pyruvate (phosphoenolpyruvate) carboxylating enzymes found in plants and came to the following conclusions: 1) Phosphoenolpyruvate carboxylase constitutes one of the most effective carboxylating mechanisms known. In higher plants it seems probable that a great deal of oxalacetate is synthesized in the reaction catalyzed by phosphoenolpyruvate carboxylase and stored as malate, or possibly aspartate, if not used immediately. 2.) It is improbable that the malic enzyme is concerned with carboxylation at all, as the equilibrium position favors oxidative decarboxylation, but this enzyme probably functions to mobilize the malate reserved in plants to form pyruvate for needed synthesis. 3) Phosphoenolpyruvate carboxykinase can catalyze the formation of oxalacetate, but its main function may well be the decarboxylation rather than the carboxylation. Thus, the reactions catalyzed by a) phosphoenolpyruvate carboxylase and b) phosphoenolpyruvic carboxykinase and the malic enzyme could be said to constitute mechanisms for a) entry to and b) exit from the tricarboxylic acid cycle. Wood and Stjernholm (1962) reviewed the literature on all heterotropic $CO_2$ assimilation reactions and reached the following conclusions: 1) It was formerly believed that the equilibrium of the reaction catalyzed by the malic enzyme favored malate decarboxylation, but it is now believed that malate synthesis is favored. 2) Direct evidence for the malic enzyme in heterotrotrophic bacteria is still lacking[1]. 3) The interactions of the enzymes catalyzing the reactions which carboxylate phosphoenolpyruvate and pyruvate probably are dependent upon their physical location within the cell; for example, phosphoenolpyruvate carboxykinase is located in the mitochondria and the malic enzyme is found in the cytoplasm. The possibility of phosphoenolpyruvate being synthesized from reactions catalyzed by the combined action of the carboxykinase and malic enzymes therefore seems unlikely because of their intracellular localization. Utter and Keech (1963) have shown that pyruvate carboxylase has a high affinity for $CO_2$ and, in liver tissue, is located in the mitochondria; these authors also suggested that the combined action of pyruvate carboxylase and phosphoenolpyruvate carboxykinase in mitochondria may provide a mechanism for synthesis of phosphoenolpyruvate from pyruvate. Papers presented by Lardy _et al_. and Utter (Connecticut, U., Bulletin, 1963) have demonstrated that conditions which stimulate increased carbohydrate synthesis

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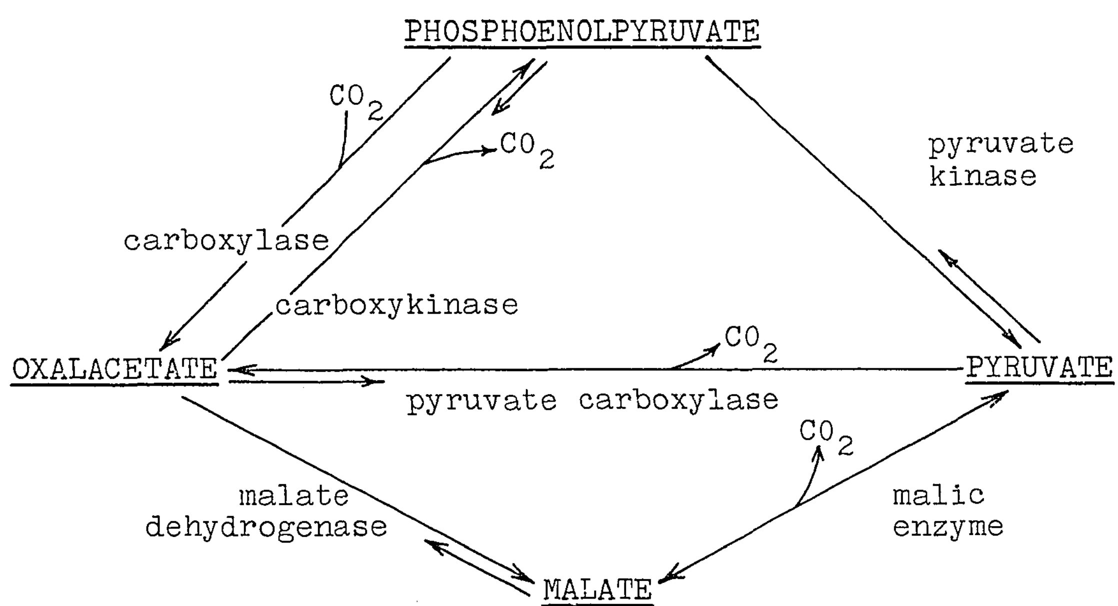
[1]This statement is contradictory to the view held by Vishniac _et al_. (1957).

in animal tissue also stimulate increases in the activity of phosphoenolpyruvate carboxykinase but not the malic enzyme. In the same Bulletin, Utter presented evidence to demonstrate that pyruvate carboxylase in the presence of phosphoenolpyruvate carboxykinase catalyzes net formation of phosphoenolpyruvate from pyruvate. These data suggest that, under certain conditions in animal tissue, the malic enzyme functions primarily to catalyze the formation of malate and the equilibrium of the phosphoenolpyruvate carboxykinase-catalyzed reaction primarily favors the formation of phosphoenolpyruvate.

The following scheme summarizes the current data on the function of the phosphoenolpyruvate and pyruvate carboxylating enzymes as indicated by the reviews of Walker (1962) and Wood and Stjernholm (1962):



Thus, one should not consider the theoretical or observed thermodynamic data alone, but also give full consideration to the amounts, properties and physical location of both enzymes and metabolites within a cell before the overall function of an enzyme system can be accurately predicted. Since such extensive data is not yet available on the bacterium Acetobacter suboxydans, certain hypotheses as to the function of its phosphoenolpyruvate carboxylating enzyme can be presented only after consideration of the limitations stated above.

The presence of the phosphoenolpyruvate carboxylase-like enzyme and the apparent lack of other phosphoenolpyruvate (pyruvate) carboxylating enzymes in A. suboxydans is consistent with the distribution and known function of this enzyme in other bacterial and plant systems. If one assumes an adequate source of energy and considers the proposed lack of a tricarboxylic acid cycle, it would then appear that a mechanism for entrance into the nitrogen pool would be more important during active growth than escape from it.

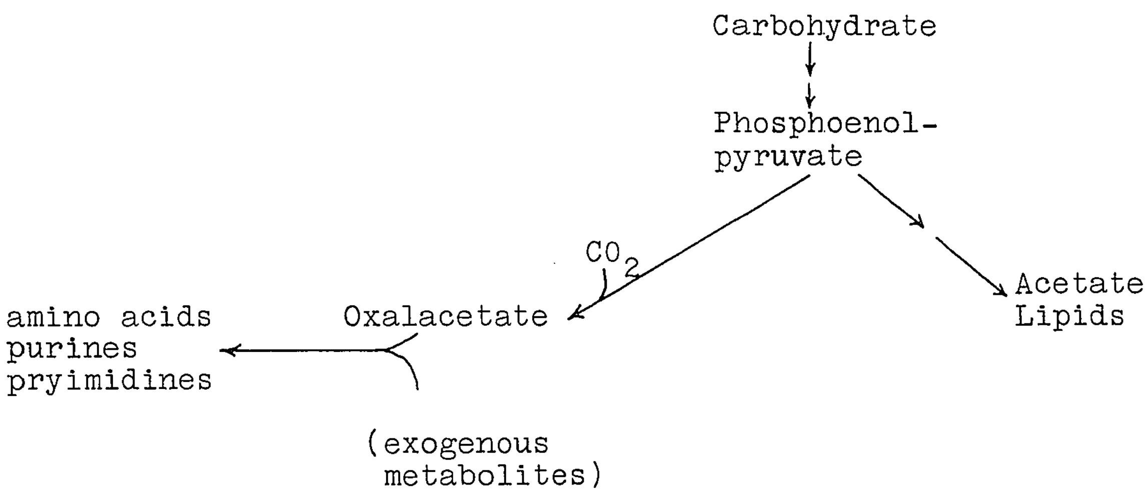
The original published description of A. suboxydans (Kluyver and De Leeuw, 1924) described the amino acids essential for growth as valine, alanine, isoleucine, histidine, cystine and proline. More recently, Stokes and Larsen (1945) reported that these same amino acids would stimulate growth of ATCC strain 621 to levels obtained with medium containing 20 amino acids; however, growth "to a limited extent" was observed with only valine, isoleucine, alanine and histidine. In contrast to the latter report, Cheldelin (1961) reports the unpublished results of Shamberger and Kewar listing only serine plus either glutamate, histidine or proline as necessary for "healthy growth" using strain ATCC 621 and again using glycerol as the sole carbohydrate. In any event, an exogenous source of aspartate is not required for growth and one might conclude that this bacterium can synthesize a sufficient amount of this amino acid to satisfy its growth requirements.

The mechanism of aspartate synthesis in _A_. _suboxydans_ as proposed by Cheldelin and his collegues (reactions 12 through 14) seems thermodynamically possible, since the overall standard free energy change at pH 7.0 should be _ca_. -20.2 kcal. per mole (Burton and Krebs, 1953; Burton, 1955); seven enzymes are needed for this mechanism of synthesis from phosphoenolpyruvate, and a glyoxylate-acetyl coenzyme A condensation reaction is involved. Cheldelin (1961) reports on unpublished observations by Baich which indicate that "...glyoxylate has only a transient existence, due to the presence of a very active glyoxylate reductase (glycolic dehydrogenase) in the organism". The mechanism of aspartate synthesis from phosphoenolpyruvate suggested by the present study is also thermodynamicaly feasible (standard free energy change calculated after Burton and Krebs to be ca. -6.93) and requires only two enzymes for the synthesis.

In order to completely synthesize aspartate from phosphoenolpyruvate, the present data suggest that another amino acid such as glutamate is needed for enzymatic transamination. Kluyver and De Leeuw (1924) and Stokes and Larsen (1945) report that histidine is an absolute requirement for growth of A. suboxydans. Cheldelin (1961) reports either glutamate, histidine or proline is necessary for growth. In many microorganisms, it is known that histidine and proline are metabolic precursors to the formation of glutamate (Meister, 1957). If one or more of these amino acids are essential for growth (multiplication) of his bacterium, one might assume that the organism cannot synthesize enough glutamate for its metabolic needs; thus, an exogenous source of glutamate for transamination with oxalacetate is implicated. On the other hand, Cheldelin (1961) states that when isotopic glucose or $C^{14}O_2$ are furnished for growth of the microorganism, the label appears first (among amino acids) in aspartate and glutamate; one might assume from these results that some glutamate is formed either 1) by direct synthesis from glucose catabolic intermediates or 2) by interconversion of the formed radioactive aspartate. In any event, from the nutritional studies, one must assume that the multiplying-cell's need for aspartate can be provided by biosynthesis but the majority of its requirement for glutamate (or immediate precursor) must be exogenously provided.

The importance of slight glutamate synthesis for the growth of A. suboxydans remains to be determined. Of the two pathways proposed, Cheldelin and his collegues (Sekizawa et al., 1962; Cheldelin et al., 1963) present strongest evidence that the primary path of glutamate synthesis is from an initial condensation of pyruvate and acetyl Co A (reactions 3 through 6). In studying glutamate formation in the anaerobic bacterium Clostridium kluyveri, Tomlinson (1954) found that cells grown in a synthetic medium, with ethanol, acetate and $CO_2$ as carbon sources, formed radioactive glutamate when either acetate-1-$C^{14}$ or $C^{14}O_2$ were added to the medium. The location of the radioactive carbon in glutamate was such that its precursor could not have been formed via the usual tricarboxylic acid cycle reactions; instead, several proposals of glutamate synthesis were presented, one of which involved conversion of the radioactive aspartate to α-ketoglutarate which could then form radioactive glutamate by transamination. This latter proposal seems strengthened by the recent findings by Stern (1963) that radioactive oxalacetate can be formed by extracts of <u>C. kluyveri</u> by a pyruvate carboxylase-like enzyme; the β-position of the radioactive carbon in aspartate formed by transamination would allow for the position (γ) of the radioactive carbon found in glutamate. When one considers the nutritional requirements for growth of <u>A</u>. <u>suboxydans</u> and the findings in another bacterium known not to have an active tricarboxylic acid cycle, the possibility that aspartate is first formed then interconverted to glutamate (to a slight extent) seems more attractive than direct synthesis from the catabolic intermediates of glucose; the exact mechanism, whether it be one of those proposed by Cheldelin or one similar to the proposal of Tomlinson, will await radiotracer analysis.

The cells used in this study were grown in large amounts of nutrients and harvested just prior to their entrance into the stationary phase of growth. It is presumed that these cells were not being deprived of nutrients essential for their growth. Any enzymes found to be active in such cells might then be considered intrinsic to the metabolism of logarithmically growing cells, as opposed to having their syntheses induced by limitation of growth factors. The fact that $C^{14}$-aspartate can be formed from phosphoenolpyruvate and $C^{14}O_2$ (in the presence of glutamate and pyridoxal phosphate) in soluble extracts indicates that it is both thermodynamically and kinetically possible in whole cells. The literature of this microorganism's nutritive requirements and intermediary metabolism indicate that an aspartate-forming mechanism is an important connecting link between carbohydrate catabolism and amino acid anabolism. It might be visualized that control of the fate of phosphoenolpyruvate determines whether the cell synthesizes amino acids or acetate. Whether the control mechanism is inhibition or repression, the cells might be expected to exhibit more activity




of the phosphoenolpyruvate carboxylating enzyme in the absence of the nonessential amino acids (e.g. aspartate). Such an avenue of investigation is currently being followed in an attempt to enhance specific activities so that a more critical identification and analysis of the carboxylating enzyme can be made.

# PART II

## FINE STRUCTURE OF *ACETOBACTER SUBOXYDANS*

# INTRODUCTION

Fine structural studies of bacteria have been limited to relatively few species; however, in the past few years, enough data have become available so that general conclusions on the comparative fine structure of microorganisms can be made. Much evidence on the chemical nature of certain componants of the bacterial cell have also been recently accumulated. Only very recently have the structural and chemical evidence of a single component been compared; this dual approach is necessary before the function of the component is understood.

The following study deals primarily with morphological features of the cell wall and plasma membrane, the protoplasmic constituents, and the flagella as observed by thin sectioning and negative staining in the electron microscope. Primary emphasis has been given to the structure of the cell wall and flagella in comparison with their reported chemical nature.

The gram-negative cell wall has previously been studied by sectioning, ghost isolation, and chemical analysis. Sections of Escherichia coli (Birch-Anderson et al., 1953; Hofschneider and Lorek, 1962; Kellenberger and Ryter, 1958; North, 1961; Starr and Williams, 1952) and other gram-negative genera (Beer, 1960; Murray, 1960; Murray, 1962) have demonstrated a membrane that lies outside of the plasma membrane, is 6 to 10 mμ thick, and is usually implied to be the cell wall of the bacterium. "Macromolecular spheres" have been observed to lie within the smooth outer layer of shadowed, isolated, gram-negative cell walls (Houwink, 1953; Salton, 1960; Weidel et al., 1960); however, sectioned bacteria generally have not shown a comparable structure between the membrane of the cell wall and the plasma membrane. Chemical analyses of isolated cell walls generally yield about 20 per cent lipid, a large percentage of protein, and some mono- and poly- saccharides in addition to a mucopeptide complex (Salton, 1960); such analyses suggest that the cell wall thickness is about 25 mμ (Mitchell, 1959) and, therefore, is structurally more complex than a single membrane.

The possibility of flagellar hollowness was proposed as early as 1941 (Polevitzky). Proposed models of bacterial flagella based on x-ray diffraction pattern analysis (Astbury et al., 1955; Burge, 1961), and on ultracentrifuge analysis and electron microscopy (Kerridge et al., 1962), illustrate a hollow central region of the fibril. Although observations of sections containing bacterial flagella indicate hollowness (Kerridge et al., 1962), observations with negatively stained flagella are possible only after rigorous physical treatment.

# MATERIALS AND METHODS

## Cell Growth

Stock cultures of Acetobacter suboxydans (ATCC 621) were maintained on Acetobacter agar slants containing 0.1 per cent each of yeast extract and $CaCO_3$, 0.15 per cent agar, and 0.03 per cent glucose under conditions employed by the American Type Culture Collection (Weiss, 1957). A medium containing 0.2 per cent each of glucose and yeast extract and 0.3 per cent peptone was used to subculture the bacteria at $30^{\circ}C$. for 24 hours with slight agitation. Five ml. of the subculture were used as an inoculum for Roux flasks containing 150 ml. of Acetobacter agar. Growth was accomplished at $30^{\circ}C$. for 24 hours to produce cells for sectioning, or at $20^{\circ}C$. for 120 hours to produce flagellated cells for flagellar isolation (Mitchell, 1959).

## Fixation

The cells were prepared for fixation by a technique similar to the plasma clot method described by Glauert et al. (1961). Cells were scraped from the agar surface with a glass rod and suspended in enough freshly drawn chicken plasma to make a turbid suspension. One part of fresh chick embryo extract was added to 4 parts of the plasma-bacteria suspension, and this mixture was spread thinly on a glass surface and allowed to clot. The clot was placed in an osmium tetroxide-tryptone medium solution which was buffered at pH 6.1 with acetate-veronal buffer (Ryter *et al.*, 1958) and left at room temperature for 15 minutes. The clot was removed, cut into cubes (*ca.* 1 $mm.^3$), and returned to the fixation solution for 16 hours. The cubes were then placed in two 15-minute changes of 0.5 per cent uranyl acetate solution maintained at pH 5.2 with acetate-veronal buffer (Ryter *et al.*, 1958), and in a third change of this solution for 2 hours.

## Dehydration and Embedding

Dehydration was performed by 20-minute changes of 50, 75 and 95 per cent ethanol with two 20-minute changes in absolute ethanol. The methacrylate used was a mixture of 2 parts ethyl and 3 parts n-butyl methacrylate with 1 per cent benzoyl peroxide; this mixture was dried immediately before use by filtering through anhydrous, powdered $Na_2SO_4$. Infiltration was accomplished with 3 changes of the methacrylate mixture (20 and 45 minutes and 16 hours at room temperature). Each cube was transferred to a separate gelatin capsule and covered with methacrylate which was polymerized at 60°C.

## Sectioning and Staining

Sections were cut on LKB or Porter-Blum microtomes, mounted on 400-mesh grids covered with a parlodion membrane, and overlaid with a methacrylate membrane; however, a few of the sections were mounted on methacrylate membranes and overlaid with carbon membranes. Sections were stained with uranyl acetate (Gibbons and Bradfield, 1957) at a concentration of 0.5 per cent for 2 hours at pH 4.5 (Glauert *et al*., 1961) before the overlay was added, or for 1 minute with a 1/100 dilution of a stock lead hydroxide stain (Karnovsky, 1961), or for 1 hour with 1 per cent potassium permanganate (Lawn, 1960).

## Flagella Isolation and Disruption

Two Roux flasks containing 5-day-old cultures of *A. suboxydans* grown at 20°C. were each washed with 15 ml. of distilled water. The resulting cell suspension was placed in a stoppered flask and shaken vigorously for 5 minutes. The bacterial cells were removed by centrifugation with a Servall superspeed angle centrifuge model SS-1 at 14,000 x g for 15 minutes, and the flagella were removed from the supernate by centrifugation with a Spinco model L preparative ultracentrifuge at 75,000 x g (average) for 45 minutes. The pellet was resuspended in distilled water and centrifuged at

25,000 x g for 15 seconds in order to remove excess contaminating material (Iterson and Robinow, 1961); no visible pellet was produced. After further centrifugation at 75,000 x g for 45 minutes, a pellet was formed and was resuspended in 0.8 ml. of distilled water. This flagellar suspension was stored at $4^{o}$C. and used without further dilution.

The isolated flagella were partially disrupted by ultrasonic vibration (Schoeller ultrasonic spray dispenser, Brinkman Co., Great Neck, New York). An Erlenmeyer flask containing the suspension was lowered to the bottom of the treatment chamber which was partially filled with ice water. Samples of the suspension were taken after treatments of 30, 60, 120 and 300 seconds. After each of these time intervals, the 1 megacycle transmitter was turned off, the aliquot removed, the surrounding water replaced with ice water and the treatment continued.

## Negative Staining

A stock solution of 2 per cent phosphotungstic acid was adjusted with 1 N KOH (Brenner and Horn, 1959) to pH 5.3. Equal volumes of the potassium phosphotungstate and the flagella or flagella-Tobacco Mosaic Virus (TMV) suspensions were mixed and a droplet placed on a supporting membrane where the particle-phosphotungstate suspension dried in 20 to 30 minutes. The flagella suspension was used either without dilution or was mixed with an equal volume of TMV suspension containing $10^{10}$ particles/ml.

Supporting membranes of parlodion with small holes were formed in high humidity atmosphere and lightly filmed with carbon before the specimen-phosphotungstate suspension was added, in order to increase contrast of the holes. The presence of the holes in the membrane served as a focusing aid and a constant check for astigmatism.

## Electron Microscopy

An RCA EMU 3F with a standard single condenser lens was operated at 100 kv with a 60-μ objective aperture. Negatives were produced at magnifications up to 20,000 times. Higher instrumental magnifications of 37,000 to 49,000 were obtained by inserting a smaller bore pole piece in the intermediate lens (RCA high magnification kit). The high magnification kit was used (with a 30-μ objective aperture) primarily for the observation of negatively stained flagella.

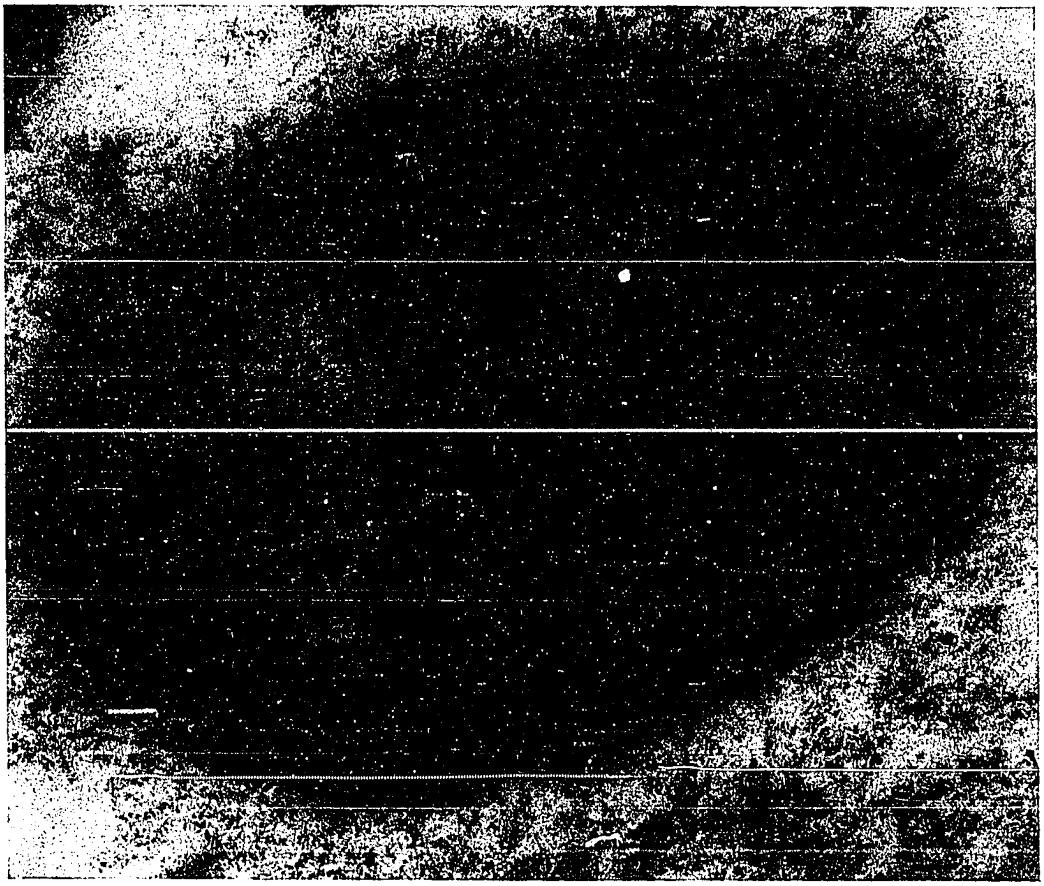
# OBSERVATIONS

## Cell Wall and Plasma Membrane

Three separable structures are observed between the capsular material and the protoplasm of Acetobacter suboxydans. In cross-section, the outermost structure appears as two layers of high electron scattering, each 3 mμ thick, separated by a space about 2.5 mμ thick (Figs. 1, 2 a and b, OM). This structure has the appearance of a unit membrane and will be referred to as the outer membrane. Another similar membrane, located beneath the outer membrane and having slightly thinner separation of layers, appears to surround the protoplasm and is referred to as the plasma membrane (Figs. 1, 2 a and b, PM). Between these membranes is a layer of material that appears homogeneous, is less dense than the high electron-scattering layers of the membranes, and is 5 to 10 mμ thick; this is referred to as the intermediate layer (Figs. 1, 2 a and b, IL).

The outer membrane usually appears more obvious than the plasma membrane because it has a thicker electron-transparent layer and is bound only on one side by cellular material. Frequently, where the protoplasm seems well preserved, the outer membrane appears serrated, although still unbroken. Although the outer membrane usually appears as a continuous

Fig. 1a. Relatively short cell stained with uranyl acetate, demonstrating aggregated chromatin areas (C) with chromatin strands (CS) and ribosome-like particles (R). The protoplasm is surrounded by an outer membrane (OM), an intermediate layer (IL), and the plasma membrane (PM). A frequently observed anomaly occurring in the outer membrane is shown (OMA). X 162,000



envelope, several anomalies have been observed: a) the outer membrane appears to branch toward the region of the plasma membrane, b) perpendicular "bridges" or striations appear between the outer membrane and the plasma membrane or protoplasm (Fig. 7), and c) the outer membrane appears to branch and rejoin giving the appearance of an additional membrane (Fig. 1, OMA).

The region of the intermediate layer is also subject to some structural variation. This layer often appears to be thicker at the polar region of a cell (Fig. 2 a, IL) without any difference in density regardless of the stains used. However, two types of cells lack the intermediate layer and may have increased separation of the two membranes: a) those in which the shape of the cell and the appearance of the protoplasm seem normal (Fig. 4), and b) those in which the cell is more spherical and the protoplasmic constituents are largely missing (Fig. 5 a). In both instances, the inner layer of the outer membrane has a greater thickness which appears to be due to the adherence of material of the same density as the intermediate layer (Figs. 4, 5 a and b, A).

Since both the outer membrane and the intermediate layer are outside the plasma membrane and both appear to be distinct, separable layers, the term cell wall will be used to include both of these layers.

Fig. 2a. Section stained with lead hydroxide demonstrating the outer membrane (OM), plasma membrane (PM) and the varying thickness of the intermediate layer (IL). Polar regions (P) of the elongated cell lack ribosome-like particles. X 84,500

Fig. 2b. Enlargement of adjacent cells in Fig. 2a, again demonstrating the outer membrane (OM) and intermediate layer (IL) of the cell wall, and the plasma membrane (PM). X 148,000

Fig. 3. Permanganate-stained section demonstrating the cell wall, the plasma membrane and a structural anomaly (A) in the outer membrane. Distinct ribosome-like particles are not demonstrated. X 73,500

Fig. 4. An atypical cell demonstrating an absence of most of the intermediate layer; that which remains appears as a thickening of the inner layer of the membrane (A). The protoplasm of the cell is normal, showing aggregated chromatin (C) with chromatin strands (CS) and ribosome-like particles (R). X 79,000

Fig. 5a. Cell has atypical shape and a protoplasmic area that contains only chromatin strands (CS) and a few ribosome-like particles (R). The absence of most of the intermediate layer of the cell wall is demonstrated; that which remains (A) appears as a thickening of the inner layer of the outer membrane (OM), yet separated from the plasma membrane (PM). The adjacent cell appears typical. X 83,000

Fig. 5b. Enlargement of Fig. 5a, demonstrating atypical appearance of the intermediate layer (A). X 175,000

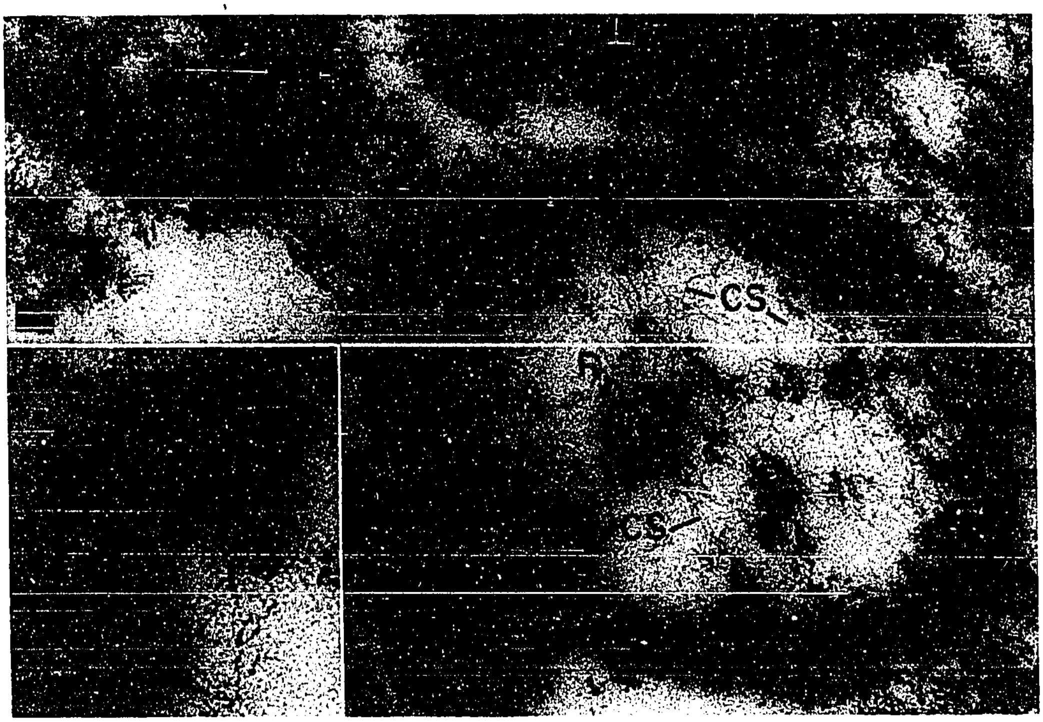

CS
A
CS

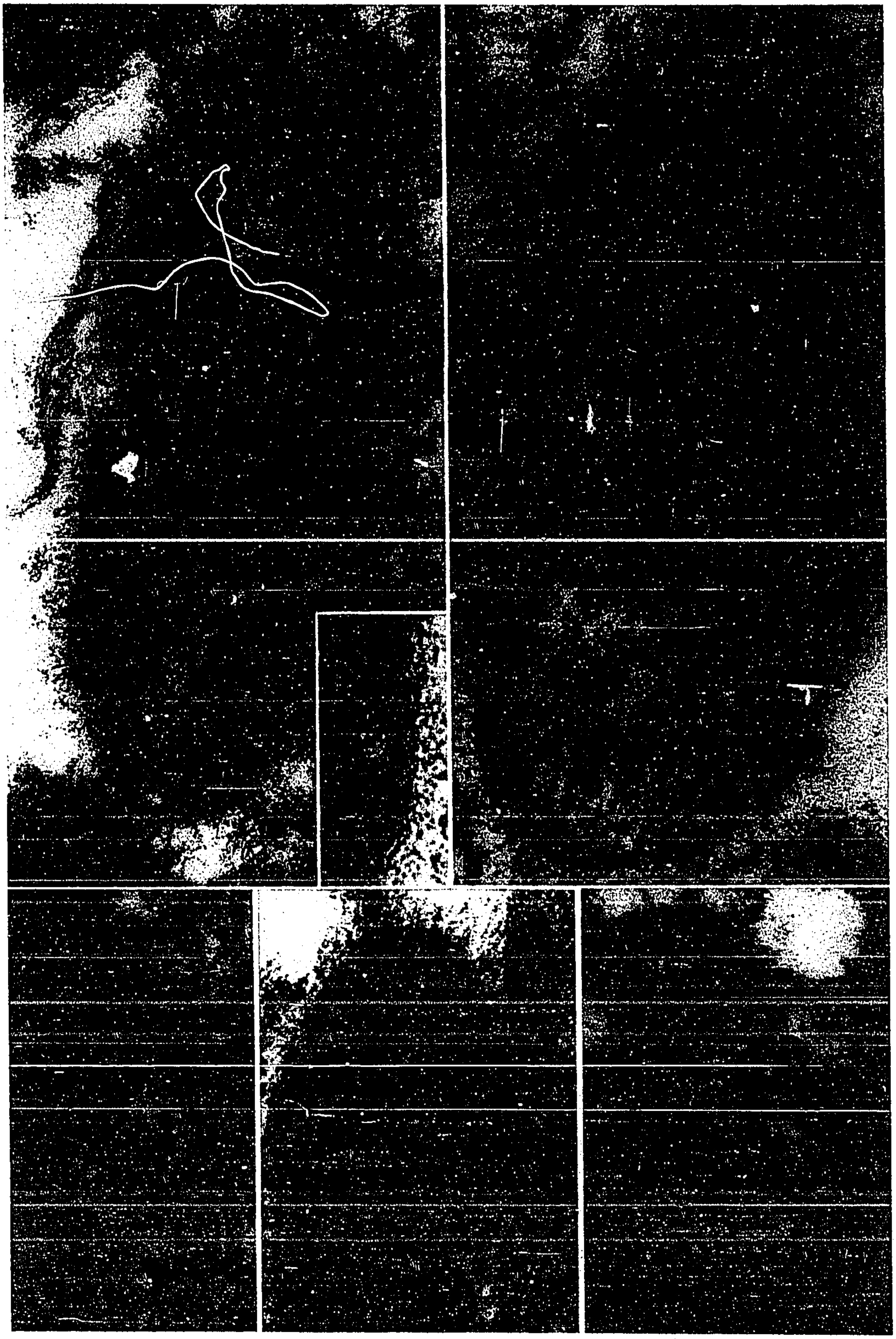
Fig. 6a. Dividing cell which demonstrates the invagination of plasma membrane (PM) and the extramembranous material (M) between the plasma membrane and the outer membrane (OM). X 62,000

Fig. 6b. Enlargement of the extramembranous area of Fig. 6a. X 149,000

Fig. 7. Demonstration of membranous invaginations (M) at the polar end of uranyl acetate-stained cell. Chromatin (C) is partially dispersed, and the ribosome-like particles (R) are distinct. X 87,000

Fig. 8. Differentiation of polar region demonstrates electron-transparent area, electron-opaque parallel lines, and the lack of ribosome-like particles. Lead stained. X 108,000

Figs. 9 and 10. The plasma membrane is occasionally observed to invaginate into the cytoplasm (Fig. 9, arrows). Membrane-like inclusions of the cytoplasm which cannot be traced to the plasma membrane are also observed (Fig. 10, M). Fig. 9, lead hydroxide stained, X 110,000. Fig. 10, uranyl acetate stained, X 62,000



The plasma membrane occasionally appears to be folded into the cytoplasm. In general, these intracytoplasmic membranes are found in higher concentrations at the ends of the cells, either as single membranes (Figs. 7 M, and 9, arrows) or as whorls (Figs. 11 and 12), W); however, such material can occasionally be seen along the sides of the cells in regions other than were the plasma membrane would be expected to invaginate during the initial stages of division (Figs. 6 and 10, M).

A layer that varies in thickness and appears as a loose aggregation of undifferentiated material is present outside the cell wall (Figs. 1 through 14) and is thought to be the slime layer or capsule.

Protoplasmic constituents

Structures appearing almost spherical with a diameter of ca. 14 mμ were found in great numbers distributed randomly throughout the cytoplasm. A slight accumulation of these particles occasionally occurred at the polar region of elongated cells (Fig. 13, R). The contrast between these particles and the remainder of the cytoplasm was greatly improved by staining with uranyl acetate (Figs. 1, 4, 7, 11 and 13, R). Such particles were also observed in sections stained with lead hydroxide, but the cytoplasm was also stained so that the particles contrasted more poorly with their surroundings (Figs. 9 and 11, R). In cell sections stained with permanganate, the particles were almost unobservable (Fig. 3). In their staining affinity and structural appearance, these particles are characteristically similar to ribosome-like particles found in other cells.

Uranyl acetate-staining strands measuring about 1.8 mμ in diameter are seen in electron-transparent localizations varying in number and size. These strand-containing areas are assumed to represent the chromatin of the cells and will be thus denoted, although definitive proof is not yet available. The relative distribution of the chromatin material seems to correspond with the length of the cells: the chromatin of short cells (ca. 1 μ) appears aggregated (Figs. 1, 4 and 17, C); that in cells of intermediate length (ca. 1.5 μ) demonstrates a more randomly dispersed appearance (Fig. 7, C); and that in elongated cells (ca. 2 μ) or cells in the process of division seems most dispersed (Figs. 6 a and 11). In cells stained with uranyl acetate the contrast between the chromatin and the cytoplasm is greater (Figs. 1 and 4, C) than it is in cells stained with permanganate (Fig. 3) or lead hydroxide (short cell, Fig. 2). The 1.8 mμ strands of the chromatin area (Figs 1 and 4, CS) are more readily apparent in sections stained with uranyl acetate. Lead hydroxide occasionally demonstrates the intrachromatin strands (Fig. 12, CS), whereas with permanganate staining the stranding seems virtually unobservable (Figs. 3 and 17). Strands of the same diameter are readily visible in uranyl acetate-stained cells that are devoid of most of their protoplasm (Fig. 5 a, CS).

The polar regions of most elongated cells show a specialized low density region. In uranyl acetate-stained longitudinal sections, this area appears as a cluster of non-membrane-limited spheres which demonstrate a diameter of ca. 7 mμ (Figs. 11 and 13, P). A few distinctly separated spherical structures were occasionally found at the polar region of cells presumably cut at a more oblique angle. Virtually all uranyl acetate-stained sections showing cell elongation or division and dispersed chromatin material also demonstrate the polar cluster of spheres (Figs. 11 and 13, P), whereas sections through relatively short cells demonstrating accumulated chromatin do not (Figs 1 and 4). The non-membrane-limited spheres (or the cluster) differ from the remainder of the cytoplasm in that they are free of 14-mμ particles; they also differ from the chromatin material by their location within the cell and the absence of 1.8 mμ strands (Figs. 11 and 13). When sections are stained with lead hydroxide, the polar region seems to be distinct only

Figs. 11 and 12. A characteristic polar appearance (P) as found with uranyl acetate staining (Fig. 11) and lead staining (Fig. 12). Occasionally observed whorl of membrane material (W) and ribosome-like particles (R) are demonstrated by both figures, but chromatin strands (CS) are more apparent in Fig. 12. Fig. 11, X 74,500. Fig. 12, X 78,000

Fig. 13. Concentration of ribosome-like particles (R) around polar area (P), demonstrated with uranyl acetate-stained section. X 44,500

Fig. 14. Polar region as demonstrated by lead hydroxide staining. X 92,000

Fig. 15. Palladium-shadowed, plasmolyzed cell demonstrating protruding body at the polar end of the cell near the base of the flagellum. X 20,700


W
P

in that a) it lacks the 1.8-mμ strands and the 14-mμ electron-opaque particles, and b) its electron scattering is often similar to that of the chromatin material (Figs. 2 and 12, P).

When whole cells are partially plasmolyzed, dried on grids and shadowed with palladium, a basal granule or blepharoplast-like enlargement can be seen at the polar region of the cell near the area of attachment of the flagellum (Fig. 15).

Flagella

Cell preparations stained for light microscopy according to Leifson (1951) demonstrate up to 4 polar flagella and aggregations of detached flagella. The present studies demonstrate that formalin-fixed cells shadowed with ca. 15 A of palladium at an angle of $10^{o}$ and observed in the electron microscope usually show no more than one flagellum ca. 12 to 15-mμ diameter without visible periodicity (Fig. 15). Cells fixed with formalin and negatively stained with potassium phosphotungstate have multiple polar flagella with the same diameter and without periodicity. Staining for the light microscope and both shadowing and negative staining for the electron microscope show that the flagella have a wave length of 1.4 μ and an amplitude of 0.3 μ, which are in agreement with the observations of Leifson (1954).

In sections, flagella are occasionally observed in high concentrations that are believed to be aggregates of detached flagella similar to those observed in preparations stained for the light microscope. Longitudinally sectioned flagella appear as two parallel dense lines, each 3.5 mμ thick, separated by a space of lesser scattering that is ca. 4.5 mμ thick, so that the total thickness is ca. 12 mμ (Figs. 16 and 21); oval or circular profiles that are oblique or cross-sections are noted less frequently (Figs. 16 and 17). When sections are observed which are cut through areas of the plasma clot not containing bacterial cells, these filamentous structures are almost never seen, while the cell-containing sections almost invariably demonstrate evidence of the 12-mμ filaments.

Permanganate (Figs. 16 and 17) and lead-hydroxide (Fig. 18) staining increase the electron scattering of the outer portion of the flagellum; permanganate staining appears to result in more contrast between the outer and inner regions of the flagellum. Uranyl acetate appears to cause no increase in electron scattering (Figs. 19 and 20). The diameter of 12 mμ is not strictly consistent; occasionally 14- to 15-mμ oblique and cross-sections and 10- to 16-mμ longitudinal sections are observed.

Negatively stained, isolated flagella were studied after having been dried on carbon-coated support membranes containing small holes. TMV particles are added to the flagellar suspensions in order to aid the focusing and to provide a particle with a similar diameter and a known central core (ca. 4 mμ). When the darkened central core of the TMV particle is best demonstrated, the supporting membrane appears slightly underfocus. In order to be assured that optimum focus is achieved in preparations, either the TMV or the width of the underfocus fringe around the membrane hole can be utilized.

Negatively stained, isolated, but otherwise untreated, flagella do not show a darkened central region like that of TMV (Fig. 23). After ultrasonic treatments of 30 and 60 seconds, the flagella are shorter, occasionally demonstrate a subtle central darkening (Figs. 21, 22 and 24), and have a more irregular appearance along their length. The darkened central region is not apparent in all treated flagella and, when apparent, it can only be detected along short portions of the flagellum. The width of this central region is about 1.5 mμ. Occasionally, a region giving the impression of partial degradation of the flagellum appears as a void in the structure (Fig. 24, large arrow). Flagella

Figs. 16 and 17. Longitudinal (arrows) and oblique or cross-sections (circles) of flagella as demonstrated by permanganate staining. The structure labeled (A) demonstrates an atypically small dimension. Aggregated chromatin (C) may be observed in adjacent cell-sections. Fig. 16, X 110,000; Fig. 17, X 107,000

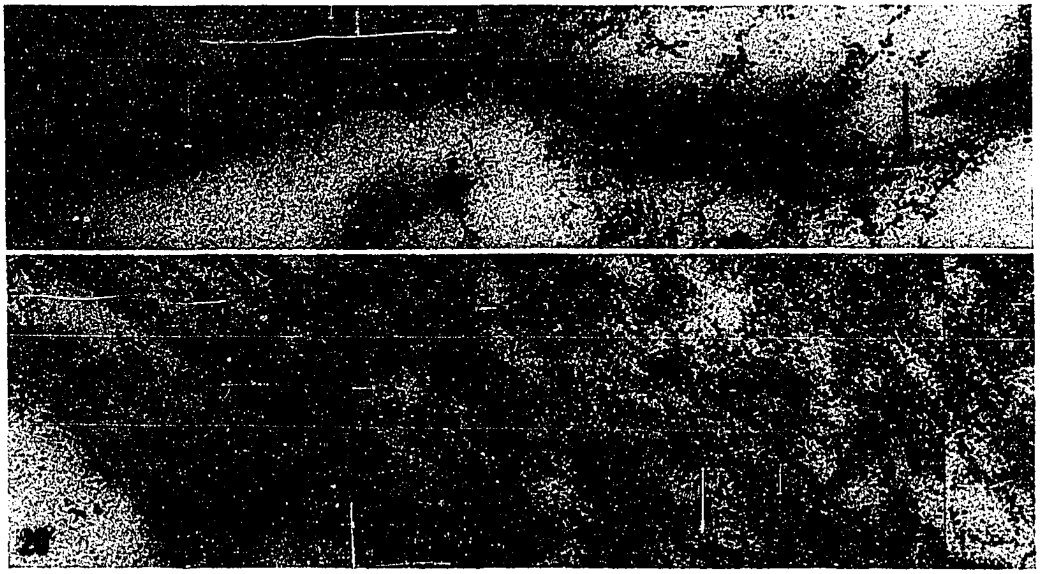
Fig. 18. Lead-stained section demonstrating longitudinal (arrows) and oblique or cross-sections (circles) of flagella. X 97,000

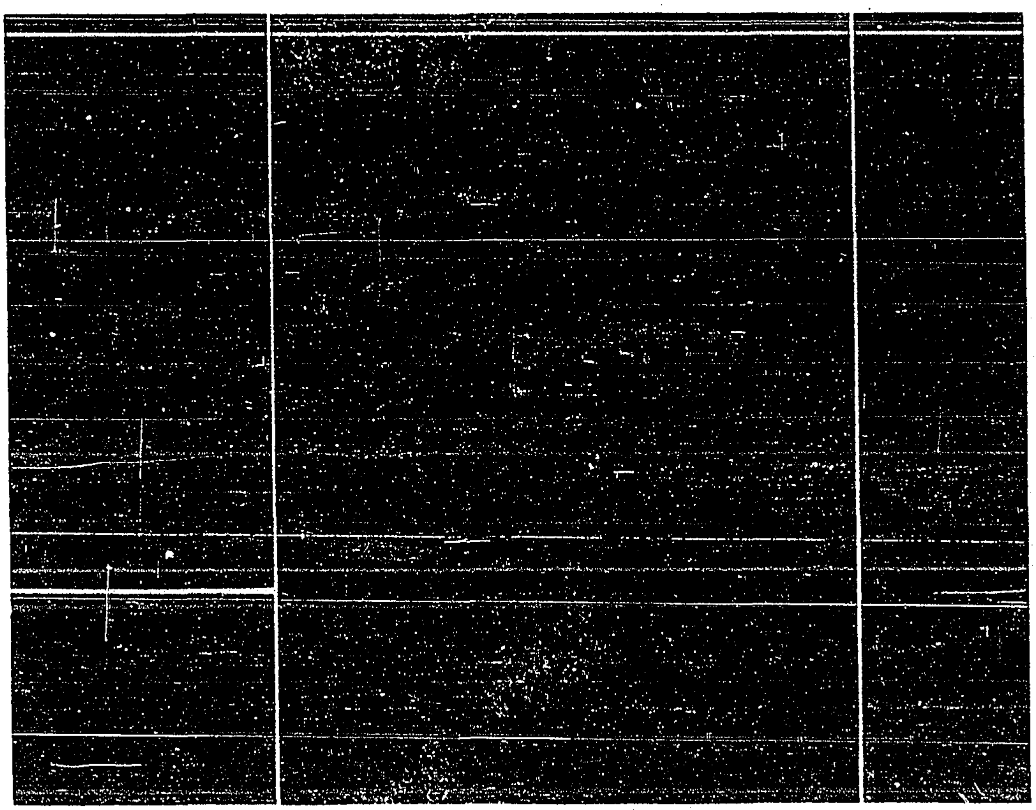
Figs. 19 and 20. Appearance of longitudinal (arrows) and oblique or cross-sectioned (circles) flagella in an unstained section (Fig. 19) and in one stained with uranyl acetate (Fig. 20). Fig. 19, X 91,000; Fig. 20, X 85,000

Figs. 21 and 22. Flagella negatively stained with phosphotungstate after treatment with ultrasonic vibrations for 30 and 60 seconds (Figs. 21 and 22, respectively). Central darkening is apparent (C) in Fig. 21; Fig. 22 demonstrates two parallel rows of subunits (outer lines) separated by a darkened central region (C). Fig. 21, X 366,000; Fig. 22, X 334,000

Fig. 23. Flagellum, not previously treated with ultrasonic vibration, and two particles of Tobacco Mosaic Virus, the latter demonstrating central darkening due to hollowness. Negatively stained with phosphotungstate. X 134,000

Fig. 24. Flagellum, previously treated for 60 seconds with ultrasonic vibration, demonstrating darkened central region along part of its length (C), apparent void (large arrows). Negatively stained with phosphotungstate. X 435,000





treated for 120 and 300 seconds are severely altered so that they appear as only slight darkenings of the supporting membrane.

# DISCUSSION

## Cell Wall and Plasma Membrane

A detailed concept of the gram-negative cell wall and plasma membrane structure has arisen from various studies. Prior to 1959, little evidence (Weibull and Bergstrom, 1958) had been presented to support the occurrence of membranes which are structurally similar to the "unit membrane" described by Robertson (1959). Since Glauert and Hopwood's convincing demonstration (1959) of a multilayered plasma membrane in hyphae of _Streptomyces coelicolor_, similar evidence has been given for gram-negative and gram-positive bacteria which seem to vary only in the thickness of the electron-transparent layer (Conti and Gettner, 1962; Fukushi _et al._, 1962; Hofschneider and Lorek, 1962; Ogura, 1963; Robinow, 1960; Voelz and Dworkin, 1962). A similar additional membrane has been observed outside the plasma membrane of certain gram-negative bacteria (Beer, 1960; Hofschneider and Lorek, 1962; Kellenberger and Ryter, 1958; Murray, 1962; North, 1961; Thorsson and Weibull, 1958). The clearest observations of the cell-wall membrane and the plasma membrane have come from studies of phage-infected and induced spheroplasts of gram-negative bacteria (Hofschneider and Lorek, 1962; Kellenberger _et al._, 1958; Murray, 1962; Thorson and Weibull, 1958) where two membranes are present but separated by an apparently empty space. When gram-negative cell-wall fragments are observed in the electron microscope, "macromolecular" spheres having diameters of 8 to 12 mμ (Salton, 1960) have been observed to lie within, or occasionally outside, a smooth layer (Murray, 1962; Murray, 1963; Salton, 1960; Weidel et al., 1960); such a "macromolecular" layer in sectioned gram-negative bacteria has been conspicuous only by its absence, as noted by Chapman (1959). Cohen-Bazire and Kunisawa (1963) have recently indicated that the cell wall of Rhodospirillum rubrum may consist of two layers. Ogura (1963) has also very recently described a cell wall consisting of two layers; however, the lack of consistent demonstration of a plasma membrane leaves the observer without a point of reference with which to interpret the whole cell envelope. Murray (1963) has recently described a gram-negative cell wall containing a thin layer between the cell-wall membrane and the plasma membrane; "macromolecular" spheres were described on the outside of the cell-wall membrane.

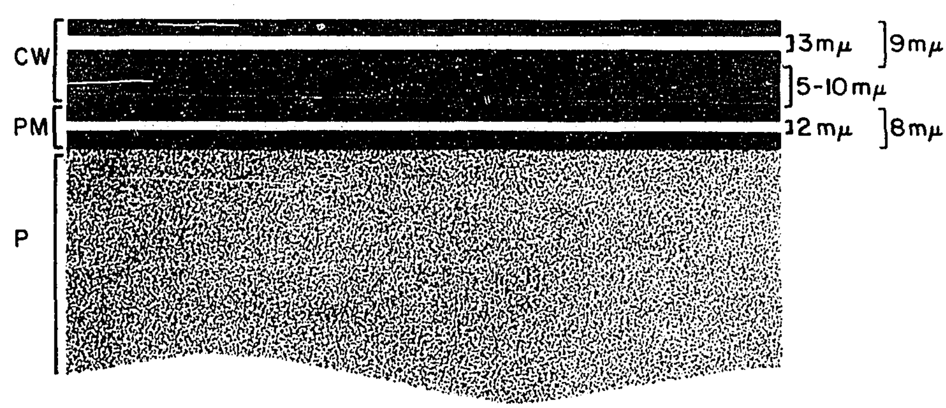
The sectioned cell wall of A. suboxydans has been found to consist of more than an outer membrane. An inner, more homogeneous layer having a thickness similar to that described for the layer of "macromolecular" spheres is also present. The plasma membrane of A. suboxydans is structurally similar to the cell-wall membrane and separates the cell-wall inner layer from the protoplasm.

A structural concept of the gram-negative cell wall and plasma membrane can be formulated on the basis of this and the several other studies cited above. Fig. 25 is a diagrammatic presentation of the structure of the cell envelope found in *A. suboxydans* and is thought to be representative of the morphological features generally expected in gram-negative bacteria.

Bladen and Waters (1963) have recently published micrographs of the anaerobic genus *Bacteroides* which strengthens this generalized structural concept of gram-negative cell walls. They have demonstrated that cells suspended in agar prior to fixation show a "space" between the cell-wall outer membrane and the plasma membrane; however, this "space" is more dense than the embedding medium and appears very similar to the inner cell-wall layer of *A. suboxydans*.

The chemical composition of gram-negative cell walls also indicates that they consist of more than a single membrane. The cell walls are reported (Salton, 1960) to contain about 20 per cent lipid, a large percentage of protein, and some mono- and polysaccharides in addition to a mucopeptide complex. From such studies, it has been estimated that the gram-negative cell wall has a thickness of *ca.* 25 mμ (Mitchell, 1959). This estimate corresponds closely with

Fig. 25. Diagrammatic representation of the sectioned cell wall (CW), consisting of a membrane covering a more homogeneous inner layer, and the plasma membrane (PM). This structural concept of the sectioned cell wall and plasma membrane, as found surrounding the protoplasm (P) of *Acetobacter suboxydans*, is thought to be characteristic of gram-negative bacteria in general


CW
PM
P
13 mμ
9 mμ
5-10 mμ
12 mμ
8 mμ

the combined thickness of the cell-wall membrane and inner layer of _A_. _suboxydans_. A difference in the number of carbon atoms in the fatty-acid chains of the electron-transparent lipid layer of membranes may account for the variable thicknesses reported in bacterial membranes (Hughes, 1962); such a difference may also account for the slight variation noted in electron-transparent layers of the cell-wall membrane and plasma membrane of this bacterium. Chemical studies also indicate that the cell walls consist of a thin network of "mucocomplex" (Hughes, 1962), similar in composition to the cell wall of the gram-positive organisms, and that this complex is covered on the outside by a plastic film of lipoprotein (Mitchell, 1959; Salton, 1960; Work, 1961). Such a concept is in keeping with the previous observations of isolated cell walls and the appearance of sectioned cell walls in _A_. _suboxydans_.

Chemical studies suggest that part of the "mucocomplex" portion is responsible for the structural rigidity of the cell wall (Salton, 1960; Work, 1961). The gram-negative cell-wall membrane and the plasma membrane of phage-infected cells and induced spheroplasts are separated by an empty space (Hofschneider, and Lorek, 1962; Kellenberger _et al_., 1958; Murray, 1962; Thorson and Weibull, 1958). Furthermore, evidence (Work, 1961) suggests that phage infection enzymatically destroys the material necessary for the structural rigidity of the cell wall, and that phage have affinity for the "macromolecular" spheres isolated from cell walls of E. coli (Weidel and Kellenberger, 1955). The morphological evidence of abnormal cells in this study (Figs. 4 and 5 a) similarly suggest that the lack of the cell-wall inner layer is correlated with the loss of structural rigidity.

Thus, the structural evidence that the cell wall in sections of A. suboxydans consists of an outer membrane and an inner, homogeneous layer agrees with the information from chemical and ghost isolation studies of the gram-negative cell wall. In addition, the inner homogeneous layer of the cell wall probably corresponds structurally to the "mucocomplex" portion that, in part, is functionally vital to the structural rigidity of the cell wall.

One possible explanation for the previous lack of preservation of the inner layer in sections of gram-negative cell walls may be that which Weidel et al. (1963) recently suggested for the erroneous interpretations in physical and chemical studies of cell wall ghosts, that is, the extremely rapid action of the autolytic enzymes which destroy the continuous rigid mucopolymer layer in cell walls of Salmonella gallinarum. The use of a plasma clot suspension in this study may have better preserved the conditions necessary for cell maintenance and thereby greatly decreased the time during which the cells were subjected to nonphysiological conditions prior to inhibition of the autolytic enzymes.

Although the chemical composition of the "mucocomplex" varies, gram-negative and gram-positive bacteria have mucopeptide constituents in common (Salton, 1960; Work, 1961), a fact which has led to the idea of a "basal" chemical structure being present in all bacterial cell walls (Work, 1957). Sectioned gram-positive cell walls appear as a non-differentiated layer (Chapman, 1959; Fitz-James, 1962) similar to the cell-wall inner layer of A. suboxydans. From the chemical considerations and from the electron microscope studies, it can be postulated that the cell-wall inner layer of gram-negative bacteria is morphologically similar to the complete cell wall of gram-positive bacteria.

Protoplasmic constituents

When bacteria are fixed by various methods (Chapman, 1959; Ryter et al., 1958) and sectioned, the chromatin material appears as a non-membrane-limited, relatively electron-transparent region that contains fibrillar material in an unorganized (Kellenberger et al., 1958) or organized (Giesbrecht, 1962; van Iterson and Robinow, 1961) mass; the fibrillar material in the chromatin of A. suboxydans appears unorganized. Under the experimental conditions of the studies reported here, the chromatinic material of short cells is much more aggregated than that of elongated cells, which would suggest a possible correlation between chromatin dispersion and cell growth. These observations are not in keeping with those on other bacteria in which continuously aggregated chromatin is observed (Chapman, 1959; Chapman and Hillier, 1953; Conti and Gettner, 1962; Mason and Powelson, 1956); however, Glauert (1962) has reported similar findings in Clostridium welchii and Lactobacillus acidophilus.

A differentiation in the polar region is typically present in A. suboxydans. Although its chemical nature or function is not known, the region was not observed in permanganate-stained sections and was not stained by uranyl acetate or lead hydroxide (see Huxley and Zubay, 1961; Watson, 1958). Typically, elongated cells with highly dispersed chromatin best demonstrate these polar structures, whereas short cells do not show them with certainty. Similar inclusions have been reported in the polar region of E. coli and B. megaterium (Murray, 1960; Murray, 1962) and have been described as polysaccharide accumulations; however, these inclusions are less specifically localized and are fewer in number than those reported here.

Leifson (1954) characterizes A. suboxydans as having polar multitrichous flagella. In the present study, shadowed preparations of plasmolyzed cells have demonstrated the appearance of a protruding body near the base of the flagellum, while sections show the polar differentiations described above and occasionally some intracytoplasmic membranous material. Recently, the presence of extramembraneous material and the lack of ribosome-like particles in the region of flagellar insertion of *Spirillum serpens* has been reported (Murray, 1963). The possibility that the polar differentiation found in *A. suboxydans* is structurally or functionally correlated with the flagella seems reasonable, but is not established.

## Flagella

Since their description by Cohn in 1872 (see Houwink and van Iterson, 1950), bacterial flagella have been described as uniform filaments (Weibull, 1960) having diameters of 12 mμ (Robinow, 1960), although diameters of from 12 to 19 mμ (Astbury and Weibull, 1949; Kerridge *et al.*, 1962; Starr and Williams, 1952) have been reported. Helical subfibrils have been reported upon occasion in the sheathed and unsheathed flagella (De Robertis and Franchi, 1952; Gordon and Follett, 1962; Labaw and Mosley, 1955; Weibull, 1960; Williams and Chapman, 1961). Studies of the chemical nature of flagella indicate that they consist almost entirely of proteinaceous material of the keratin-myosin-elastin-fibrinogen group (Weibull, 1960). The flagellin (Astbury et al., 1955) macromolecular subunit has been reported by Erlander, Koffler, and Foster (1960) to have a molecular weight of from 30,000 to 40,000 (Kerridge et al., 1962).

The examples of structures presented as flagella in Figs. 16 through 20 are believed to be the flagella of A. suboxydans for the following reasons. First, the diameter of the linear, circular, or oval structures is the same as the flagella diameter determined by shadowing or negative staining; the fact that the over-all diameter is greater than that of either the outer membrane of the cell wall or the plasma membrane eliminates the slight possibility that these structures are membranous debris. Secondly, the finding of scattered areas of high concentration of flagella in sections correlates well with the light microscope observation of their detachment and aggregation; the presence of little or none of these structures in sections of the plasma clot devoid of cells strongly indicates they are not components of the clot. Finally, the appearance of these structures closely resembles the appearance in sections of isolated flagella from S. typhimurium (Kerridge et al., 1962). Thus, from sections of A. suboxydans the structure of flagella appears to consist of a dark outer region surrounding a region which is either hollow or filled with material having an electron-scattering property similar to that of methacrylate.

Negative staining of isolated flagella does not readily demonstrate hollowness. This fact is accentuated when negatively stained, isolated flagella are compared with TMV particles (Fig. 23). Since the diameter of the central region of the flagellum as observed in sections is similar to the known luminal diameter of the TMV particle, one would expect the flagellum to also show the penetration of phosphotungstate if this central region is hollow. Rigorous physical treatment of the flagellum appears necessary for such penetration into the central region, and even then the diameter of this region is very small.

The second flagellar model suggested by Burge (1961), which consists of 6 subunits surrounding a central subunit, seems unlikely in this case, since the phosphotungstate penetration, when seen, appears centrally located. Erlander *et al*. (1960) report that sedimentation values for one form of flagellin indicate that it is ellipsoid in shape. An arrangement of such subunits would result in a much smaller central diameter than would an arrangement of essentially spherical subunits suggested by Kerridge *et al*. (1962). Correlation of the present negative staining and sectioning results along with the chemical and physical studies on other flagella suggests the hypothesis that the large protein molecules are polymerized in such an orientation that osmium can react only at the periphery of the molecule, while the remainder of these macromolecules, surrounding a nearly closed central region, is left unaffected.

Similar filaments, though slightly larger in diameter, are observed in sections of the mitotic apparatus, cilia, centrioles and the protozoan infraciliature, as recently reviewed (Roth, 1963). Regarding the function of such filaments, Slautterback (1963) has emphasized their "tubular" nature by suggesting that they are utilized for fluid passage and transport. The present study suggests, however, that the luminal diameter demonstrable from sections may also be much smaller for these filaments.

# SUMMARY AND CONCLUSIONS

1. Cells of <u>Acetobacter</u> <u>suboxydans</u> (ATCC 621) were grown in a medium containing glycerol and yeast extract and harvested prior to entrance into the stationary phase of growth. Cell-free extracts were prepared and found to catalyze the fixation of $C^{14}O_2$ when phosphoenolpyruvate was added as the substrate.

2. In well dialyzed extracts, radioactive oxalacetate is the only product of the phosphoenolpyruvate catalyzed carboxylation reaction. Pyruvate and NADPH or pyruvate and ATP will not replace phosphoenolpyruvate as the substrate in dialyzed cell-free extracts.

3. The addition of diphosphonucleotides, to extracts treated with ion-exchange resins, inhibits $CO_2$ assimilation in the presence of phosphoenolpyruvate. Such assimilation is inhibited more by purine than by pryimidine nucleotides. The reaction requires a divalent cation. $Mn^{++}$ stimulates four times more enzyme activity than $Mg^{++}$. Oxalacetate formation is 4 to 5 times greater at pH 7.4 than at 7.0.

4. The $CO_2$-exchange reaction stimulated by a triphosphonucleotide is so slight that it can be accounted for by the nonenzymatic decarboxylation of oxalacetate, combined by slight formation of phosphoenolpyruvate, and followed by nucleotide dependent carboxylation.

5. It is concluded that the primary enzymatic mechanism for the formation of oxalacetate from phosphoenolpyruvate, in extracts of _A_. _suboxydans_ grown under the present conditions, is nucleotide independent, irreversible, primarily stimulated by Mn++, and is more reactive at pH 7.4 than 7.0. Such a mechanism is similar to the reaction catalyzed by phosphoenolpyruvic carboxylase; an enzyme found in other microorganisms and higher plants.

6. The apparent lack of a reversible phosphoenolpyruvate (or pyruvate) carboxylating enzyme(s) is unique in heterotrophic bacteria studied to date.

7. _A_. _suboxydans_ extracts will catalyze the formation of $C^{14}$-aspartate from phosphoenolpyruvate and $C^{14}O_2$ in the presence of glutamate and pyridoxal phosphate. The formation of oxalacetate from aspartate and α-ketoglutarate can be shown with spectrophotometric analysis.

8. Since aspartate is reportedly not essential for the growth of _A_. _suboxydans_ (ATCC 621), it is proposed that the primary pathway for aspartate synthesis is a) formation of oxalacetate from phosphoenolpyruvate and $CO_2$ by an enzyme similar to phosphoenolpyruvate carboxylase, then b) subsequent transamination in the presence of glutamate and pyridoxal phosphate.

9. Even though cells reportedly yield radioactive glutamate from $C^{14}$-glucose, glutamate or its metabolic pre-

cursor (proline or histidine) is reported to be essential for the growth of this bacterium. It is concluded that an exogenous source of glutamate is necessary to assure synthesis of aspartate (and subsequent metabolities) from phosphoenolpyruvate.

10. The cell wall of A. suboxydans (ATCC 621), as observed in the electron microscope, can be distinguished as two layers; a) an outer membrane similar to the classical unit membrane, and b) an inner, more diffuse layer which exists between the outer membrane of the cell wall and the plasma membrane.

11. It is proposed that the general cell-wall structure found in A. suboxydans typify the gram-negative cell wall. It is also proposed that a) the outer membrane corresponds to the lipoprotein layer described in chemical studies, and b) the inner layer of the cell wall corresponds to the polysaccharide-mucopeptide (mucocomplex) portion of the gram-negative cell wall.

12. Particles having a diameter of ca. 14 m are found in great numbers in the cytoplasm of the sectioned bacterium. These particles are characteristically similar to ribosome-like particles because of their staining affinity and structural appearance.

13. The chromatinic material of short cells is much more aggregated than that of elongated cells. The intra-

chromatin strands, measuring ca. 1.8 m in diameter, collectively appears as an unorganized mass.

14. A. suboxydans has been characterized as having polar multitrichous flagella. Plasmolyzed cells demonstrate a blepharoplast-like protrusion near the base of the flagellum. Sectioned cells demonstrate polarly-oriented, nonmembrane-limited areas which are devoid of ribosome-like particles. The possibility that the polar differentiation is structurally or functionally correlated with the flagella seems reasonable, but exacting proof is not yet available.

15. The structure of flagella from sections appears to consist of a dark outer region surrounding a region which is either hollow of filled with a material having an electron-scattering property similar to that of methacrylate.

16. Negative staining of isolated flagella does not readily demonstrate hollowness. Rigorous physical treatment is necessary in order to observe slight phosphotungstate penitration of the flagella.

17. Although previously proposed flagellar models suggest a hollow central region, this study suggests that flagellin subunits may be polymerized in such a way that osmium can react only at the periphery of the molecule, while the remainder of these macromolecules, surrounding a nearly closed central region, is left unaffected.

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