THE ISOLATION AND PROPERTIES OF FORMIC DEHYDROGENASE FROM ESCHERICHIA COLI.

THESIS PRESENTED FOR THE DEGREE

of

MASTER OF SCIENCE

in the

UNIVERSITY OF SYDNEY.

by

COLIN W. WRIGLEY.

January, 1961.

PREFACE

The work reported in this thesis has been in progress since the beginning of 1959, under the supervision of Dr. A. W. Linnane. The experimental results and their interpretation are original work by the author.

Grateful thanks are due to Dr. A. W. Linnane for his constant help and encouragement throughout the course of this work. The author also wishes to thank Professor Still and the members of the Biochemistry Department, University of Sydney, for helpful advice and discussion.

This work was supported by a research grant from the Australian National Health and Medical Research Council.

[REDACTION]

COLIN W. WRIGLEY

January, 1961.
Biochemistry Department,
University of Sydney.

THE ISOLATION AND PROPERTIES OF FORMIC DEHYDROGENASE FROM ESCHERICHIA COLI.

ABBREVIATIONS

DPN	Oxidized form of diphosphopyridine nucleotide.
DPHN	reduced form of diphosphopyridine nucleotide.
RNA	ribose nucleic acid.
DNA	deoxyribose nucleic acid.
BSA	bovine serum albumin.
DOC	deoxycholate.
DEAE-cellulose	diethylaminoethyl-cellulose.
OD_{280}	optical density at 280 mu.
mu.	mμ.
umoles	μmoles.
ug	μg.
cyt.	cytochrome.
tris	tris(hydroxymethyl)aminomethane.

INTRODUCTION

Some three decades have passed since the classical work of Keilin initiated the investigation of the class of compounds which he called "cytochromes". Since that time, the cytochromes have been shown to be closely associated with the terminal dehydrogenating enzymes in the electron transport system, which catalyses the aerobic oxidation of hydrogen atoms arising from substrate dehydrogenation. This system has been shown to be a highly organized structure located in the particulate material of the cell. Studies during the last decade have been largely concerned with investigating the manner in which these components are arranged in the electron transport system. In the main, these studies have been carried out on mammalian tissue and very little work has involved bacterial cells. This thesis is an attempt to examine these problems in a typical bacterium, Escherichia coli.

These studies on E.coli have been particularly concerned with the enzyme formic dehydrogenase which has been reported to be associated with cytochrome b_1 in the particulate material of the cell. Information on this enzyme so far accumulated is very sparse, and even less is known about the cytochrome with which it is associated. Neither this enzyme nor cytochrome b_1 has yet been isolated

from the particulate material in soluble form. Indeed, there has been no report of any of the b cytochromes being prepared from the electron transport system in true solution.

The work described in this thesis has involved a close investigation of formic dehydrogenase in E.coli. Methods have been developed for the culture and disintegration of the cells so that a maximum yield of formic dehydrogenase is obtained in the resulting particulate material. A procedure has been devised for the isolation of this enzyme and its associated cytochrome from the particulate material in considerable purification. Evidence is advanced that in this preparation, formic dehydrogenase and cytochrome b_1 have been isolated in soluble form. Investigation of this preparation has provided some interesting information on the nature of the formate oxidation system of E.coli.

Part of this work has been published in Biochemical and Biophysical Research Communications 4, 66 (1961).

I. THE CYTOCHROMES.

The cytochromes are a class of chromoproteins which occur in almost all living cells. The first observations of this type of haemochromogen were made around about 1880 by several workers, by Sorby (1876) in snail intestine and by MacMunn (1885 and 1886) in Actiniae and mammalian muscle. These were but fleeting glimpses of a class of compounds that was later to prove one of the main components of the cellular respiration system.

These early studies were almost forgotten by 1925, when Keilin rediscovered this type of compound. This was the first of a series of investigations by Keilin into the chemical structure and biological significance of this class of compounds, which he called "cytochromes". Using the visual spectroscope, Keilin (1933), observed certain compounds in yeast and mammalian heart muscle. These compounds, in the reduced state, showed 4 distinct absorption bands which Keilin called a, b, c and d bands. The a, b and c bands occurring at about 605, 564 and 550 mu respectively, were shown to be due to three different haemochromogens which he called cytochromes a, b and c. The alternative possibility that cytochrome is a single protein, with three separate haematin prosthetic groups, was discounted because of changes in the spectrum under various conditions

and the ability of each cytochrome to undergo reduction and oxidation independently of the others. The d band was due to the fused secondary bands of cytochromes b and c. A tertiary band was later discovered by more refined techniques. This band was difficult to distinguish in the visual microspectroscope because it occurred at much shorter wavelengths than the other bands. These three bands, termed the α-, β- and γ- bands, are characteristic of porphyrin - containing compounds. The values of these bands for the three cytochromes are set out in Table I. Cytochrome c was the only one of the three that could be extracted and isolated as a water-soluble haemoprotein.

TABLE I. Positions of absorption bands (in mu) of the reduced cytochromes in mammalian heart muscle.

	α	β	γ
Cytochrome a	605	-	452
Cytochrome b	564	530	432
Cytochrome c	550	521	415

Reproduced by Green (1959) from Keilin and Hartree (1939).

The use of the recording spectrophotometer makes possible quantitative evaluation of absorption bands with an accuracy that cannot be obtained with the visual microspectroscope. The automatically recorded absorption spectrum of oxidized and reduced cytochrome c (Figure I) shows the position and relative absorbancies of the bands spoken of above. The

Soret or γ- peak is the only well-defined band appearing in the spectrum of the oxidized cytochrome. On reduction, α-, β- and γ- peaks appear and in addition a fourth peak, the δ-peak, out of the visible range at 316 mu.

Since the discovery of these three cytochromes by Keilin, a number of other cytochromes have been recognized, and additional cytochromes have been named with reference to the three compounds first described. On the basis of the absorption sprectra, prosthetic groups and reactions with various inhibitors, the cytochromes have been classified in groups, such that Groups A, B and C contain the compounds related to cytochromes a, b and c, respectively. Subscript numbers (e.g., a_1, b_2, c_1) have been assigned arbitrarily to the various cytochromes, generally in the order of discovery after the reference compound. The general characteristics of each of the various groups are tabulated in Table 2.

The cytochrome from mammalian tissues have been studied more thoroughly than those from other sources.

TABLE 2. General characteristics of cytochromes of groups A, A-2, B, C and D. (reproduced from Morton, 1958).

Group.	α- band of reduced compound (mu)	α- band of reduced pyridine haemochromogen (mu)	Prosthetic group.	CO-binding activity at pH.
A	588-612	583-585	haem a	Varies
A-2	630	613	haem a	combines
B	556-564	557-558	protohaem	not reactive
C	549-557	550-551	haem c	not reactive
D	550	550-551	haem c	combines

A large number of different cyts. have been discovered in plants and microorganisms. The mammalian cytochromes will be first discussed and the occurrence of cytochromes in bacteria will then be reported.

FIGURE 1. Absorption spectrum of oxidized and reduced cytochrome c from horse heart muscle. (Reproduced by Fruton and Simmonds (1958) from Keilin and Slater (1953).)

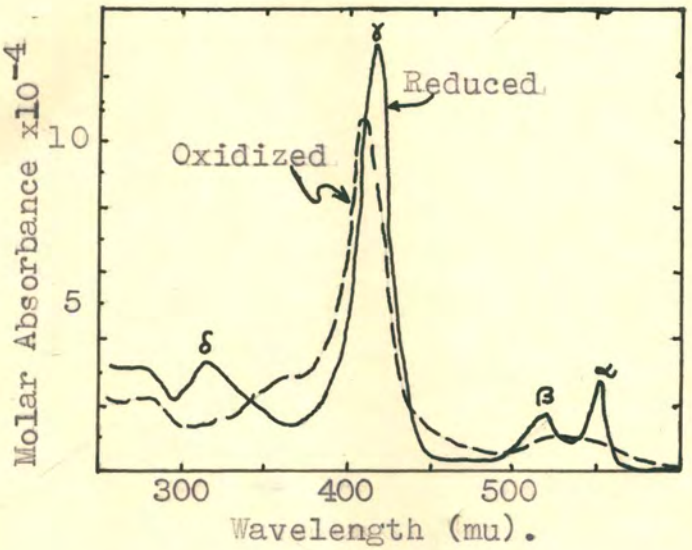
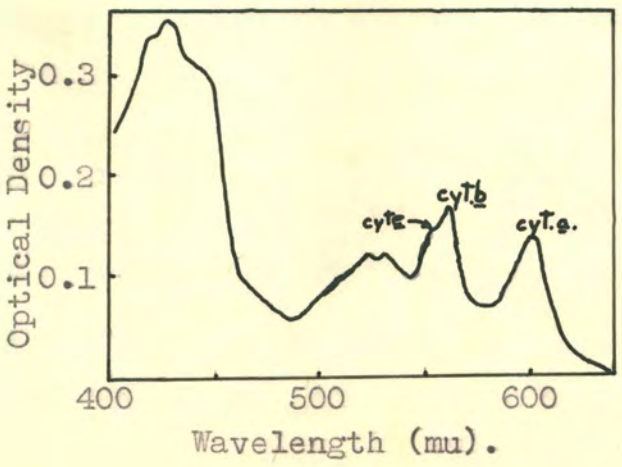


FIGURE 2. Difference spectrum between electron transport particle (ETP) in oxidized state and ETP reduced with dithionite. Low temperature recording. (Reproduced from Green, 1958.)



I. MAMMALIAN CYTOCHROMES.

The positions of the main spectral bands of a mammalian heart muscle preparation are shown on Table I. The original differentiations of cytochrome into the three compounds, cytochromes a, b and c, is now generally accepted. There has been evidence brought forward for the further differentiation of cytochromes a and c into cytochromes a and a_3 and cytochromes c and c_1, respectively. These latter distinctions have not been so widely accepted and are still the subject of controversy.

Cytochromes c and c_1.

Evidence for cytochrome c_1 arose largely from observation of the cytochrome spectrum at low temperature. At the temperature of liquid air or thereabouts, the cytochrome absorption bands are sharpened, thus permitting more precise definition of the absorption maxima. Figure 2 shows the difference spectrum of the electron transport particle (ETP) from beef heart muscle recorded at low temperature. The electron transport particle is a submitochondrial particle which contains the bulk of the mitochondrial cytochromes. The positions of the absorptions peaks are only slightly changed at low temperature from the positions occupied at room temperature. However, an additional peak appears at 554 mu between the α-peaks of cytochromes c and b. Keilin and Hartree (1949) ascribed this peak to a new cytochrome which they named cytochrome e. Cytochrome e was later

found (Keilin and Hartree, 1955) to be identical with cytochrome c_1, a compound isolated much earlier by Yakushiji and Okunuki (1940) from heart muscle, after treatment with cholic acid and ammonium sulphate. The α - band of cytochrome c_1 is indistinguishable from that of cytochrome c in the spectrum recorded at ordinary temperatures because the bands are fused into one peak.

Cytochrome c_1 has since been isolated in a water-soluble form, free from other cytochromes. Green et al.,(1959) The cytochrome was also isolated in the form of a water-soluble lipoprotein complex containing 50% lipid on a dry weight basis. This latter compound probably approximates more closely to the form of the cytochrome in intact tissue. The isolated cytochrome c_1 was further distinguished from cytochrome c by the fact that it did not replace cytochrome c in any of several catalytic assay systems. The two cytochromes have nothing in common except their spectral characteristics and probably the chemical nature of the prosthetic haem group.

Green (1959) has claimed that the physiological form of cytochrome c is not the classical water-soluble compound (Figure 1), obtained by direct extraction of the tissue. Rather he has found that in ETP, cytochrome c exists in a water-insoluble form, associated with lipid. Treatment of this lipid cytochrome c with lipid disrupting reagents, releases classical cytochrome c.

Cytochrome b.

Cytochrome b, on the other hand, is not readily extractable from tissue, as it appears to be firmly complexed with lipoprotein. This cytochrome has recently been isolated from beef heart mitochrondria, free from other cytochromes (Bomstein et al., 1960). The purified cytochrome b was insoluble in water and in solutions of bile salts, but could be dispersed with sodium lauryl sulphate. The authors suggest that the water-insolubility of cytochrome b is an intrinsic property of the haemoprotein. Morton (1958) has even suggested that cytochrome b is itself a lipoprotein. The cytochrome is very thermolabile and is readily denatured by organic solvents to produce the denatured protein protohaemochromogen with α - band at 558 mu.

Cytochromes a and a_3.

As was mentioned earlier, evidence has been brought forward suggesting that the spectral peaks that were originally attributed to one compound, cytochrome a, are actually due to two cytochromes, which have been called cytochromes a and a_3. (Keilin and Hartree, 1939). Even with low temperature recording, there is no suggestion that the cytochrome a band is the product of two bands fused together. On addition of carbon monoxide however, the α and γ- bands split, so that new bands appear at positions near the original α- and γ- bands, but at slightly shorter wavelengths. These new bands have been accounted for by

dividing the original cytochrome a into two separate cytochromes. The name cytochrome a was retained for the compound which did not react with carbon monoxide and the newly-discovered compound which reacted with the inhibitor was named cytochrome a_3. Reaction with potassium cyanide has produced further evidence of a similar nature for the existence of these two separate compounds. However, the differentiation between the two cytochromes is not generally accepted because of the failure of all attempts to isolate the cytochromes as separate haemoproteins. Lemberg (1960) has suggested that the two prosthetic groups corresponding to cytochromes a and a_3 may be attached to the same protein molecule by different linkages. There is evidence that lipid is involved in the attachment of haem a to the protein, but not in the linkage of the cytochrome a_3 prosthetic group. This explanation fits the experimental findings, but definite conclusions must await further evidence.

The function of mammalian cytochromes.

The principal role of the cytochromes in mammalian tissue is in the terminal electron transport system which catalyses the oxidation of succinate and reduced diphosphopyridine nucleotide (DPNH) by molecular oxygen. This electron transport system should be visualized as an organized chain, the components of which must be in correct

spatial arrangement to one another to allow the rapid transfer of hydrogen atoms or electrons. The activity of the system is therefore a function not only of the activity of the individual components, but also of the manner of arrangement of the components with respect to one another. A great deal of work has gone into elucidating the arrangement of the components in the chain. As was mentioned earlier, succinate and DPNH are the initial hydrogen donors to the system.

Cytochrome b appears to react nearer to these substrates than the other cytochromes. This was shown in 1925 when Keilin found that, in the presence of narcotics, cytochrome b remained reduced while cytochromes c and a were oxidized. The reduction of cytochrome b by succinate is much faster than by DPNH. Whether this reduction rate is sufficiently high as to warrant its inclusion in the main respiratory chain is still debated. Some reviewers have placed this cytochrome in a side reaction apart from the main pathway.

The fact that the reduction of cytochrome c_1 is blocked by narcotics and antimycin (Keilin and Hartree, 1949 and 1955) shows that is reacts after cytochrome b. Preparations of DPNH—and succinic - cytochrome c reductase contain cytochrome c reducible by substrates, (Basford et al.,1957). The location of cytochrome c_1 between cytochromes b and c is also in agreement with the oxidation-reduction potentials of these cytochromes (Keilin and Hartree, 1955).

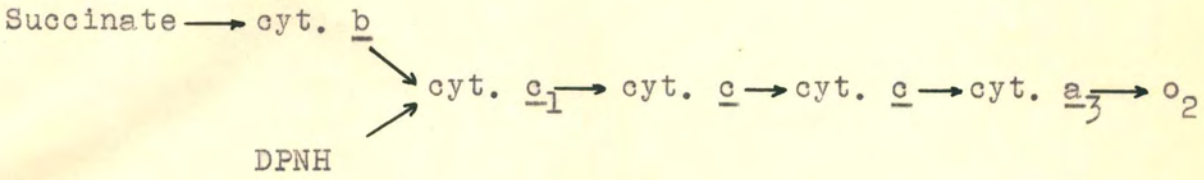
Cytochrome a_3 was identified by Keilin and Hartree (1939) to be the same as Warburg's respiratory enzyme, i.e., with the enzyme that finally reacts with oxygen. This enzyme is now known as cytochrome oxidase. Since then, much evidence has accrued to support this finding, including the isolation of a submitochondrial particle which catalyses the oxidation of reduced cyt. c by molecular oxygen (Mackler and Penn, 1957). This particle contained exclusively cytochromes a and a_3.

The elucidation of the arrangement of the electron transport chain intermediates has also been attacked by fragmenting the chain into smaller particles which have been examined individually in order to discover how they fit into the intact system. The cytochrome oxidase particle mentioned above, is an example of the application of this technique. The number of such particles that have been prepared is already considerable and a great deal of work is yet to be done before any definite conclusions can be reached. Reviews of this work are available: Green (1959), Slater (1958), Green (1956), Green and Lester (1959).

Results obtained from these studies have led to the formulation of tentative schemes of electron transport. A scheme representing current ideas on the arrangement of the cytochromes in the electron transport chain is shown in Figure 3. The cytochromes appear to be embedded in a

lipoprotein matrix to form the integrated particulate complex as it occurs in the mitochondrion.

Figure 3. Tentative scheme of arrangement of electron transport system intermediates (after Slater, 1958).



2. BACTERIAL CYTOCHROMES.

As has been noted in the foregoing section the cytochrome content of mammalian tissues of different origins is remarkably constant. However, such is not the case with other types of living organisms, and bacteria especially are noted for the diversity of their cytochrome content. Almost every conceivable assortment of cytochrome spectra occurs in bacteria, ranging from the mammalian cytochrome combination, through odd assortments of unusual cytochromes, to complete lack of cytochromes. The subject of bacterial cytochromes is therefore a very complex one. A complete review of all the bacterial cytochromes so far reported and of the organisms in which they have been found, will not be attempted here, but information of this nature can be obtained in articles by Smith (1954, b), Morton (1958), and by Kamen (1955).

For the main part our knowledge of bacterial cytochromes has so far accumulated from spectrophotometric observations of whole cell suspensions and crude extracts, and in only several cases from observation of the isolated cytochrome. Unfortunately, there is considerable disagreement in earlier reports of bacterial cytochrome content, largely due to the use of inferior techniques. The main bacterial cytochromes reported are tabulated in Table 3, together with several bacteria in which each cytochrome is

TABLE 3. Bacterial cytochromes.

Cytochrome	Absorption maxima			CO binding	Representative bacteria.
a	604		445	-	Bacillus subtilus, Sarcina lutea.
a_1	590		440	+	Acetobacter pasteurianum, Escherichia coli.
a_3	605		445	+	Bacillus subtilus, Sarcina lutea.
a_4	628			+	Acetobacter peroxydans
a_2	630			+	Azotobacter vinelandii, Escherichia coli
b	564	530	430	-	Bacillus subtilus, Staphylococcus albus.
b_1	560	533	429	-	Escherichia coli, Pseudomonas denitrificans.
b_4	554	521	418	-	Halotolerant coccus
c	550	521	415	-	Pseudomonas fluorescens
c_1	553	523	419	-	Pseudomonas spp.
c_2	550	520	417	-	Rhodospirillum rubrum
c_3	553	525	419	-	Desulphovibrio desulphuricans.
c_4	551	523	416	-	Azotobacter vinelandii
c_5	555	526	420	-	Azotobacter vinelandii

Based on data of Lemberg (1960), Morton (1958) and Smith (1954). The positions of absorption peaks are shown in mu for the direct spectrum of the reduced compound.

Whether or not the cytochrome combines with carbon monoxide at physiological pH is shown by + or - signs.

located. The results so far accumulated do not indicate that the occurrence of cytochromes in bacteria is very systematic, but some general observations can be made.

(i) Some bacteria show absorption spectra in the visible region, which are very similar to the mammalian cytochrome spectrum. On the basis of spectrum, therefore, the cytochrome content of these bacteria seems to be the same as that of mammalian tissue. Bacillus subtilus and Sarcina lutea are bacteria falling into this category.

(ii) Cytochrome a is often found to be replaced by cytochrome a_1, usually accompanied by cytochrome a_2. It appears that the classical type a cytochrome never occurs together with cytochromes a, and a_2. The absorption spectrum of cytochrome a_2 shows a α-peak at about 630 mu which is far removed from the position of the other a cytochromes between 588 and 612 mu. Cytochrome a_2 is further distinguished from the other a cytochromes by having a dihydroporphyrin (chlorin a_2) as its prosthetic group. The properties of cytochrome a_2 have prompted Morton (1958) to place cytochrome a_2 in a separate group from the other a cytochromes (Table 2). Escherichia coli and Azotobacter chroococcum are examples of bacteria containing cytochromes a_1 and a_2.

(iii) Some bacteria which do not contain cytochromes b and c have a single band at about 560 mu which has been

attributed to the presence of cytochrome b_1. It has been suggested that this band is a result of the bands of cytochrome b and c fused together. Keilin and Harpley (1941), however, showed that this suggestion was false by low temperature observations of the spectrum of E.coli, one of the organisms in which this cytochrome appears. Smith (1954,a) also showed that this peak is not due to the fused bands of cytochromes b and c in Proteus vulgaris by warming the bacteria at 70° for 10 minutes, a procedure which destroys cytochrome b. The cytochrome b_1 peak remained unchanged after warming.

(iv) As mentioned earlier, some bacteria contain c cytochromes that appear to be the same as mammalian cytochrome c. However, organisms were also found that contain cytochromes spectroscopically similar to, but enzymically different from mammalian cytochrome c. Rhodospirillum rubrum, for example, contains a soluble cytochrome whose absorption spectrum closely resembles that of mammalian cytochrome c. This compound, however, is not oxidized by heart muscle cytochrome oxidase and so the name cytochrome c_2 has been suggested for it (Elsden et al., 1953). Similar discoveries led to the naming of several other new c cytochromes that are shown in Table 3.

(v) At least one bacterium, Streptococcus faecalis, contains no cytochromes at all, yet can respire, apparently via a flavin oxidase.

The function of the bacterial cytochromes.

Just as the cytochrome content of bacteria varies, so also does the function of these compounds. In mammalian tissue, the sole function of the cytochromes, with possibly a few minor exceptions, is in terminal oxidative respiration. In bacteria, on the other hand, the cytochromes are involved in a number of processes.

(i) The main function of the bacterial cytochromes is in the terminal oxidative processes of the cell. The b and c cytochromes of bacteria are thought to act as electron carriers and cytochrome a_3 as terminal oxidase. However, many bacteria do not contain cytochrome a_3 and it is thought that cytochrome a_2 may function as terminal oxidase in many bacteria.

(ii) Sato and Egami (1949) have reported the involvement of cytochrome b_1 in the reduction of nitrate to nitrite by a system called "nitrate reductase" in E. coli. Another bacterial reduction system, by which sulphate is reduced, has been found in Desulphovibrio desulphuricans to involve cytochrome c_3 (Postgate, 1956).

(iii) The remarkable persistence of c cytochromes has been noted in a number of bacteria having little in common other than the ability to grow photosynthetically. These observations suggest that a c cytochrome may be connected with the photosynthetic processes of these bacteria. Furthermore, evidence has been reported supporting the involvement

of a cytochrome, thought to be cytochrome c_2, in the photometabolism of Rhodospirillum rubrum (Vernon and Kamen, 1953; Duysens, 1954).

(iv) There have also been reports of the possible involvement of cytochrome in nitrogen fixation by bacteria (Lemberg, 1960; Lindstrom et al., 1950).

Location of bacterial cytochromes.

It has been shown that the site of action of the bacterial cytochromes is in the particulate material of the cell. Weibull (1953) has shown this by digesting away the cell wall of Bacillus megaterium by lysozyme, and lysing the resulting protoplasts in hypotonic salt solution. The particulate material separating on centrifugation of the lysate has been identified as the cytoplasmic membrane of the cell. In this material was concentrated the entire cytochrome system of the cell together with the particulate dehydrogenases. For the most part these cytochromes and dehydrogenases are firmly bound to the particulate material and relatively severe treatment is required to solubilize them from this material. In fact, it is only in several isolated cases that this has been achieved. Work of this nature will be described in this thesis involving the solubilization of a particulate dehydrogenase and an associated cytochrome. The enzyme involved is formic dehydrogenase which is associated with cytochrome b_1 in the particulate material of E.coli. The system in which these two components are involved will now be discussed.

II. FORMIC DEHYDROGENASE IN ESCHERICHIA COLI.

Formic dehydrogenase activity has been recognized in E. coli since the early days of enzymology (Quastel and Whetham, 1925), but even now comparatively little is known about it. Gale (1939) was the first to investigate its properties in any detail. He described the enzyme as being very firmly bound to the particulate material of the cell. This finding was confirmed in the early stages of the present studies (Wrigley, 1958). Indeed so strongly is it attached to the particulate material, that attempts by several subsequent workers to extract the enzyme in a soluble form have been unsuccessful (Pinsent, 1954; Wolin and Lichstein, 1956).

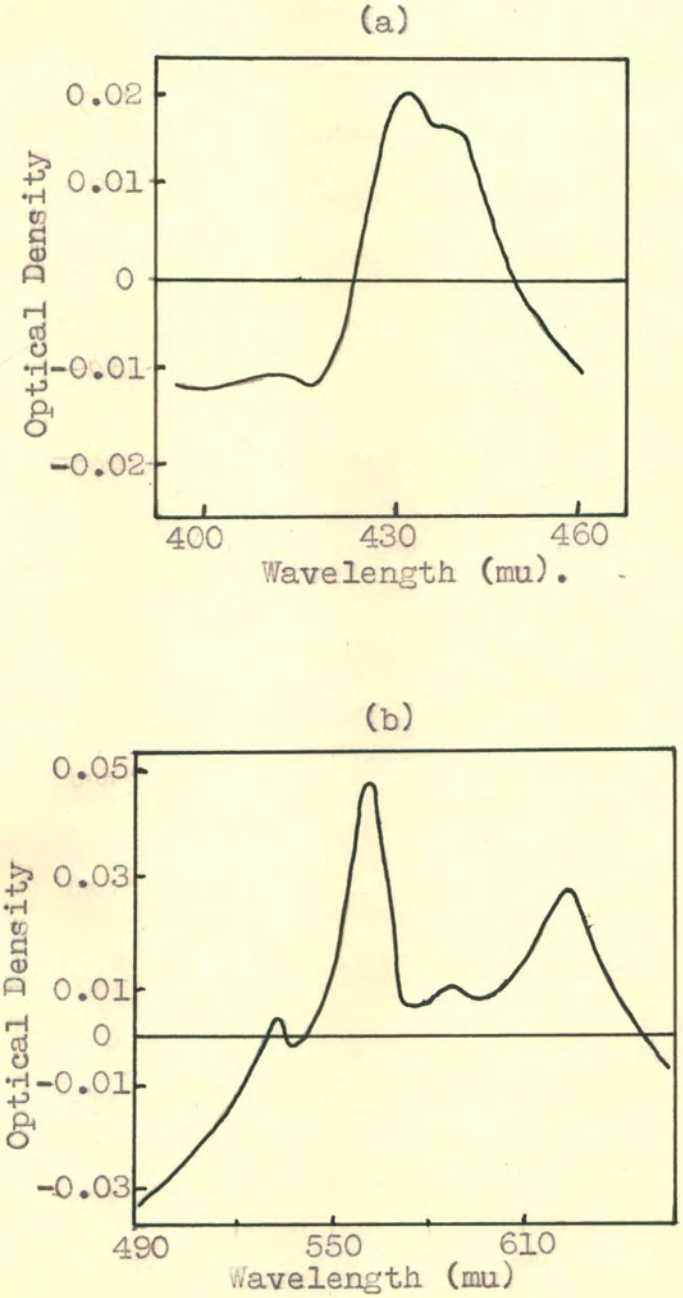
Cytochrome b_1 involvement.

An important finding of Gale (1939) was that the enzyme was closely associated in the particulate fraction with cytochrome b_1, which was observed to be reduced by formate. This cytochrome occurs in E. coli together with cytochromes a_1 and a_2, as is seen in the difference sprectrum of the whole cells in Figure 4 (Smith, 1954,a).

Generally speaking the b cytochromes are very firmly attached to the particulate material of the cell. Mammalian cytochrome b has not yet been extracted in water-soluble form A cytochrome b_2 has been purified in crystalline form from yeast cells and is readily soluble in water (Appleby and

Morton

FIGURE 4. Difference spectrum of E. coli whole cells. The cell suspension used to record the visible region of the spectrum (b) was three times more concentrated than that used to record the Soret region (a). (Reproduced from Smith, 1954, a.)



Morton, 1959). However, this b cytochrome is exceptional in that it is only loosely attached to the yeast mitochondria.

There have been several attempts to extract cytochrome b_1 of E.coli into soluble form. Since the development of the cytochrome b_1 solubilization procedure that will be described in this thesis, Williams and Hagar (1960) reported the solubilization of cytochrome b_1 from E.coli by treatment with trypsin. However, the α-peak of the reduced preparation appears at 556 mu, 4 mu removed from the usually reported position of 560 mu. This shift in the α-band may be due to modification of the protein moiety of the cytochrome by trypsin treatment.

Association with nitrate reductase.

Gale (1939) reported that in the unfractionated particulate material of E.coli, formate was oxidized to carbon dioxide and water by molecular oxygen. Taniguchi and co-workers have reported an alternative pathway in E.coli, whereby formate oxidation is coupled to the reduction of nitrate (Taniguchi et al., 1958; Iida and Taniguchi, 1959). They have called this system "nitrate respiration" in contrast to the normal oxygen respiration system. The primary enzyme in this system is nitrate reductase which catalyses the reduction of nitrate to nitrite in the presence of a suitable electron donor. This enzyme is linked to formic dehydrogenase by cytochrome b_1 and is firmly bound to the particulate fraction of the cell.

The studies to be described in the following sections, involve the investigation of formic dehydrogenase and its relation to the molecular organization of the particulate material of the cell. This problem was attacked by extracting the enzyme from the particulate material with a view to isolating with it some of the neighbouring components of the respiratory chain. The task was therefore undertaken of solubilizing formic dehydrogenase and investigating the properties of the resulting preparation.

METHODS.

Organisms.

The strain of E.coli used throughout these investigations was one designated "20.101". This organism was chosen in a review of organisms available in the Biochemistry Department, Sydney University (Wrigley, 1958). This strain was found to have the best combination of high formic oxidase activity, large yield of cells and low blank respiration. Stock cultures were maintained on agar slopes composed of the medium normally used for liquid culture with agar added to 2.5%. The organisms were subcultured every 4 weeks by inoculating sterile slopes. The slopes were incubated at 37° for 24 hours to allow adequate cell growth and were then stored at 4°.

Growth conditions.

The medium giving best cell growth and formic oxidase activity was found to be a medium denoted as "Nutrient Broth". (See Results section for discussion of this medium). Its composition was as follows:-

0.3% beef extract (Riverstone Meat Co., Sydney).
0.5% microme peptone (E. Gurr, Ltd.)
0.5% NaCl
1.0% glucose
in 0.03 M potassium phosphate.

The medium was brought to pH 6.5 by the addition of NaOH or

H Cl and autoclaved at a pressure of 10 pounds per square inch for 30 minutes.

Organisms were routinely grown aerobically at 30°in a continuous culture apparatus. A flask containing 200 ml. of medium was inoculated from an agar slope and grown overnight at 30° on a circular shaker. This cell suspension was used to inoculate 4 litres of medium in the culture flask of the apparatus. The contents of the culture flask were stirred vigorously and aerated at a rate of about 10 litres per minute. After about 6 hours' growth, fresh medium was run into the culture flask at the rate of about 1.5 litres per hour. The cell suspension was collected in two 18 litre glass containers. When nearly filled, these containers were removed to the cold room to await harvesting. Usually a total of 60 litres of medium was used for each growth experiment. Care was taken that all growth medium, containers and connecting tubing were kept sterile during cell growth. All air passed through the culture flask was sterilized by entrance and exit filters. At various stages of the growth process a sample of the culture was examined under the microscope as a check on purity.

Preparation of Organisms for Disintegration.

Cells were harvested as soon as possible after growth by centrifuging in the Sharples continuous super-centrifuge at about 60,000 x g. Cells were washed once in a large volume of 0.01M KCl (10 - 15 litres). This washing was

found to be sufficient to reduce to a neglibible level, the blank respiration of the cells (with respect to oxygen uptake or ferricyanide reduction). Sixty litres of medium yielded about 150-200 g. wet weight of cells. The organisms were resuspended by blending the packed cells with two volumes of the suspending medium in a Waring blendor. The cells were harvested at room temperature and stored at about 0°. All subsequent operations were conducted at 0° unless otherwise stated.

Cell rupture and fractionation.

The conditions of cell breakage adopted were the best of a variety of breaking media and methods tested. (See Results Section). The packed cells, after harvesting, were suspended in two volumes of 0.03M potassium phosphate buffer, pH 6.8. A volume of 120 ml. of this cell suspension was homogenized for 10 minutes with 120 ml. of ballotini glass beads in an overhead-drive blendor. A dry ice-alcohol bath at - 10°was used to maintain the temperature of the homogenate close to 0°. The design and use of the blendor is discussed more fully by Linnane and Vitols (1961, in press). Sixty litres of growth medium usually provided enough cells for four such disintegrations.

After cell rupture, the glass beads were separated from the homogenate by low speed centrifugation. Trapped material was removed from the glass by washing the beads three times in 0.02M phosphate buffer, pH 6.8. Centrifugation of the glass-free homogenate at 5,000 x g. for 20 minutes served to

remove any remaining whole cells and large cell debris. The resulting supernatant was then centrifuged at 55,000 x g. for 30 minutes. The material deposited by this centrifugation is the fraction used in the following studies and is denoted as the "cell residue" or "particulate fraction". This material was washed once in 0.02M KCl and resuspended in 0.02M KCl by homogenizing in a Waring blendor or Potter and Elvejham homogenizer.

Ballotini glass beads.

The glass beads used for cell disintegration were obtained from Ballotini Manufacturing Co., Barnley, Yorkshire, England. Before use, the beads were sieved and the beads ranging in size from 0.10 - 0.18 mm. in diameter were collected and boiled in several changes of concentrated H Cl. After thorough washing in tap and distilled water the beads were dried in an oven at 100° and then stored in a cold room ready for use. After each use the beads were recovered for further use by cleaning in H Cl (about 3 N), followed by washing with water and drying once again.

Assay of the formate oxidizing enzyme system.

The formate oxidizing enzyme system was assayed by several methods: by measuring the rate of oxygen uptake or by following the reduction of potassium ferricyanide manometrically or spectorphotometrically. The development of these assay methods is described in the Results Section. All assays were conducted at 30° unless otherwise stated.

All formate enzyme assays were carried out at pH 6.0, the optimum assay pH of the enzyme (Gale, 1939). Specific activity was expressed as umoles of formate oxidized per minute per mg. of protein. Total activity was the product of the specific activity and the total protein.

Formic oxidase assay.

The rate of oxygen uptake during formate oxidation was measured manometrically using flasks without a side arm. The assay mixture was as follows:-

Main compartment.

0.1 ml. Magnesium sulphate, 0.05M. (5 umoles)

0.5 ml. Potassium phosphate buffer, 0.1M, pH 6.0 (50 umoles)

0.2 ml. gelatin, 2.5%. (5 mg.)

0.1 ml. formate, 0.4M. (40 umoles)

water to a vol. of 3.0 ml.

Enzyme preparation (sufficient to give a change in momometer fluid height of about 20 mm./5 minutes)

Centre well: 0.2 ml. 20% potassium hydroxide.

The flasks were kept on ice during addition of the reactants. The reaction was initiated by removing the flasks from the ice into the manometer bath (30°). After 5 minutes' equilibration the taps were closed and readings taken every 5 minutes for 30 minutes. The initial oxygen uptake rate was used to calculate the formic oxidase activity after correcting for blank respiration.

Manometric formic dehydrogenase assay.

The rate of ferricyanide reduction by formic dehydrogenase was followed manometrically by measuring the rate of acid production in bicarbonate buffer. The manometer flasks used had one sidearm and contained the following mixture:-

Main compartment:

0.1 ml. magnesium sulphate, 0.05 M (5 umoles)

0.7 ml. sodium bicarbonate, 0.06 M (42 umoles)

0.2 ml. gelatin, 2.5% (5 mg.)

Water to a volume of 2.8 ml.

Enzyme preparation (sufficient to give a change in manometer fluid height of about 20 mm./5 minutes).

Side arm:

0.1 ml. sodium bicarbonate, 0.06M (6 umoles)

0.1 ml. sodium formate, 0.4M, (40 umoles)

potassium ferricyanide (as specified below)

water to a volume of 0.4 ml.

The flasks were kept cold by standing them in ice whilst additions were made. When all reactants were added the flasks were gassed for 5-10 minutes with pure carbon dioxide and then placed in the manometer bath for equilibration at 30°. Commencing at the second minute after tipping, readings were taken at 5 minute intervals for 30-40 minutes. The specific activity was calculated from the initial rate of carbon dioxide evolution, correcting readings for blank

respiration. Three moles of carbon dioxide evolved represented one mole of formate oxidized.

Two procedures were adopted with relation to ferricyanide concentrations:

(i) The assay was conducted in the present of one particular ferricyanide concentration, usually 20 umoles of ferricyanide per manometer. Where this method of assay was used the ferricyanide concentration will be quoted in the text.

(ii) Several manometers were set up containing different concentrations of ferricyanide. Usually concentrations of 50, 75, 100 and 200 umoles of ferricyanide per flask were used together with appropriate substrate blanks. The rate of oxidation of formate was calculated for each ferricyanide concentration. The rate of formate oxidation at infinite ferricyanide concentration was determined from these values, using the double reciprocal plot method. (Singer and Kearney, 1957). This rate at infinite ferricyanide concentration was called the maximum velocity or V_{max}.

Spectrophotometric formic dehydrogenase assay.

The rate of reduction of ferricyanide by formic dehydrogenase was followed by measuring the decrease in optical density at 410 mu. The assay was conducted at room temperature (20° - 25°). Reactants were added to the 1 cm. light path cuvette in the following order:

0.5 ml. phosphate buffer, 0.1M, pH 6.0 (50 umoles)
0.2 ml. potassium ferricyanide, 0.01M (2 umoles)
0.1 ml. magnesium sulphate, 0.05M (5 umoles)
0.2 ml. gelatin solution, 2.5% (5 mg.)
water to a total volume of 2.7 ml.
0.1 ml. fresh potassium cyanide, 0.01M (for cell residue assay only) (1 umole)
Enzyme preparation (to give an optical density change of about 0.02 per 30 seconds).
0.2 ml. sodium formate, 0.2M (40 umoles)

The reactants were quickly mixed and optical density readings were taken at 30 second intervals for about 5 minutes. A substrate blank was set up and assayed simultaneously with the test cuvette. The average initial rate of decrease of optical density (blank corrected) was used to calculate specific activity. The extinction coefficient of potassium ferricyanide was taken as being 1.0×10^3 cm^2/millimole. The extinction of potassium ferrocyanide at 410 mu is negligible.

Formic dehydrogenase - phenazine methosulphate assay.

Formic dehydrogenase activity with respect to phenazine methosulphate was assayed by measuring oxygen uptake manometrically in presence of the dye which is highly autooxidizable. The reaction mixture was as follows:

Main compartment:

0.1 ml magnesium sulphate, 0.05M (5 umoles)
1.0 ml potassium phosphate, 0.1M, pH.0 (100 umoles)
0.2 ml gelatin, 2.5% (5 mg.)

water to a volume of 2.8 ml.

Enzyme preparation (sufficient to produce a change in manometer fluid height of about 20 mm per 5 minutes).

Side Arm:

Phenazine methosulphate, 1%, as specified

0.1 ml sodium formate, 0.4M. (40 umoles)

water to a volume of 0.4 ml.

Centre well:

0.2 ml. potassium hydroxide, 20%.

Phenazine methosulphate was made up in distilled water and stored at -10°C in the dark. Under these conditions the dye solution is stable for several months. As the solution is light sensitive, it was added to the manometer flask as one of the last components. The flasks were iced during the addition of the reactants, after which the flasks were equilibrated for five minutes in the manometer bath (30°). After tipping, readings were taken at five minutes intervals for about thirty minutes.

Singer and Kearney (1957) have reported that hydrogen peroxide is produced as a result of the autoxidation of phenazine methosulphate. So that the possible inhibitory effect of hydrogen peroxide on the reaction would be reduced to a minimum, calculations were based on the initial rate of oxygen uptake. The specific activity was calculated by equating umoles of oxygen utilized to umoles of formate

oxidized. The reaction rate at infinite dye concentration ($V_{max.}$) was determined by the double reciprocal plot method, using phenazine methosulphate concentrations of 0.5, 1.0, 2.0 and 3.0 mg. per flask.

DPNH oxidase assay.

DPNH oxidase activity was assayed by following the rate of decrease of optical density at 340 mu in the system described below. Reactants were added to a 1 cm. light path cuvette in the following order:

1.0 ml. 0.2M phosphate, pH 7.5 (200 umoles).

0.3 ml. 5 x 10^{-4}M Versene (0.15 umoles).

Water to a total volume of 2.7 ml.

0.2 ml. 5% B.S.A. (10 mg.)

enzyme solution (sufficient to produce an optical density change of about 0.02 per 30 seconds).

0.1 ml. 0.2% DPNH (0.2 mg.)

The reaction was initiated by mixing in the substrate and optical density readings were taken at 30 second intervals for about 5 minutes thereafter. A blank cuvette containing no DPNH was examined simultaneously. The assay was conducted at room temperature (20° - 25°).

The rate of DPNH oxidation was calculated from the initial rate of decrease of optical density, assuming an extinction coefficient for DPNH at 340 mu of (6.25 x 10^3) x cm^2 per millimole.

DPNH dehydrogenase assay.

DPNH dehydrogenase was assayed by a similar method to DPNH oxidase, with the further addition of 0.1 ml. of 0.02M potassium ferricyanide (2 umoles) to act as artificial electron acceptor. For the assay of the cell residue, it was necessary to make the further addition of 0.1 ml. of 0.02M potassium cyanide (2 umoles) in order to depress DPNH oxidase activity.

Nitrate reductase assay.

Nitrate reductase activity was assayed by estimating the nitrite produced after incubating the enzyme preparation with nitrate and an artificial electron donor. The assay was carried out in Thunberg tubes using the following reaction mixture:

Main tube:

0.4 ml potassium phosphate buffer, 0.2M, pH6.8 (80 umoles).
0.1 ml magnesium sulphate, 0.05M. (5 umoles).
0.3 ml sodium hydrosulphite, 0.5% (FRESH) (1.5 mg.).
0.1 ml. potassium nitrate, 0.1M (10 umoles).
Water to a volume of 1.4 ml.

Side arm:

0.2 ml. gelatin, 2.5%
0.2 ml. benzyl viologen, 0.1% (0.2mg.)
Water to a volume of 0.6 ml.

As the side arm contained the enzyme preparation, it

was kept chilled with ice. The Thunberg tubes were evacuated and flushed repeatedly with oxygen-free nitrogen, adding the hydrosulphite solution immediately before gassing. The tubes were removed from the ice and immersed in the water bath (30°) at one minute intervals in order to allow time for subsequent manipulations on each tube. After each tube had been in the bath for three minutes, the side arm contents were tipped in and the reaction was allowed to proceed for exactly five minutes. The reaction was stopped by removing the stopper, and placing each tube in a boiling water bath for two minutes, aerating to remove excess hydrosulphite. Nitrite was determined on a 1.0 ml. aliquot, which was centrifuged clear before making spectrophotometric measurements. Nitrite formation (blank corrected), was equated to nitrate reduction in calculating nitrate reductase activity. Specific activity was expressed as umoles nitrate reduced per minute per mg. protein.

Nitrite was estimated by the method of Snell and Snell (1949), measuring the optical density at 540 mu of the red colour formed by the addition of sulphanilamide (diazotizing agent) and N-(1- naphthyl) ethylenediamine hydrochloride (coupling agent) to the nitrite-containing solution. This method was suitable for samples containing from 2 to 70 m umoles of nitrite. The colour produced was stable for several hours. A linear relationship was found to exist between nitrite concentration and optical density

such that 6 mu moles of nitrite corresponded to an optical density of 0.100. This relationship was reproducible from one determination to another.

Formate-nitrate reductase assay.

The formate-nitrate reductase system was assayed by a procedure similar to the nitrate reductase assay method except that 0.1 ml. 0.4M sodium formate (40 umoles) in the main compartment replaced the dye-reductant mixture.

Protein determination.

Protein concentration was routinely determined by a modification of the Biuret method of Gornall et al. (1949). An aliquot of the protein solution for assay was taken containing between 0.5 and 3.0 mg. protein. To this was added either 1.0 ml. of IN or 0.2 ml. of 5N sodium hydroxide and the solution diluted to 1.2 ml. with water. If at this stage the protein did not dissolve easily, the solution was heated in a boiling water bath for up to 2 minutes, taking precautions to prevent loss of water by evaporation. Clarification was further aided by the addition of 0.3 ml. 1% sodium deoxycholate. Biuret reagent, 1.5 ml. was then added. After incubation at 37° for 15 minutes the solutions were cooled to room temperature and clarified by concentration if necessary. The optical density at 540 mu was measured in a 1 cm. light path cell with a reagent blank as reference cell.

Using dry bovine serum albumin a straight line relationship was found to exist between optical density and protein concentration using up to 3 mg. protein, in the proportion of 1.0 mg. protein to an optical density of 0.095. A similar relationship existed for protein isolated from the soluble formic dehydrogenase preparation after removal of lipid and other contaminants by extraction with chloroform-methanol (1:1). Unless otherwise stated it should be assumed that protein concentration was assayed by the above method.

In very dilute solutions, protein was determined by measuring the optical density at 260 mu and at 280 mu. The formulation suggested by Layne (1957) was used in this determination, namely:

Protein concentration (mg./ml.) = $1.55 \times OD_{280} - 0.76 \times OD_{260}$

OD_{260} where OD_{280} and OD_{260} are the optical densities at 280 mu and 260 mu.

This method is only of use when the ratio $OD_{280}:OD_{260}$ did not fall below about 0.55. Values obtained by this method were regarded as being only approximate. This method was only used with the fractions resulting from purification of the solubilized preparation with tricalcium phosphate gel and DEAE - cellulose.

Dry weight determinations

The dry weight of a solution or suspension was determined by evaporating a salt-free aliquot to dryness

in a tared 30 ml. beaker in the oven at 100°. The beaker was then dried to constant weight in vacuo over fresh phosphorus pentoxide.

For rapid approximate determination of the dry weight and protein concentrations of cell suspensions, a turbidity graph was set up, plotting the relationship between the optical density at 650 mu and the dry weight and protein concentration of the suspension.

Manometry.

Volumes of the manometer flasks and U-tubes were determined gravimetrically using mercury. Flasks with and without a side-arm were used. The average volume of each type of flask was about 32 and 23 ml., respectively. The flasks were shaken at about 100 oscillations per minute in a circular water bath maintained at 30°.

Centrifugation.

An International refrigerated centrifuge, Model PR2, was used for routine centrifugation in the cold. High speed centrifugation was carried out in a Spinco, Model L, Preparative Ultracentrifuge.

Photometric Determinations.

Generally spectrophotometric measurements were carried out using a Hilger Uvispec spectrophotometer. A Cary, Model 14, recording spectrophotometer was used for recording all absorption spectra.

Cytochrome b_1 estimation.

As no values are available for the extinction coefficients of cytochrome b_1, it was necessary to use data from other b cytochromes in the estimation of cytochrome b_1.

1. Estimation based on mammalian cytochrome b.

The method of Chance (1952) was used in the estimation of cytochrome b_1, using the extinction coefficient for mammalian cytochrome b. The formula suggested is

$$\text{Cytochrome b concentration} = \frac{OD_{561} - OD_{575}}{20} \text{ mM.}$$

where OD_{561} and OD_{575} refer to the absorbancies of the difference spectrum (reduced minus oxidized) at 561 and 575 mu for a 1 cm. light path cuvette.

2. Estimation based on yeast cytochrome b_2.

A further value for cytochrome b_1 concentration was obtained by using the direct extinction coefficient for reduced yeast cytochrome b_2, at 557 mu of 38.8×10^3 $cm.^2$ per millimole (Appleby and Morton, 1959,b).

3. Preparation of pyridine haemochromogen.

The method used for the preparation of the pyridine haemochromogen was that described by Basford *et al.* (1957). Lipid was first removed from the preparation by extracting with neutral acetone and with a chloroform-methanol mixture. The protein precipitate was then extracted with acid acetone in order to isolate the cytochrome b_1 prosthetic group,

protohaem, which was coupled to pyridine in alkaline solution.

Throughout the procedure, care was taken to keep the preparation cool (0^{o}- 4^{o}) and shielded from the light. It was important that neutral acetone be used in the initial step of lipid removal, in order to prevent loss of protohaem, which is extracted by acid acetone. The absorption spectrum of the pyridine haemochromogen was recorded as soon as possible after the addition of alkaline pyridine to the extracted protohaem. The concentration of cytochrome b_1 was calculated from the absorption spectrum assuming an extinction coefficient of 34.7×10^{3} cm.^{2} per millimole for the α - peak (557 mu) of the reduced pyridine haemochromogen direct spectrum.

Flavin estimation.

Flavin was estimated by the procedure described by Green et al. (1955) by extraction with dilute perchloric acid. Flavin extracted after digestion was termed "total flavin". Care was taken throughout the procedure to protect solutions from the light.

Isolation of coenzyme Q and vitamin K.

Coenzyme Q and vitamin K were isolated by direct extraction with iso-octane as described by Lester and Crane (1959). In the finalized procedure, the preparation for examination was dried in the oven at 100^{o}, before repeated extraction with iso-octane. In order to assay each of the

quinones in the resulting iso-octane extract it was necessary to to separate them by Decalso chromatography in the following manner:

An iso-octane extract of the material isolated from the dried preparation was applied to a column of Decalso resin, 5 cm. high and 1.7 cm. in diameter. When dealing with whole cells, the material extracted from 0.5 g. dried cells was applied to a column of this size. The column was eluted with 50 ml. volumes of each of four solvent systems. Elution with these solvents was carried out in the following order: iso-octane, 5% ether in iso-octane, 10% ether in iso-octane and 10% ethanol in iso-octane The column was eluted at a rate of about 0.5 ml. per minute and fractions of 10 ml. volume were collected. The resulting fractions were examined in the recording spectrophotometer for the presence of coenzyme Q and vitamin K. Care was taken that none of the solvents used in the above procedure contained impurities absorbing above 230 mu. Only glass apparatus was used, as other materials were found to interfere profoundly with spectrophotometric measurements.

The level of coenzyme Q was estimated by measuring the decrease in optical density at 275 mu on reduction with potassium borohydride in ethanol. For this estimation an extinction coefficient of 142 was assumed for a 1% solution of coenzyme Q and a light path of 1 cm. (Crane et al., 1959). The level of vitamin K was estimated from its absorption

spectrum, assuming an extinction coefficient of 435 at 249 mu for a 1% solution and a 1 cm. light path. (Ewing et al., 1943). No attempt was made in these studies to determine which vitamin K homologue was extracted, so that the general name of vitamin K will be used throughout.

Lipid estimation.

Lipid was estimated by direct weighing of chloroform - soluble material. An aliquot of the preparation that contained about 70 mg. protein in approximately 3 ml. distilled water, was mixed with 7.5 volumes of methanol in order to precipitate the protein. Chloroform (7.5 volumes) was then added and after stirring for 15 minutes at 40° the precipitated protein was removed by centrifugation. The protein precipitate was re-extracted in the same manner with three 30 ml. portions of chloroform-methanol (1:1). The combined chloroform-methanol extracts were evaporated to dryness on the rotary evaporator and the residue was extracted with four 5 ml. portions of chloroform by shaking for 15 minutes at 40°. Chloroform-insoluble matter was removed by centrifugation and the combined chloroform extracts were carefully evaporated to dryness in a tared beaker. The beaker and contents were dried to constant weight in vacuo over fresh phosphorous pentoxide.

In order to obtain a sufficiently concentrated solution of the solubilized material for lipid determination, the preparation was freeze-dried. The resulting freeze-

dried powder was dissolved in a small volume of distilled water for the above determination. Only glass apparatus was used in the extraction procedure. The solvents used were checked to ensure that no solid material remained after evaporation.

Pentose assay.

Pentose concentration was assayed by the method of Drury (1948). The calculation of RNA content from pentose concentration was based on the fact that only the pentose attached to the purines reacts in the assay, so that phosphorus content was double the number of moles of pentose indicated. The phosphorus content of RNA was assumed to be 9%.

Hydrolysis and chromatography of the polynucleotide material.

The chloroform-insoluble residue (12 mg.) was hydrolysed in IN HCl at 100° for one hour and the components of the hydrolysate were identified by paper chromatography using an isopropenol - HCl solvent system. Both these procedures are described in full by Markham and Smith (1951).

RESULTS AND DISCUSSION.

PART I. THE ISOLATION OF FORMIC DEHYDROGENASE FROM ESCHERICHIA COLI.

The first part of the results section will deal with conditions for the growth and disintegration of cells, and will go on to describe the development of the enzyme solubilization procedure and attemps to further purify the solubilized enzyme.

GROWTH MEDIUM

Several media were investigated with a view to determining which produced the best conditions for growth and enzyme production. In these investigations, a medium denoted "nutrient broth" was used in association with a medium described by Bunting (1940). The composition of nutrient broth is shown in the Methods Section. This medium was used in the early stages of the formic dehydrogenase studies (Wrigley, 1958) but was found rather difficult to work with as it became infected easily. Dilution with Bunting's medium produced a mixture which was more convenient to handle. Preliminary experiments showed that there was very poor cell growth on Bunting's medium alone. A mixture of equal volumes of each of the two media was therefore used for comparison of cell growth with undiluted nutrient broth.

Formate was also added to the medium, as growth on relatively

high substrate concentration has, with a number of other bacterial enzymes, been observed to stimulate enzyme formation.

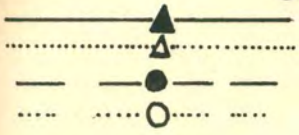
Four basic media were tested: nutrient broth, with and without 0.15% sodium formate and a mixture of Bunting's medium and nutrient broth (1:1), also with and without 0.15% formate. Each of these mixtures was set up at pH values of 5, 6, 7 and 8. Each flask was inoculated with the same quantity of cell suspension and aerated on the rotary shaker for 18 hours at 30°. At the end of the growth period the pH of the medium was in all cases lower than the initial pH. Final pH values of approximately 4.9, 5.2, 5.8 and 6.0 were obtained for media having initial pH values of 5,6,7 and 8 respectively. The protein yield and enzyme activity of the resulting cells were determined. These results are represented by the graphs appearing in Figure 5.

Dilution of nutrient broth with Bunting's medium increased cell yield at the higher pH values, but decreased enzyme formation. The addition of formate to the media also caused a slight stimulation in cell growth at the higher pH values, but completely inhibited growth at pH 5. In no case was enzyme formation enhanced by the addition of formate to the medium. The highest total oxidase activity was found in cells grown on nutrient broth alone at pH6 and pH7. The medium that was therefore adopted for routine growth of cells was nutrient broth, adjusted to pH 6.5.

FIGURE 5. Effect of culture medium on
(a) formic oxidase specific activity of cells
(b) yield of cells (total protein)
(c) total formic oxidase activity of cells.

A number of culture media were tested over a range of pH values. A 200 ml. portion of each medium was inoculated with a uniform cell suspension and was shaken for 18 hours at 30°. The resulting cells were assayed for protein and formic oxidase activity.

Culture media were denoted as follows:



Nutrient broth
Nutrient broth + 0.15% formate
Nutrient broth and Bunting's medium (1:1)
Nutrient broth and Bunting's medium (1:1) + 0.15% formate.

FIGURE 5(a). Formic oxidase specific activity of cells was expressed as umoles of formate oxidized per minute per mg. protein.

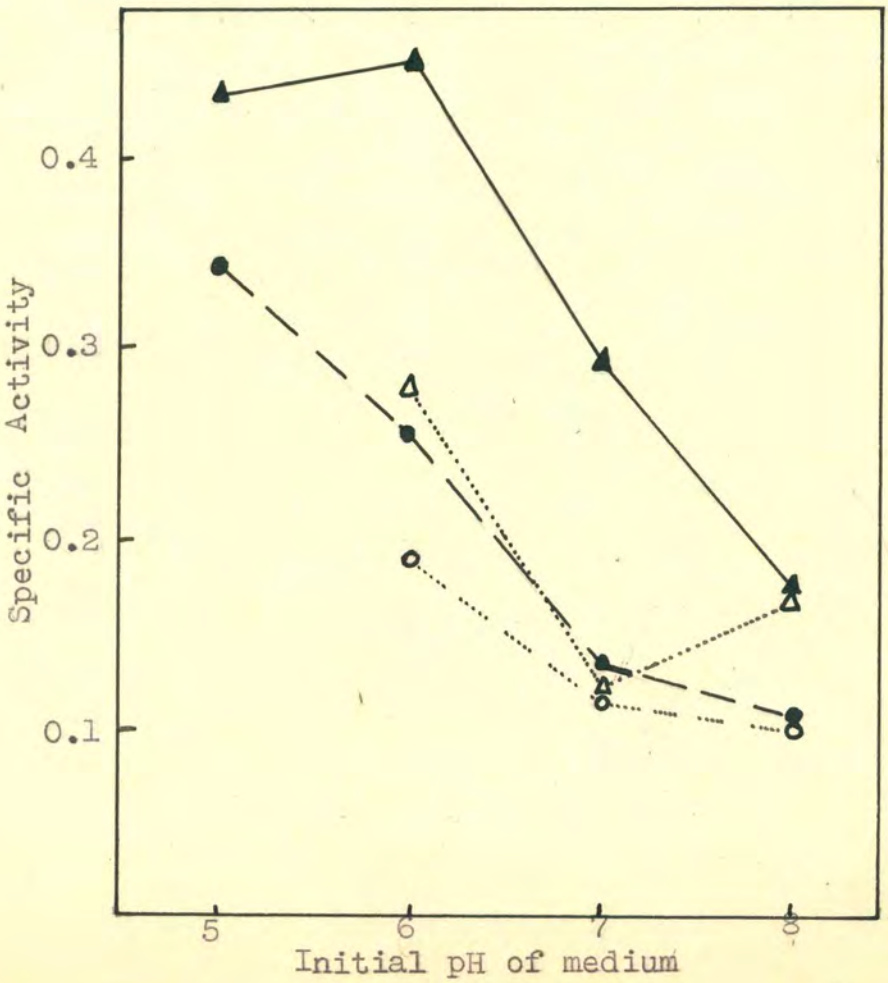


FIGURE 5(b). The yield of cells was expressed as mg. of protein (biuret determination).

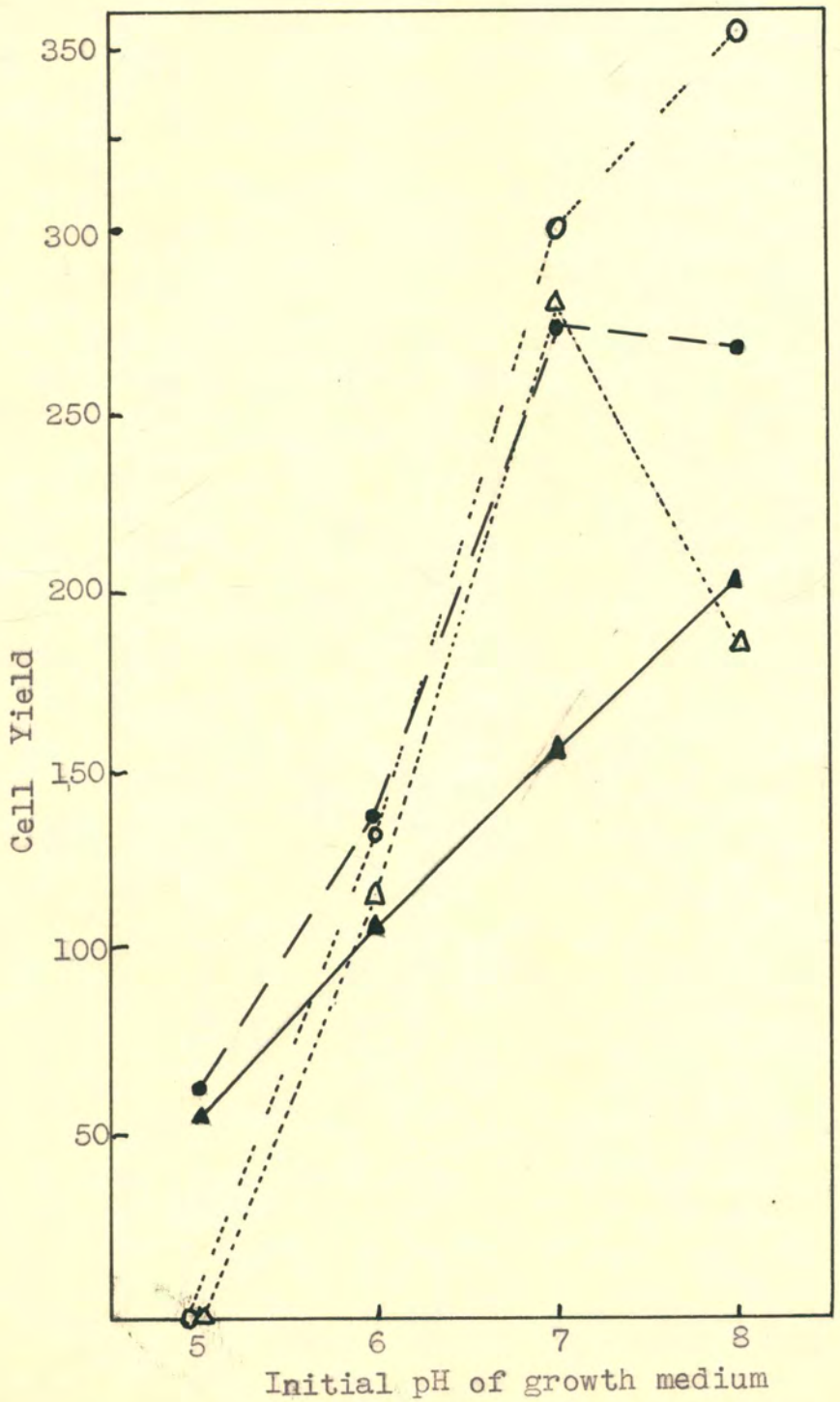
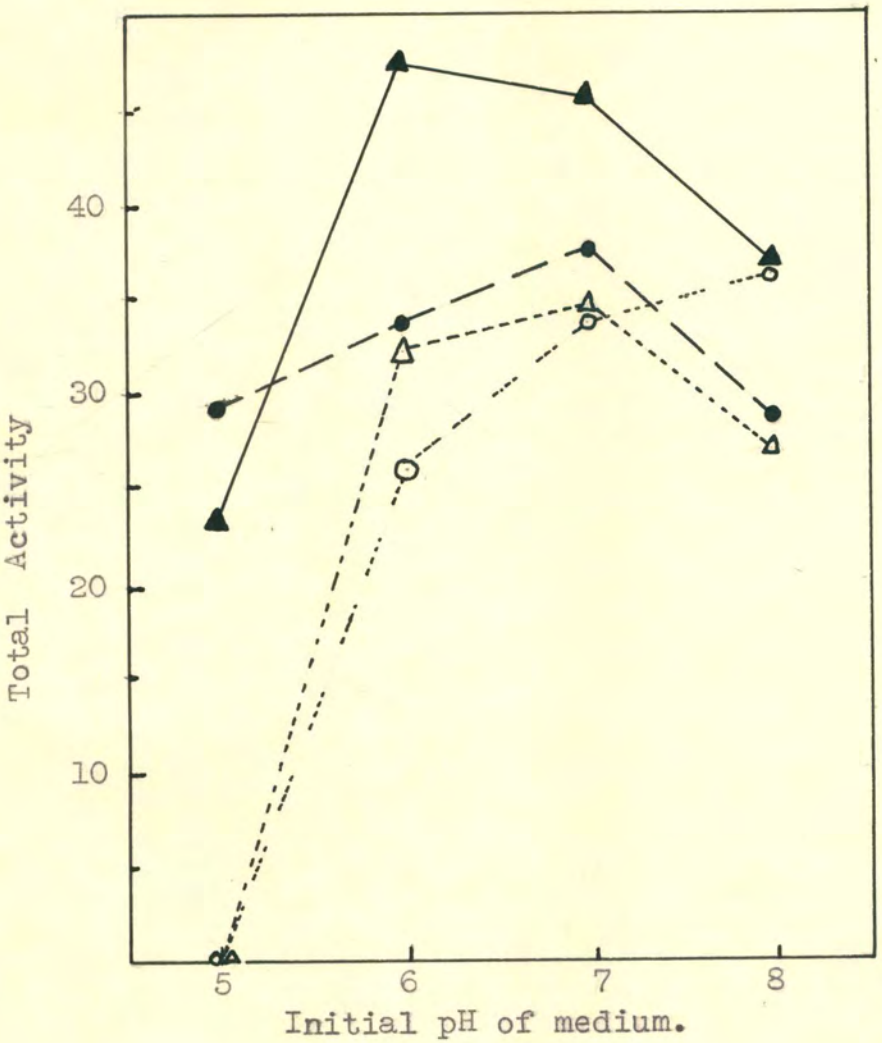


FIGURE 5(c). Total formic oxidase activity of cells was calculated as the product of the specific activity of the cells and the total protein.



These findings do not agree with those of Gale and Epps (1942), who found that in E.coli cells grown on casein digest, production of formic dehydrogenase was minimal at its optimum activity pH of 6.0. These discrepancies in results are possibly due to the fact that the basic medium used by these workers was different to that used in these present studies, and may also be attributable to strain differences.

CELL DISINTEGRATION

Suspending medium for disintegration.

Investigations were carried out to determine the most suitable suspending medium for cell disintegration. Four solutions were tested: 0.02M potassium phosphate buffer, pH 6.8; 0.02M potassium chloride; 0.5M sucrose; and 0.5M sucrose plus 0.01M sodium β-glycerophosphate, pH 7.3. The salt concentrations quoted are the approximate final concentrations present after cell rupture. The packed cells were suspended in two volumes of buffer solution which was 50% more concentrated than those above, to compensate for dilution by the cell contents released on disintegration.

The homogenate resulting from cell disintegration was divided into three fractions by differential centrifugation:

(i) unbroken cells and large particulate material ("cell debris").

(ii) smaller particulate material ("cell residue").

(iii) material not sedimented by high speed centrifugation ("soluble material").

As shown in Table 4, formic oxidase activity was completely confined to the particulate material in accordance with the work of Gale (1939) and confirming the results obtained in the earlier stages of these present studies (Wrigley, 1958).

TABLE 4. Effect of disintegration medium on protein recovery and formic oxidase activity of fractions resulting from cell disintegration.

Disintegration Medium	0.02M Potassium chloride	0.02M Potassium phosphate pH 6.8	0.5M Sucrose	0.5M sucrose 0.01M sodium β-glycero-phosphate.
Cell debris	39% (0.4)	35% (0.4)	40% (0.2)	32% (0.2)
Cell residue	5% (1.04)	9% (1.10)	8% (0.16)	15% (0.17)
Soluble material	30% (0.04)	27% (0.04)	28% (0.02)	32% (0.03)

The percentage figures above represent the proportion of the original cell suspension total protein isolated in each fraction. The values in brackets refer to the formic oxidase activity of the fraction expressed as umoles of formate oxidized per minute per mg. of protein. The cell suspension (10 ml.) together with an equal volume of glass beads, was shaken in the Nossal disintegrator for 30 seconds. After removal of the beads, the cell debris was collected by centrifugation at 5,000 x g. for 20 minutes. The resulting supernatant was centrifuged at 55,000 x g. for 30 minutes to separate the cell residue from the soluble material.

The results obtained using the various disintegration media are shown in Table 4. The total protein recovered in the fractions from cell disintegration was only about 75% of the whole cell suspension total protein in each case. This low protein recovery can be explained by the fact that a considerable amount of material was occluded by the glass beads and the quantitative removal of this material was rather difficult. As a result of the low protein recoveries, care was needed in drawing any conclusions from the results, such as relative recovery of cell residue.

However, the most striking aspect of the results was the fact that the cell residue resulting from disintegration in either of the salt solutions was had some six times higher enzyme activity than the cell residue prepared in sucrose solution. Phosphate buffer was chosen for routine use in cell disintegration but there was little to choose between phosphate buffer or potassium chloride as disintegration media on the basis of this experiment.

Method of cell disintegration.

A large number of methods have been developed for the disruption of bacterial cells, each having some special merit. Some of these methods require quite complicated and expensive apparatus whilst others can be performed using the simplest of laboratory equipment.

Grinding with glass powder or alumina is a method which requires no more complicated apparatus than a mortar and pestle. Kalnitsky and Werkman (1943) prepared a subcellular fraction containing an active formic dehydrogenase by grinding a paste of E.coli cells with glass powder. Grinding with either of these abrasives was the method routinely used for breaking E.coli cells in the early stages of these investigations (Wrigley, 1958). However, this method does not usually give a high proportion of breakage and is not suitable for large quantities of cells.

A number of machines have been devised for the disintegration of bacterial cells. The Hughes press depends upon the application of extremely high pressure to cells which are precooled to about -30°. This method provides efficient breakage but the apparatus is rather cumbersome and is only suitable for small quantities of cells.

Another machine which provides good breakage is the Mickle disintegrator (Mickle, 1948). In this machine, cells are disrupted by vigorous agitation with small glass beads about 0.15 mm. in diameter in a cylindrical glass capsule. This capsule is attached to a metal arm which is caused to vibrate in an alternating electric field. Using this disintegrator, E.coli cells suffered 80% disruption in 25 minutes. Nossal (1953), has described a similar machine in which beads and cells are agitated by mechanical shaking at 5,600 cycles per minute. In both these machines, however, effective temperature control is difficult and only small quantities of cells can be processed.

Additional methods of cell disintegration are described in a review of the subject by Hugo (1954). It is apparent that each of the cell breaking methods considered has its own particular advantages and failings. An ideal method would be considered to provide a combination of the following characteristics: The apparatus should be relatively simple, inexpensive and easily operated giving effective breakage in a short time with efficient cooling. The method should be

suitable for both small and large quantities of cells.

Several disintegration procedures were applied to the breaking of E.coli cells in an effort to develop a method providing these ideal characteristics. In co-operation with Dr. A.W. Linnane and Mr. E. Vitols a suitable method was devised. Like the Mickle and Nossal disintegrators, this method involved high speed agitation of the bacterial cells with small glass beads. The method of agitation, however, was different. The cell suspension together with an equal volume of glass beads (0.10 to 0.18 mm. in diameter), were placed in a serrated molybdenum steel bowl and agitated by means of rotating metal blades. The blades were driven by a high speed motor mounted over the bowl, so leaving the bowl free for immersion in a cooling bath. The overhead position of the motor also served to keep the glass beads clear of the motor bearings. The abrasive action of the beads was a serious problem encountered in other types of blendors used. The bowl routinely used had a total capacity of about 400 ml. and contained 120 ml. of thick cell suspension for each disintegration. The routine method of use of the machine is described in the Methods Section.

The results tabulated in Table 5, show that excellent cell breakage was produced by this machine. There was almost complete loss of viable cells after five minutes' blending. However, cells were routinely disintegrated for ten minutes, as a higher yield of cell residue resulted. The additional

five minutes' blending probably serves to further comminute large particulate material and partially damaged cells. Furthermore, the smaller particles so produced are more amenable to the solubilization procedure. There are no significant loss of formic oxidase activity as a result of the longer blending period.

TABLE 5. Efficiency of cell breakage by overhead drive blendor and Nossal disintegrator.

	Overhead drive blendor		Nossal disintegrator
Disintegration time	5 minutes	10 minutes	30 seconds
Cell residue yield	25%	34%	9%
Specific activity	1.1	1.0	1.0
Viable cells remaining	0.1%	0.0%	-

For disintegration in the overhead drive blendor, 120 ml. each of cell suspension and beads were used. For the Nossal disintegrator, 10 ml. of cell suspension were processed together with an equal quantity of beads.

The cell residue yield is expressed as the percentage of the cell suspension total protein isolated in the cell residue. The specific activity values represent the formic oxidase activities of the cell residue fractions, expressed as umoles of formate oxidized per minute per mg. protein.

The viable cells remaining are expressed as the percentage of the total original viable cell count remaining in the uncentrifuged homogenate immediately after cell disintegration. No values for viable cell count are available for the Nossal disintegrator.

In the earlier stages of these studies, the Nossal disintegrator was used. In this machine a small capsule containing cell suspension and glass beads is agitated at high speed. The results obtained using this method are also shown in Table 5. Disintegration for thirty seconds give a yield of only 9% of cell residue from whole cells. Disintegration times greater than 30 seconds could not be used conveniently, as there was no way of cooling the capsule during the operation of the machine. The main difficulty with this machine, however, was that it was only suitable for small quantities of cells.

Both of these difficulties have been overcome in the new overhead drive blendor which provides a relatively inexpensive method for the quantitative disintegration of large quantities of cells with efficient cooling.

It should be emphasised that this procedure of cell disintegration does not solubilize the enzyme, but merely permits the separation of the particulate material from the other components of the whole cell. The solubilization of the enzyme from the particulate material was next attempted.

SOLUBILIZATION OF THE ENZYME.

Introduction.

Most purified nonconjugated proteins are soluble in dilute salt solutions at pH values away from their isoelectric points. This is especially true of enzymes, which are generally albumins or globulins of relatively small molecular size. However, in the environment of the cell, enzymes are closely associated with many other compounds - other proteins, lipids, polysaccharides and nucleic acids. The properties of a particular enzyme are modified by the presence of these compounds. In particular, the solubility of the enzyme is changed. The extent to which the solubility of an enzyme is modified will depend upon its localization within the cell.

Enzymes can be divided into two main categories according to their intracellular localization, viz., those remaining in the supernatant after high speed centrifugation of the ruptured cells, and those associated with the particulate material of the cell. Those enzymes falling in the former category are released into the dispersing medium on cell rupture and can then be purified from the contaminating material by classical methods of protein fractionation. Enzymes of the latter group are generally firmly bound to the particulate structures and cannot be extracted by dilute salt solutions. It is mainly association with lipid material that prevents this extraction. Therefore, in order to extract

the bound enzymes into solution, it is often necessary to dissociate them from the interfering lipid material.

Dissociation of lipoprotein complexes so that the protein is recovered in the native form is one of the main problems in preparing such enzymes in true solution. To this end a number of procedures have been devised including the use of lipolytic enzymes, lipid solvents and detergents. These methods were investigated in an attempt to find a suitable method for the solubilization of formic dehydrogenase from the particulate material of E.coli.

Phospholipase treatment.

The use of lipolytic enzymes as a means of disrupting lipoprotein bonds has the advantage of being much milder on the protein material than other methods such as treatment with organic solvents or detergents. Singer and his associates have been very successful in the use of phospholipase A from snake venom as a solubilizing agent. Two particulate enzymes that have been extracted in soluble form as a result of this method are α- glycerophosphate dehyrogenase (Ringler and Singer, 1958), and choline dehydrogenase (Rendina and Singer, 1958). In the solubilization of the latter enzyme from rat liver mitochondria, Rendina and Singer used the venom of the tropical rattlesnake (Crotalus terrificus) at a level of 1 mg. venom per 100 mg. mitochrondrial protein. In applying this method to the

solubilization of formic dehydrogenase, the conditions used by Rendina and Singer were used as a guide.

The cell residue was treated with rattle snake venom (Crotalus adamanteus) in the proportions of 0.5 mg. and 5.0 mg. weight of venom per 100 mg. protein in the following digestion mixture:

1.0 ml. tris buffer, pH 7.4, 0.1M.

0.5 ml. Ca Cl_2 solution 0.03M.

Suspension of particulate material in 0.02M K Cl.

Snake venom in dilute tris buffer.

Water to a total volume of 5.0 ml.

[redacted]

Calcium ions were included for activation of phospholipase.

The digestion was carried out at 37° for one hour, after which the solutions were dialysed for several hours against 0.01M K Cl, in order to remove buffer and calcium ions in readings for enzyme assay. Part of the digestion mixture was centrifuged at 144,000 x g for 10 minutes. Even in the case of the higher venom level, the resulting supernatant was found to contain only 25% of the original particulate protein and this solubilized material was completely inactive. It is also worthy of note that even in the uncentrifuged digestion mixture, there was considerable loss of formic dehydrogenase activity. It was therefore concluded that this method was unsuitable for the solubilization of formic dehydrogenase.

Treatment with organic solvents.

Organic solvents are quite commonly used to break lipoprotein bonds and so to facilitate the solubilization of particulate proteins. However, care must be taken in the choice and use of solvents, because rapid protein denaturation can result. When using the more polar solvents such as acetone, the presence of water accelerates enzyme inactivation. Water content is not so critical when using some of the less polar solvents and the intermediate chain length aliphatic alcohols, such as n - butanol, iso -butanol and amyl alcohol, are often used. Three solvents were tested for ability to solubilize formic dehydrogenase: butanol, acetone and alcohol.

(a) Butanol

In the earlier stages of these studies (Wrigley, 1958), experiments were performed testing the ability of butanol to solubilize formic dehydrogenase from the particulate material. However, these attempts were not successful.

(b) Acetone.

The preparation of acetone powders has been frequently used for the disruption of lipoprotein bonds. The risk of protein denaturation is minimized by the use of low temperature (about -15°) and a large excess of acetone, so that the water present during the preparation is kept below about 15%. The precipitated protein is filtered and dried as quickly as possible. Earlier experiments using this method on the cell

on the cell residue (Wrigley, 1958), resulted in considerable loss of formic dehydrogenase activity and negligible protein solubilization. Subsequent repetition of this method has confirmed these earlier results.

(c) Alcohol.

A satisfactory method for the solubilization of DPNH - cytochrome c reductase has been devised by Edelhoch et al., (1952), and by De Bernard, (1957). The method consists of incubating the insoluble protein material with 9% alcohol at elevated temperature (44°) and at reduced pH. Guided by the conditions used by these workers, this method was applied to the present problem.

Three tubes were set up containing particulate material (17 mg. protein per ml.). One of the tubes was adjusted to pH 5.4 and another to pH 4.8. The remaining tube was treated as a control. Cold absolute alcohol was slowly added to each tube to a concentration of 9% and the tubes were quickly warmed to 44°. After 15 minutes at this temperature, the tubes were cooled and centrifuged for 10 minutes at 144,000xg.

The "cell sediment" was however, unlike heart mitochondria, resistent to this procedure and no significant amount of the enzyme or other protein was solubilized.

Treatment with detergents.

The disruption of lipoprotein complexes is frequently achieved by the use of detergents. One of the dangers in their use, however, is that they may form stable micelles with the lipoprotein complex, so producing a physical dispersion of the water-insoluble material. These miscelles amy well be resistant to sedimentation by high centrifugal forces and so appear soluble. However, material of this kind is generally precipitated by the addition of a relatively low concentration of ammonium sulphate after which redissolution is not possible.

Despite these difficulties in the use of detergents as solubilizing agents, some successful work has been carried out involving their use. The bile salt deoxycholate has been used with considerable success by Green and co-workers in the solubilization of a number of preparations from beef heart mitochondria. Guided by these instances of the successful use of deoxycholate, further solubilization studies were carried out along these lines.

(a) Treatment of the particulate material with deoxycholate and tris.

Crane and Glenn (1957), working in Green's laboratory, have described an extraction procedure involving the use of sodium deosycholate and tris buffer which proved quite severe with heart mitochondria. It was decided to first of all attempt deoxycholate solubilization following a

procedure similar to that used by Crane and Glenn.

A suspension of the cell residue in 0.02M potassium chloride was homogenized with deoxycholate (DOC) solution and tris buffer, pH 8.0, in such proportions as to produce the following final concentrations:

Cell residue, 14 mg. protein/ml.

Sodium deoxycholate, 1 mg./mg. protein.

Tris buffer, pH 8.0, 0.1M.

After homogenizing for several minutes, the mixture was centrifuged at 144,000 x g. for 15 minutes. The resulting supernatant and the resuspended sediment were dialysed separately overnight against several changes of 0.01M KCl solution. After dialysis, the supernatant fraction was spun clear at 144,000 x g. for 15 minutes. The dialysed fractions were assayed for protein content and formic dehydrogenase activity.

TABLE 6. Effect of DOC-tris treatment on the cell residue.

	Total protein.	Total Protein Recovery.	Specific activity.	Total activity Recovery
Sediment	250 mg.	53%	0.035	23%
Supernatant	155 mg.	33%	0.125	51%
		86%		74%

Cell residue (470 mg. protein; 0.08 specific activity) was treated with DOC and tris buffer as described in the text. The mixture was centrifuged, and the resulting supernatant and resuspended sediment were dialysed overnight. Material precipitated by dialysis of the supernatant fraction was removed by centrifugation, before determining enzyme activity. The precipitated material, which was less than

5% of the total protein, it was discarded.

Formic dehydrogenase activity was determined manometrically using 20 umoles of ferricyanide. Specific activity was expressed as umoles formate oxidized per minute per mg. protein.

The results tabulated in Table 6 show that the procedure extracted into the supernatant about one third of the cell residue protein and one half of the original activity. There was only a small loss of total activity as a result of the treatment.

Although the material in the supernatant fraction was resistant to sedimentation by a high centrifugal force, when it was subjected to ammonium sulphate fractionation, the enzyme activity was almost completely precipitated by an ammonium sulphate concentration as low as 20% saturation. The precipitated material would not redissolve in dilute salt solution. This indicated that the DOC-tris treatment had not truly solubilized the enzyme. A variation was therefore sought whereby the procedure could be modified so as to produce true solubilization of formic dehydrogenase

(b) Ammonium sulphate treatment of the undialysed DOC-tris supernatant.

It was found that if the supernatant fraction resulting from the above DOC-tris treatment, was fractionated with ammonium sulphate before dialysis, the enzyme was precipitated by much higher salt concentration than after dialysis. The results of this experiment (Table 7) show that although the bulk of the protein was still precipitated by 20% ammonium

sulphate saturation, this material was almost devoid of formic dehydrogenase activity. About 70% of the enzyme activity resided in the fractions precipitated by the upper levels of ammonium sulphate (40% - 60% and 60% - 90% saturation).

TABLE 7. Ammonium sulphate fractionation of the undialysed DOC-tris supernatant.

Ammonium sulphate fraction.	Total protein	Total Protein Recovery	Specific activity	Total activity Recovery
0-20% saturation	195 mg.	59%	0.015	11%
20-40% "	50	15%	0.03	6%
40-60% "	26	8%	0.45	44%
60-90% "	17	5%	0.35	23%
		87%		84%

The cell residue was treated with DOC and tris buffer and the mixture centrifuged (as described above in sub-section (a)). The undialysed supernatant (330 mg. protein) was fractionated with ammonium sulphate to yield the fractions in the above Table. The fractions were dialysed overnight against 0.01M KCl before specific activity determination.

A small portion of the unfractionated DOC-tris supernatant was dailysed overnight, after which it was found to have a specific activity of 0.08.

Formic dehydrogenase activity was determined manometrically using 20 umoles of ferricyanide. Specific activity was expressed as umoles of formate oxidized per minute per mg. protein.

As only 13% of the protein was precipitated in these fractions, formic dehydrogenase was purified some fivefold. Because of the high salt concentration required to precipitate the enzyme, it seemed quite likely that it had been prepared

in soluble form. This was further borne out by the fact that the ammonium sulphate precipitated enzyme redissolved readily in dilute salt solution. Refractionation of this solution with ammonium sulphate again precipitated the enzyme at high salt concentration.

While this procedure appeared to solubilize the enzyme, the total yield of solubilized enzyme was low. Although 70% of the activity of the DOC-tris supernatant was solubilized by the ammonium sulphate treatment, this represented only some 30% of the activity of the original cell residue fraction. It was therefore decided to modify the procedure in an attempt to extract more of the enzyme from the cell residue.

(c) Ammonium sulphate-DOC-tris treatment of cell residue.

It appeared that the key to the solubilization achieved in the previous experiment was the high salt concentration that was introduced as a result of the ammonium sulphate fractionation. It was therefore decided to incorporate the ammonium sulphate fractionation in the initial DOC-tris treatment, in an effort to obtain a better yield of solubilized enzyme. The procedure adopted was as follows:

Sodium deoxycholate (80 mg./ml.) and tris buffer (0.4M), (pH 8.0), were added to a suspension of the cell residue (usually 25 to 35 mg. protein/ml.) so as to produce final concentrations of

Sodium deoxycholate, 1 mg./mg. protein,
Tris buffer, 0.1M,
Cell residue, 14 mg. protein/ml.

After stirring this mixture for several minutes, a saturated solution of ammonium sulphate was added during a 15 minute period to produce 40% saturation. Stirring was continued for a further 30 minutes after the addition of ammonium sulphate as this incubation time was found to produce maximum enzyme extraction. At the end of this period, the mixture was centrifuged for 20 minutes at 55,000 x g. The ammonium sulphate concentration of the resulting supernatant was raised to 70% saturation by the addition of the solid salt during a period of 15 minutes. After a further 15 minutes' incubation, the mixture was centrifuged at 4,000 x g. for 40 minutes. The resulting sediment was washed once with 80% saturated ammonium sulphate solution, resuspended in 0.01M KCl and dialysed overnight against several changes of 0.01M KCl. The material remaining undissolved after dialysis was removed by centrifugation at 144,000 x g. for 15 minutes to yield a clear supernatant.

Examination of this supernatant revealed that it contained about 5% of the total cell residue protein. It was found that formic dehydrogenase was extracted quantitatively into the supernatant by this procedure so that the enzyme was purified some twentyfold. These results indicated that the solubilization procedure was extremely satisfactory and it was therefore adopted as the routine procedure. The resulting active supernatant is

referred to in the subsequent sections as the "solubilized" preparation.

An interesting property of the solubilized preparation that was immediately noticed, was its deep amber colour. Subsequent investigation of this coloured material provided some very significant information concerning the nature of the preparation. Examination of the absorption spectrum of the preparation showed the colour to be mainly due to cytochrome $\underline{b}_1$. This work is reported in a later section of this thesis.

FURTHER PURIFICATION.

Having obtained a suitable procedure for the extraction of soluble formic dehydrogenase from the cell residue, It was decided to further purify this solubilized material. To this end, several of the classical methods of protein purification were applied to the enzyme preparation.

Ammonium sulphate treatment.

Although the preparation of the solubilized enzyme involves the use of ammonium sulphate, refractionation with this salt was considered worthwhile in order to further purify the solubilized enzyme. The fractionation was carried out by adding saturated ammonium sulphate solution to 40% saturation and thereafter the solid salt was added.

As is shown in Table 8 the bulk of the active protein precipitates between 50% and 60% ammonium sulphate saturation. However, the enzyme did not precipitate in a sharply defined band, as might be anticipated of a simple protein, but 20% of the total activity was spread throughout nearby fractions. Furthermore, this refractionation procedure eliminated only a relatively small amount of contaminating protein with the result that specific activity was increased by only 60% and only about 70% of the initial activity was recovered in this fraction. This method was therefore considered unsuitable for further purification of the solubilized material.

TABLE 8 . Ammonium sulphate fractionation of the solubilized preparation.

Ammonium sulphate fraction	Total protein mg.	Total protein Recovery	Specific activity	Total activity Recovery
0-40%	0.0	0%	0.0	0%
40-50%	12.8	16%	1.5	13%
50-60%	34.0	42%	2.9	68%
60-80%	14.2	18%	0.9	9%
80-95%	0.6	1%	0.0	0%

The solubilized preparation used in this experiment totalled 81 mg. of protein and had a specific activity of 1.8. Formic dehydrogenase activity was determined manometrically using 20 umoles of ferricyanide. Specific activity is expressed as umoles of formate oxidized per minute mg. protein.

Tricalcium phosphate gel treatment.

The use of adsorbents for enzyme purification, although introduced in the early days of enzymology, still remains an important technique in this field of study. The two adsorbents used most extensively are tricalcium phosphate gel and a CY modification of aluminium hydroxide gel. The development of an appropriate purification procedure for a given enzyme is a highly empirical process which cannot be based upon known chemical or physical properties of the particular protein. However, some general rules can be laid down for guidance in the use of these adsorbents. One of the most important factors for

consideration is the protein-gel ratio. In practice, the protein-gel ratio is ordinarily in the range 10:1 to 1:2, depending on the ease of adsorption of the ~~desired~~ enzyme. Salt concentration and pH are two of the main factors involved in controlling selective adsorption and elution. Generally speaking, a low salt concentration and a rather low pH (pH5 to pH6) favour adsorption and a high salt concentration encourages elution.

The particular adsorbent selected for use was tricalcium phosphate gel, which was first introduced for protein purification by Keilin and Hartree in 1938. The gel was prepared by the procedure described by these workers. Following the customary procedure, the gel was stored for several months before use. It is thought that during this aging process the gel undergoes changes in the degree of hydration necessary for reproducible adsorption results.

A suspension of the gel was mixed with the required proportion of enzyme solution in 0.01M potassium chloride adjusted to pH 6.5. After 15 minutes, the gel was removed by centrifugation. The resulting supernatant was assayed for protein and formic dehydrogenase activity in order to determine the extent of adsorption. These results are tabulated in Table 9. It was found that only a little of the active protein was adsorbed at protein-gel ratios of 1:1 or 1:2. However, increase of the ratio to 1:4 brought about the adsorption of one half of the activity and three quarters

TABLE 9. Adsorption of soluble material on calcium phosphate gel at protein-gel ratios of from 1:1 to 1;8.

Protein-Gel	Total protein (mg.)	Protein unadsorbed	Specific activity	Total activity unadsorbed.
1:1	82.	80%	2.4	96%
1:2	61	59%	2.9	86%
1:4	24	23%	4.3	50%
1:6	14	14%	0.7	5%
1:8	7	7%	0.0	0

All values in the above table refer to the unadsorbed material present in the supernatant after removal of gel. Protein-gel ratios were expressed as mg. protein (biuret): mg. dry weight of gel. For each of the five adsorption experiments, 103 mg. (protein) of the solubilized preparation were used. The preparation had a specific activity of 2.0. The protein content of the solubilized preparation was determined by the buiret method. Protein was determined in the supernatants after gel treatment, by the method of Layne (1957). The assay of formic dehydrogenase activity was conducted manometrically using 20 umoles of ferricyanide. Specific activity was expressed as umoles formate oxidized/ minute/mg. protein.

of the total protein. A protein-gel ratio as high as 1:6 was required before all the enzyme activity was adsorbed onto the gel. At this high proportion of gel, most of the inactive protein was also adsorbed, so that the material on the gel was only slightly purified.

These results made it clear that the enzyme could not be purified by selectively adsorbing impurities onto the gel. Therefore, purification by selective elution of the enzyme

from the gel was attempted. after adsorption of the enzyme onto the gel at a protein-gel ratio of 1:6, the gel was eluted by mixing with 30 ml. portions of phosphate buffer, pH 6.5. The concentration of the eluting buffer was increased in the following order: 0.02M, 0.1M, 0.5M, 1.0M. Elution with one particular buffer concentration was continued until no more protein was eluted, as judged by the optical density of the eluate at 280 mu and 260 mu. These optical density values were used to determine the protein concentration of the eluates (Layne, 1957). Formic dehydrogenase activity was determined manometrically using 20 umoles of ferricyanide.

The only fractions containing a significant amount of activity were those eluted by 0.5M buffer. The total activity of these fractions accounted for only 55% of the total activity initially adsorbed onto the gel. It was concluded that the activity loss was genuine because the total protein recovered in all the fractions was about 95% of that originally adsorbed onto the gel. The fractions eluted by 0.5M buffer contained 40% of the total protein, so that a purification of only 1.3x resulted. In view of the high loss of activity, it was concluded that the procedure was unsatisfactory.

An interesting observation of this experiment was that the colour of the solubilized material accompanied the formic dehydrogenase activity during purification. None of the inactive fractions was found to have any colour. This observation suggested that the compound responsible for the colour was closely associated with the enzyme.

Chromatrography on Cellulose derivatives.

Although the technique of column chromatography has long been in use for the isolation of many different types of compounds, it is only relatively recently that this technique has been applied successfully to the field of protein purification. These recent advances are mainly due to the development of suitable adsorbents. Earlier employed protein adsorbents such as calcium phosphate and alumina Cγ gels were not suitable for column chromatography because of their resistance to the flow of aqueous solutions. Ion exchange resins have been used to some extent for column chromatography of proteins, but their use has been limited to stable, basic substances of low molecular weight such as cytochrome c, lysozyme, ribonuclease and chymotrypsinogen.

The recently developed cellulose derivatives appear to be suitable for a wide range of proteins, and can be prepared in such particle size as to allow quite rapid flow rates. Peterson and Sober (1956) report a number of cellulose derivatives that reversibly adsorb protein molecules. They have used columns of these derivatives very effectively in the purification of a number of proteins. Proteins are adsorbed at low ionic strength and eluted at higher ionic strength. Change of pH can bring about further variation in adsorption conditions. The cellulose adsorbent chosen for use in the purification of the solubilized formic dehydrogenase was the anion exchanger diethylaminoethyl cellulose

(DEAE - cellulose), which was used in accordance with the recommendations of Peterson and Sober (1956).

The cellulose was washed well by decantation firstly with distilled water and later with 0.002M phosphate buffer (pH6.5). A suspension of the cellulose in buffer was then poured into a chromatography column of approximate diameter 1.2 cm. and the cellulose packed and equilibrated by running buffer solution through the column. Equilibration of the column with buffer was continued until further elution produced no pH change in the eluting buffer.

The material eluted from calcium phosphate gel by 0.5M phosphate buffer in the previous experiment was used for cellulose chromatography. After about three hours' dialysis against 0.002M phosphate buffer (pH 6.5), this material was applied to the column using a very slow initial flow rate. For the particular experiment reported below (Table 10) the column contained a packed volume of 27 ml. of DEAE - cellulose, which was substituted to the extent of 0.91 milliequivalent of base per gram. The column was eluted in the cold at a flow rate of about 0.5 ml. per minute, collecting 5 ml. fractions. The concentration of the eluting buffer (potassium phosphate, pH 6.5) was increased in the following order: 0.002M, 0.02M, 0.04M, 0.08M, 0.12M, 0.16M. 0.25M, 0.50M.

Table 10 shows the protein concentration and formic dehydrogenase activities of the combined fractions eluted at

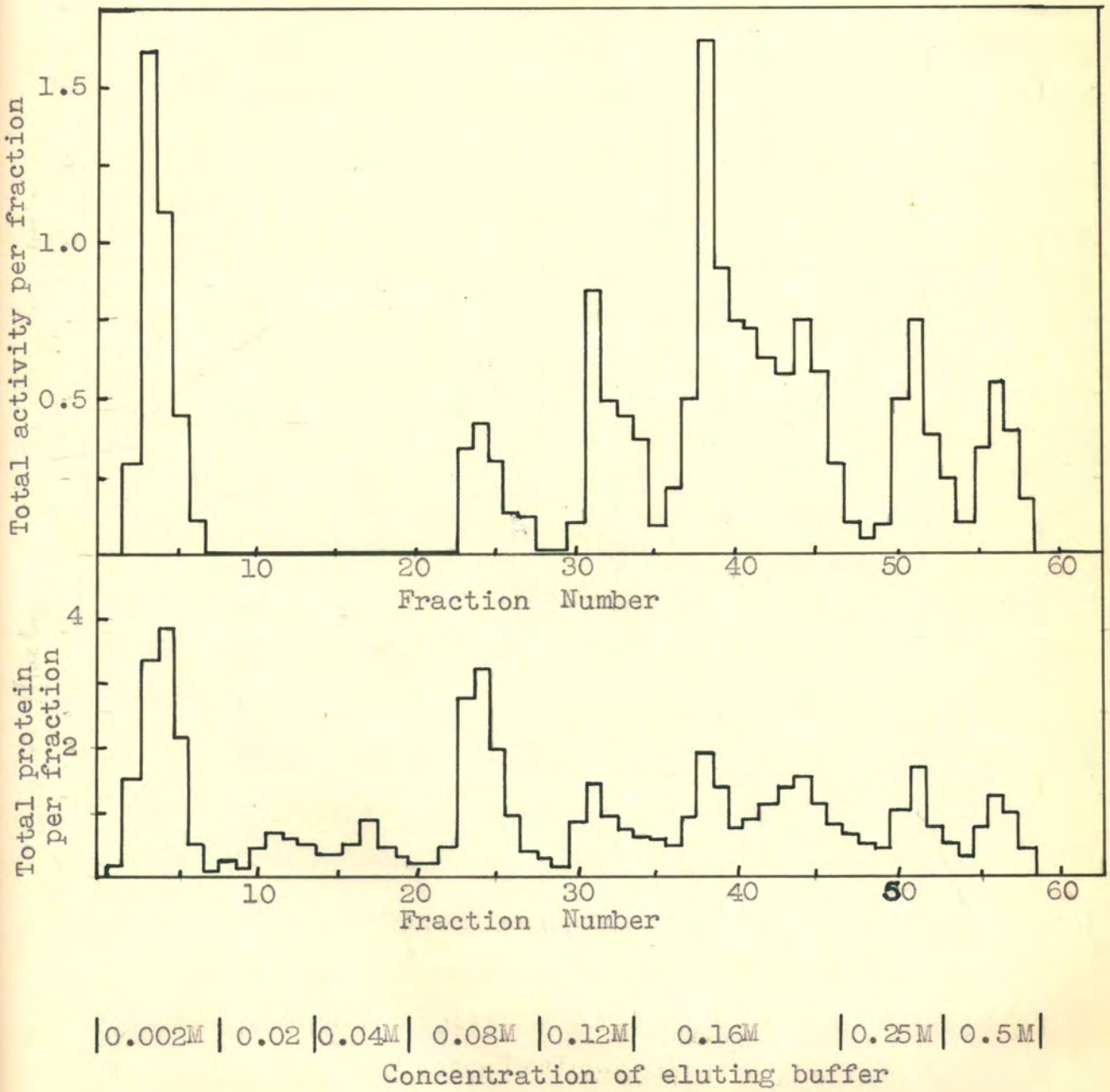
TABLE 10. Chromatography of the solubilized preparation on DEAE - cellulose.

Buffer concentration	Fractions.	Total protein. mg.	Total protein Recovery.	Average specific activity.	Total activity Recovery.
0.002M	1-7	11.3	17%	0.31	13%
0.02M	8-13	3.3	5%	0.0	0
0.04M	14-19	3.0	5%	0.0	0
0.08M	20-27	10.8	17%	0.13	5%
0.12M	28-33	5.5	8%	0.44	9%
0.16M	34-46	13.8	21%	0.57	28%
0.25M	47-52	4.3	7%	0.54	8%
0.50M	53-58	3.5	5%	0.46	6%
			85%		69%

The solubilized preparation (65 mg. protein) applied to the column had a specific activity of 0.42.

The values quoted in the table are for the combined fractions eluted at each buffer concentration (as indicated). Protein concentration was estimated by the method of Layne (1957). The average specific activity was the mean of the specific activities of the individual fractions eluted at each buffer concentration and expressed as umoles formate oxidized per minute per mg. protein. The activities were determined spectrophotometrically, using ferricyanide as artificial electron acceptor.

FIGURE 6. Chromatography of the solubilized preparation on DEAE-cellulose. This figure should be read in conjunction with Table 10. The legend of Table 10 also refers to this figure.



each buffer concentration. This table should be read in conjunction with the graphs in Figure 6 , which show the protein and enzyme activity eluted in each fraction. The bulk of the activity was eluted by 0.16M phosphate, but an activity purification of only 1.3x was obtained for the combined fractions eluted at this concentration. However, fraction number 40, eluted by 0.16M buffer, had a specific activity of 1.0 umoles formate oxidized per minute per mg. protein, which represented a purification of 2.4x. It is worthy of note that the colour that characterized the solubilized material was again purified with the enzyme activity. The fractions eluted at buffer concentrations close to 0.16M also contained quite a significant amount of active protein indicating that the elution of the enzyme was rather non-specific. Furthermore, it was noticed that a considerable amount of protein and enzymic activity was not adsorbed by the cellulose at all, but passed straight through the column in the initial elutions. This situation could not be remedied by the use of a higher proportion of adsorbent. Because of the lack of good purification together with reasonably high activity recoveries, this method was dismissed as being unsuitable for purification of the solubilized formic dehydrogenase preparation.

None of the purification procedures used in the above experiments produced better than two or possibly three-fold purification and even this was usually at the cost of

considerable loss of activity. The lack of success with these standard purification procedures suggested that the enzyme was not a simple protein. The question was therefore raised as to the form of the solubilized preparation. It was considered likely that the preparation was not a simple protein of relatively small molecular weight, but that it was rather a very high molecular weight macromolecule, possibly a complex of a number of simpler units.

It was therefore decided to examine the solubilized preparation thoroughly and the remainder of the thesis will be concerned with these studies. Examination of the solubilized preparation will be dealt with under the following headings:-

A. Evidence for true solubility.

B. Assay of the formate oxidizing system.

C. Composition of the solubilized preparation.

1. Enzyme content.
2. Nature of coloured material.
3. Coenzyme Q and vitamin K.
4. Lipid.
5. Polyribonucleotide.

PART II. PROPERTIES OF THE SOLUBILIZED FORMIC DEHYDROGENASE PREPARATION.

EVIDENCE FOR TRUE SOLUBILITY.

Introduction.

The use of detergents as protein solubilizing agents is frequently criticized on the grounds that these components merely disperse the insoluble material in the form of minute micelles without actually changing the properties of the protein or removing the hydrophobic material from the protein. These micelles, it is argued, are highly resistant to ultracentrifugation in the presence of the solubilizing agent, but are precipitated again on removal of the detergent.

Singer and his associates have been prominent in the criticism of detergents as solubilizing agents. Ringler and Singer (1958) have set out three properties of a protein that has been extracted in "true solution":

"(1) it remains in solution after extensive dialysis,

(2) it does not sediment at 144,000 x g for one hour,

(3) it may be readily fractionated with ammonium sulphate and a high salt concentration is required for precipitation, while dispersions prepared with digitonin precipitate at a relatively low ammonium sulphate concentration."

As shown in the following experiments the soluble formic dehydrogenase preparation satisfies all these requirements.

Extensive dialysis.

The procedure for the solubilization of formic dehydrogenase sets down an overnight dialysis against several changes of 0.01M potassium chloride. Although a dialysis of this length should normally remove all free deoxycholate (DOC) from the dialysed material, any DOC bound to the protein or lipid might conceivably require longer dialysis for removal. It was therefore considered advisable to dialyse the preparation exhaustively in order to ensure complete removal of both bound and free DOC.

Prolonged dialysis was carried out on a number of preparations. These preparations were dialysed for four or five days in addition to the routine overnight dialysis. On each occasion the preparation became cloudy and about 20% of the protein precipitated. However, on no occasion did any enzymically active protein precipitate, but almost complete recovery of total enzyme activity was obtained after dialysis of the solubilized preparation. This precipitation resulted in a slight purification of enzyme activity.

A similar effect was evident in the preparation on standing at 0° for some days. Aging at 0° for about 8 days caused the precipitation of approximately 20% of the total protein but none of the active material. After prolonged storage, there was some further precipitation of inactive material. One particular preparation was allowed to stand at 0° for nearly three months. Table 11 shows that there

was no significant loss of enzyme activity after four weeks' storage, but at the end of the 89-day period, there was a 35% loss of activity in the soluble material. As this was attended by a 50% precipitation of protein, there was a slight purification of activity as a result of the storage. These results showed the solubilized enzyme to be very stable. The enzyme also showed good stability in the particulate material, as there was a specific activity loss of only 30% after storage for 80 days.

The results presented above appear to satisfy the criterion for the enzyme resisting precipitation by dialysis. However, to definitely establish this point, it must be shown the dialysis removes DOC. The concentration of DOC in the solubilized preparation was therefore estimated.

TABLE 11. Effect of storage at 0° on the solubilized formic dehydrogenase preparation.

Age (days)	Total protein. (mg.)	Specific activity.	Total activity. (total units)
0	50	1.6	80
8	40	1.95	78
29	36	2.2	79
78	25	2.1	53

All results tabulated refer to the material remaining in solution after centrifugation at 144,000 x g for 20 minutes. Formic dehydrogenase was assayed manometrically using 20 umoles of ferricyanide. The specific activity represents umoles of formate oxidized/minute/mg. protein.

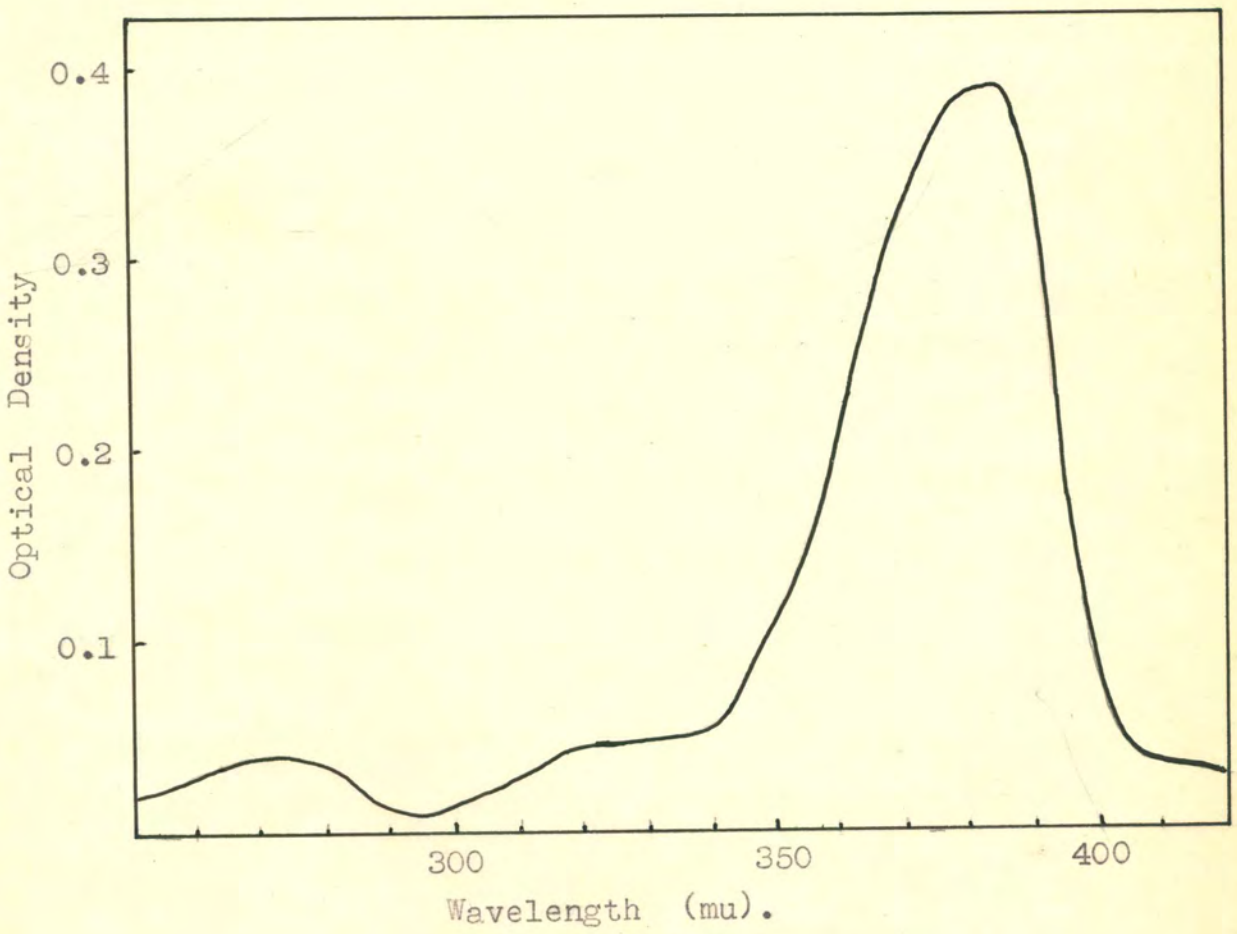
Deoxycholate determination.

The deoxycholate estimation procedure used was essentially that described by Mosbach et al. (1954) for DOC estimation in bile. This method was modified somewhat to suit the present situation. The modified procedure was as follows:

The protein (about 4 mg.) of the solubilized preparation was precipitated with ten volumes of hot ethanol and the precipitate was re-extracted several times with hot ethanol. The combined ethanol extracts were evaporated to dryness and the material remaining was taken up in water (3 ml.). Acidification with several drops of 1N HCl precipitated deoxycholic acid which was extracted with several 5 ml. portions of ether. The combined ether extracts were evaporated to dryness and the residue was heated in 5 ml. of 65% sulphuric acid at 60° for 15 minutes. The optical densities of the resulting solutions were measured at 385 mu. Standards containing from 10 to 100 mg. DOC were treated similarly.

The absorption spectrum of the standard solutions showed one major absorption peak at 385 mu. Smaller peaks also appeared at 320 mu and 275 mu (see Figure 7). The shape of the curve was identical with that presented by Mosbach et al. (1954) for chromatographically pure DOC. A similar absorption spectrum was also recorded for the material extracted from the solubilized preparation. It was found necessary to include the water and ether extractions in

FIGURE 7. Absorption spectrum of a 50 ug. DOC standard, treated as described in the text by a modification of the DOC assay procedure of Mosbach et al. (1954).



the procedure, because the alcohol-extracted material, after heat treatment in sulphuric acid, showed high absorption at about 280 mu and at 320 mu.

Using the DOC determination described above, the DOC content of the solubilized preparation was estimated to be 30 ug. DOC/mg. protein after overnight dialysis and 3 ug. DOC/mg. protein after a further five days' dialysis. These values indicated that prolonged dialysis reduced the deoxycholate content to a very low level. It was not possible to evaluate the significance of this low level of DOC, but it was felt that such a DOC concentration would contribute little to the solubilization of the enzyme.

Ultracentrifugation.

The solubilized preparation resulting from overnight and from five days' dialysis was centrifuged in a swinging bucket head at 174,000 x g (Maximum) for one hour. In neither case was any formic dehydrogenase activity sedimented as a result of this ultracentrifugation. Routine centrifugation was carried out in an angle head. The swinging bucket head produced a similar centrifugal force to that generated by the angle head but was more suitable for critical work of this kind because its design minimized mixing of the contents on deceleration.

Ammonium sulphate precipitation.

Having established the second of the solubility criteria under the stringent test conditions described above, the third criterion of solubility was examined. In the investigation of several methods for further purification of the solubilized enzyme, it was noted that after fractionation with ammonium sulphate, the material redissolves easily without loss of activity, in the same manner as would be anticipated for a soluble protein. Furthermore, the enzymic activity was precipitated at relatively high ammonium sulphate concentration, viz., above 50% saturation. However, as the enzyme does not separate cleanly into a single ammonium sulphate cut, the enzyme preparation does not lend itself to purification by this method.

The effect of acetone treatment on the solubilized material is also worthy of note at this stage. After precipitation by cold acetone, the solubilized protein redissolved readily in 0.01M KCl, but approximately 50% of formic dehydrogenase activity was lost as result.

Summary.

The results presented above provide good evidence that the deoxycholate-treated enzyme preparation was truly soluble. This was shown by the following criteria:

(1) No active protein was precipitated from the preparation by prolonged dialysis which was shown to decrease DOC concentration to a very low level.

(2) Ultracentrifugation of the dialysed material did not sediment any active protein.

(3) A high ammonium sulphate concentration was needed for precipitation of the enzyme, which redissolved readily in dilute salt solution.

However, the refractoriness of the preparation to further purification, as noted in the preceding section, appeared to indicate that the preparation cannot be looked upon as being a typical simple protein.

B. ASSAY OF THE FORMATE OXIDIZING SYSTEM.

It has already been noted that considerable purification of formic dehydrogenase with respect to ferricyanide reduction was obtained by the deoxycholate extraction procedure. The development of the assay procedure is discussed below and a comparison is made between the activities of the enzyme when coupled to several other electron acceptors.

The oxidation of formic acid can be represented by the following equations:

$$H^+ + HCOO' \longrightarrow CO_2 + 2(H^+ + e')$$

$$2(H^+ + e') + \tfrac{1}{2}O_2 \longrightarrow H_2O$$

It was of interest to investigate the effect of the solubilization procedure on the activity of the oxidase reaction and on the ability of several artificial electron acceptors to substitute for the second of these reactions, thereby replacing the cytochrome system in the cell.

Formic oxidase.

Formic oxidase is the name given to the enzyme system which catalyses the complete aerobic oxidation of formate to carbon dioxide and water. The activity of this enzyme system was determined by measuring the uptake of oxygen manometrically as described in the Methods Section.

As shown in Table 12, the formic oxidase specific activities of the cell residue and solubilized preparation were 1.0 and 0.2 umoles formate oxidized per minute per mg. protein. However, it should be emphasized that although the solubilized preparation had one fifth of the specific activity of the cell residue, the total activity values showed that only 1% of the cell residue formic oxidase was extracted into soluble form. This indicated that the complete respiratory chain was no longer intact in the solubilized preparation.

The formic oxidase activity was completely inhibited by a cyanide concentration of 3×10^{-4} M. Antimycin A, on the other hand, inhibited the oxidase only 10% at a concentration of 30 ug per ml. of reaction mixture. Antimycin A has previously been shown to inhibit only some microbial cytochrome systems.

Assay of formic dehydrogenase using ferricyanide as electron acceptor.

Potassium ferricyanide was the electron acceptor used routinely to assay formic dehydrogenase activity. This

TABLE 12. Formate oxidation rates of cell residue and solubilized preparation.

Enzyme	Cell Residue. Specific activity	Cell Residue. Total activity	Solubilized Preparation. Specific activity	Solubilized Preparation. Total activity
formic oxidase	1.0	4,750	0.20	48
formic dehydrogenase (a)	0.02	95	0.45	108
formic dehydrogenase (b)	0.11	520	2.3	550
formic dehydrogenase (c)	0.80	3,800	16.4	3,940
formic dehydrogenase (d)	-	-	13.0	3,120

The solubilized preparation (240 mg.) was prepared from 4,750 mg. of cell residue. Specific activity was expressed as umoles of formate oxidized per minute per mg. protein. Total activity is the product of specific activity and total protein. The formic dehydrogenase activities reported were obtained by the following assay procedures:

(a) spectrophotometric, ferricyanide.
(b) manometric, ferricyanide (20 umoles).
(c) manometric, ferricyanide, ($V_{max.}$).
(d) manometric, phenazine methosulphate, ($V_{max.}$).

activity was determined by measuring the reduction of ferricyanide either spectrophotometrically by the rate of decrease of optical density at 410 mu, or manometrically by measuring carbon dioxide production from bicarbonate buffer.

Manometric assay.

On reduction of one molecule of ferricyanide, one proton is formed, which in bicarbonate solution releases one carbon dioxide molecule according to the following equations:

$$HCOO^- + H^+ \longrightarrow CO_2 + 2(H^+ + e^-)$$

$$2(H^+ + e^-) + 2(Fe(CN)_6)^{+++} \longrightarrow 2(Fe(CN)_6)^{++} + 2H^+$$

$$2H^+ + 2HCO_3^- \longrightarrow 2CO_2 + 2H_2O.$$

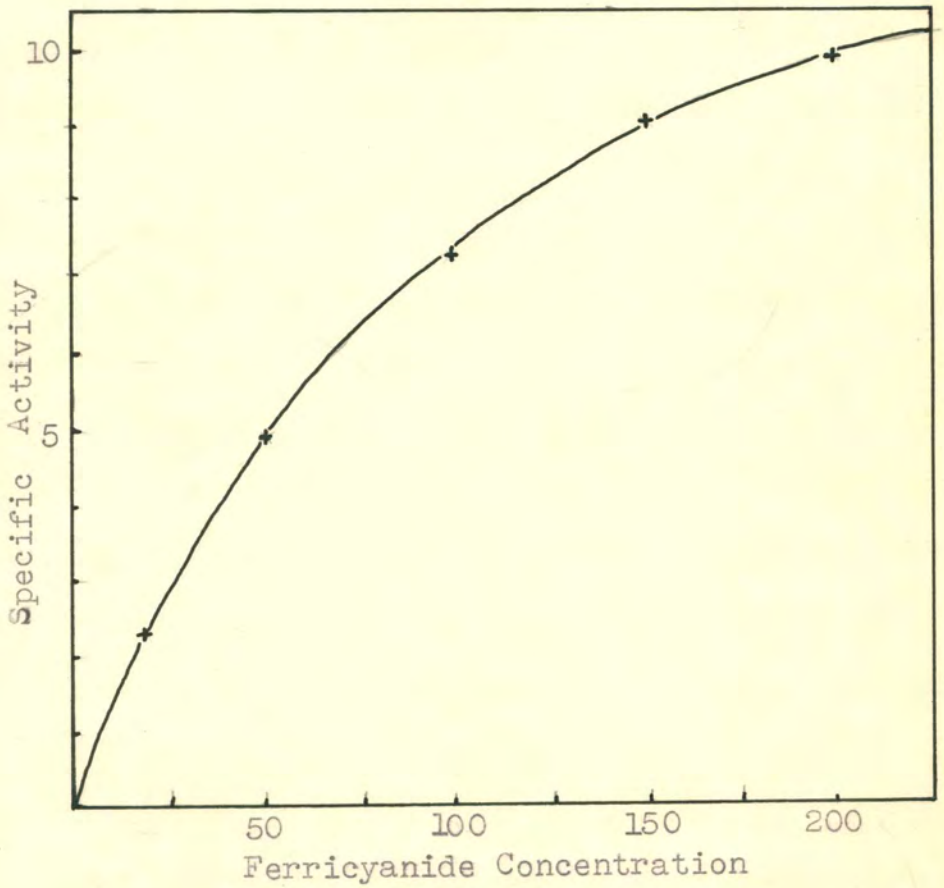
Examination of the equations above reveals that for one molecule of formate oxidized, three molecules of carbon dioxide are produced, one directly from the formate molecule and two as a result of ferricyanide reduction. The number of umoles of carbon dioxide evolved were therefore divided by three in calculating formate oxidation.

The procedure for the manometric measurement of carbon dioxide production is described in the Methods Section. In order to keep the pH at the required value at 6.0, a buffer system was set up consisting of a carbon dioxide atmosphere and bicarbonate solution. The bicarbonate concentration was calculated using the Henderson-Hasselbach equation and was verified experimentally by direct determination of the pH of the system.

In early assays a ferricyanide concentration of 20 umoles per flask was used. It was found however, that at higher ferricyanide concentrations, there was a considerable increase in specific activity such that a tenfold increase in ferricyanide concentration stimulated activity threefold (Figure 8). This effect continued so that a saturation ferricyanide concentration could not be reached. It was therefore decided to use the double reciprocal plot method (Singer and Kearney, 1957) to determine the rate of formate oxidation at infinite acceptor concentration ($V_{max.}$). The reciprocal of the ferricyanide concentration was plotted against the reciprocal of the initial rate of formate oxidation and the plot extrapolated to zero reciprocal ferricyanide concentration. $V_{max.}$ was calculated from the intercept on the ordinate (Figure 9). Table 12 shows that the specific activity determined by this method was some seven times higher than that obtained using only the one ferricyanide concentration of 20 umoles. A similar effect was observed in the cell residue assay.

The reason for this phenomenon is not known but it was thought possible that ferrocyanide formed after formate oxidation was bound to the enzyme's active site more strongly than was ferricyanide. Addition of a large excess of ferricyanide would then accelerate the dissociation of the enzyme-ferrocyanide complex, allowing faster reaction. This hypothesis has not been extensively tested, but in

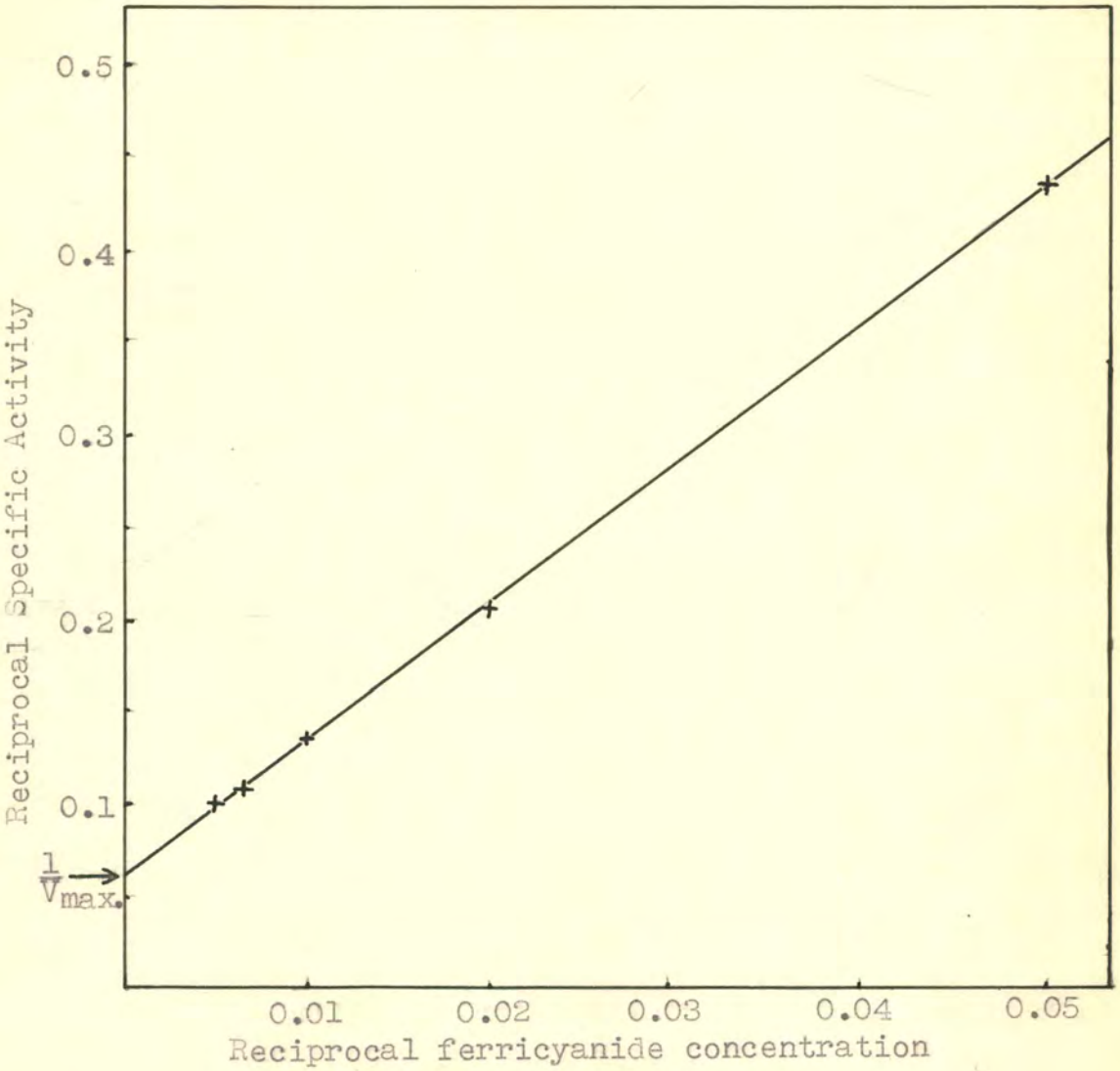
FIGURE 8. Effect of ferricyanide concentration on specific activity in the manometric assay of formic dehydrogenase.



Specific activity was expressed as umoles of formate oxidized per minute per mg. protein.

Ferricyanide concentration was expressed as umoles of potassium ferricyanide per manometer flask.

FIGURE 9. The double reciprocal plot method for the determination of formic dehydrogenase specific activity at infinite ferricyanide concentration ($V_{max.}$).



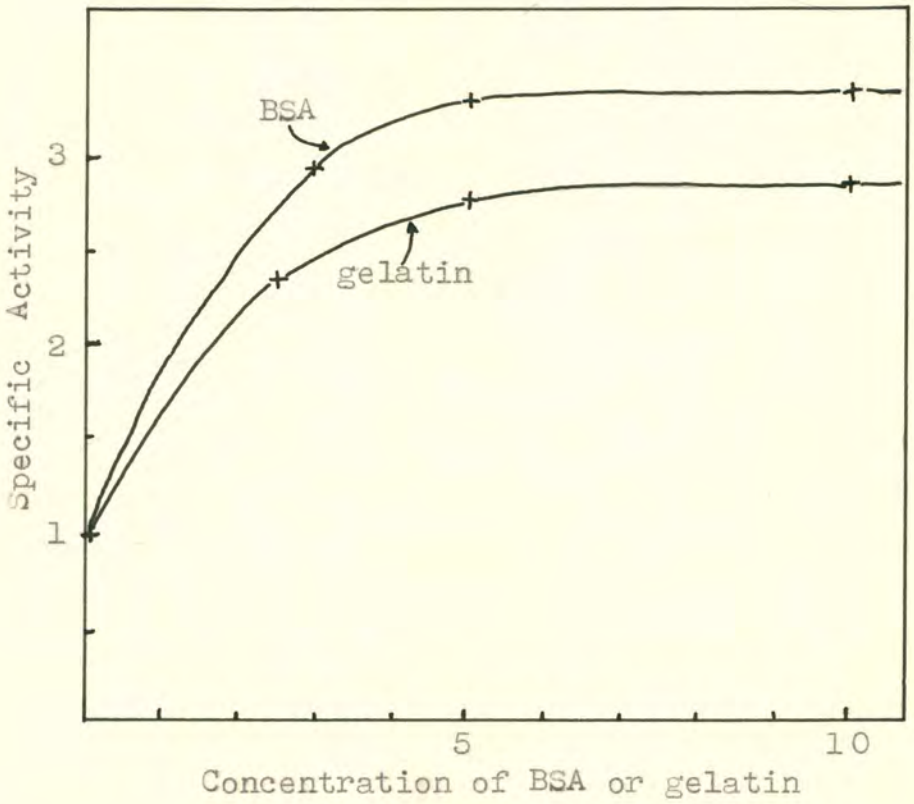
The specific activity values used in this figure are those appearing in Figure 8. From the intercept on the ordinate, the specific activity at infinite ferricyanide concentration was calculated to be 16.4. Specific activity was expressed as umoles of formate oxidized per minute per mg. protein.
Ferricyanide concentration was expressed as umoles per manometer flask.

preliminary studies, ferrocyanide, in equal concentration to ferricyanide inhibited carbon dioxide evolution by about 50%. Further addition of ferrocyanide did not however, increase inhibition.

Five umoles of magnesium sulphate are included in the assay mixture because magnesium ions were found to stimulate activity about twofold in the assay of both particulate and soluble preparations. The nature of this stimulation has not been investigated.

A threefold activation was produced in the assay of the solubilized preparation by the addition of bovine serum albumin (BSA) to the standard assay system (gelatin excluded). Figure 10 shows that optimal assay conditions were obtained with a BSA concentration of 5 mg. per flask. A similar concentration of gelatin also stimulated activity, but not to quite the same extent as did BSA. As this difference in activation was not considerable, it was decided to include gelatin (5 mg.) routinely in the standard assay system. The effect of the resulting high protein concentration produced by these additions probably served to prevent the denaturation of the soluble enzyme which was often used at as low a concentration as 10 ug per ml. in the assay mixture. Magnesium sulphate and gelatin were also included in all the assay systems for the determination of formate oxidation rates.

FIGURE 10. Stimulation of formic dehydrogenase activity by BSA and by gelatin.



Formic dehydrogenase activity was determined manometrically using 20 umoles of ferricyanide, omitting gelatin from the standard assay mixture except as shown above.
Specific activity was expressed as umoles of formate oxidized per minute per mg. protein.
The concentrations of BSA and gelatin were expressed as mg. per manometer flask.

Spectrophotometric assay.

Ferricyanide reduction by formate was also followed spectrophotometrically by measuring the rate of decrease of optical density at 410 mu. For a full description of the assay procedure, see the Methods Section. This method was not satisfactory for assaying the cell residue fraction because of its high oxidase activity. This oxidase activity had to be inhibited in some way so that formate oxidation would not proceed via the aerobic pathway, so by-passing ferricyanide reduction. In the manometric assay, aerobic formic oxidation was prevented by using an oxygen-free atmosphere. As this was not possible with the spectrophotometric assay the oxidase of cell residue was inhibited with cyanide (4×10^{-4} M final concentration). However, the manometric assay was considered more reliable for assaying the cell sediment as it appeared that cyanide did not completely inhibit the oxidase.

The soluble preparation was better suited to this method of assay, because of its very low oxidase activity relative to dehydrogenase activity. Even so, Table 12 shows that specific activity is much lower when determined spectrophotometrically than by the manometric determination. It is suspected that even in the assay of the soluble material, there is a significant aerobic formate oxidation so that ferricyanide reduction is by-passed.

Merits of the various ferricyanide-formic dehydrogenase assay methods.

As the spectrophotometric assay was considered to be not as reliable as the manometric assay, it was not used routinely. However, it was convenient to use the spectrophotometric assay in some of the protein fractionation experiments, in which a large number of fractions had to be assayed quickly and absolute specific activity values were not required. One of the main difficulties with the manometric determination, on the other hand, was that enzyme preparations could not be assayed if suspended in buffer solutions, as the buffer interfered with the carbon dioxide-bicarbonate system.

Table 12 gives the activities of both cell residue and soluble preparation using the assay systems described above for formate oxidation. Each of the dehydrogenase assay systems shows that over twentyfold purification is produced by the solubilization procedure. The oxidase, on the other hand, is almost completely eliminated by the solubilization procedure. The formic dehydrogenase recoveries of over 100% for each of the ferricyanide assay systems can probably be attributed to more efficient assay of the solubilized enzyme than of the particulate enzyme.

Phenazine methosulphate.

Although ferricyanide is the most commonly used electron acceptor in the assay of dehydrogenase systems, Singer and Kearney (1957) have pointed out that phenazine methosulphate should perhaps be the acceptor of choice.

In their experience, the rate of reaction of succinic dehydrogenase, α-glycerophosphate dehydrogenase and choline dehydrogenase is faster with phenazine methosulphate than with ferricyanide (Singer and Kearney, 1957; Ringler and Singer, 1958; Rendina and Singer, 1958). This prompted an examination of the rate of reaction of formic dehydrogenase with phenazine methosulphate. It was found, however, that formic dehydrogenase did not react as rapidly with phenazine methosulphate as with ferricyanide.

The activity of the enzyme with phenazine methosulphate as electron acceptor was assayed by measuring oxygen uptake manometrically in the presence of the auto-oxidizable dye as described in the Methods Section. The cell residue is not suitable for assay by this procedure because of its high oxidase activity. Attempts to inhibit the oxidase of the cell residue with cyanide did not prove entirely satisfactory.

Phenazine methosulphate reacts with formic dehydrogenase according to the following equations:-

$$HCOOH + \text{phenazine methosulphate} \rightarrow CO_2 + \text{leuco-phenazine methosulphate}$$

$$\text{leuco-phenazine methosulphate} + O_2 \rightarrow \text{phenazine methosulphate} + H_2O_2$$

This determination is not as sensitive as the method of ferricyande reduction in bicarbonate buffer, in which reaction three molecules of carbon dioxide are evolved for each

molecule of formate oxidized. Formic dehydrogenase activity as determined by this method is only 80% of the activity determined similarly using the ferricyanide-bicarbonate method (Table 12).

Summary.

Some interesting variations in assay methods for the formic dehydrogenase reaction have been reported, each method having its own particular merits. Formate oxidation linked to ferricyanide reduction as determined by the double reciprocal plot method is the most reliable method, but is rather cumbersome. In the assay of formic dehydrogenase, phenazine methosulphate does not appear to be as efficient an electron acceptor as ferricyanide. This is contrary to the experience of Singer and co-workers with other solubilized enzymes.

C. COMPOSITION OF THE SOLUBILIZED FORMIC DEHYDROGENASE PREPARATION. ACTIVITIES OF OTHER ENZYMES

Succinic dehydrogenase.

Succinic dehydrogenase is an ubiquitous oxidative enzyme classically associated with the terminal electron transport system in the particulate fraction of the cell and could conceivably be solubilized together with formic dehydrogenase. The activity of succinic dehydrogenase in the particulate and solubilized preparations was therefore investigated.

Succinic dehydrogenase was assayed by measuring the reduction of ferricyanide (20 umoles) in bicarbonate buffer following the procedure used for the formic dehydrogenase assay except that

(i) 40 umoles of succinate replaced formate,

(ii) a bicarbonate final concentration of 0.24M was used in equilibrium with 5% CO_2- 95% N_2 atmosphere, to produce a pH of 7.4,

The formic oxidase assay system was adapted to the assay of succinic oxidase by substituting 40 umoles of succinate for formate, and by using phosphate buffer at pH 7.3.

Both succinic dehydrogenase and oxidase were present in much lower activity in the cell residue than the corresponding formate oxidizing enzymes (Table 13). In the solubilized preparation, the rate of succinate oxidation was insignificant using either ferricyanide or oxygen as acceptors.

Reduced diphosphopyridine nucleotide (DPNH) dehydrogenase and oxidase.

DPNH dehydrogenase and DPNH oxidase are classically particulate enzymes which might also be solubilized together with formic dehydrogenase. DPNH oxidase activity was determined by measuring the rate of decrease of optical density at 340 mu. A similar system was used for the dehydrogenase, using ferricyanide as electron acceptor instead of molecular oxygen. The details of the assay procedures are described in the Methods Section.

Table 13 shows that the cell residue had a DPNH oxidase activity of 0.15 umoles DPNH oxidized per minute per mg. protein, only one sixth of the formic oxidase activity. The soluble preparation, however, had no DPNH oxidase activity at all.

DPNH dehydrogenase was assayed by a similar method to that described above for the oxidase, except that ferricyanide was added as artificial electron acceptor. When assaying the cell residue, it was found necessary to also include cyanide in the assay mixture to depress DPNH oxidase activity. Under these assay conditions, a DPNH dehydrogenation rate of 0.21 umoles per minute per mg. protein was obtained. Although the dehydrogenase should not require the mediation of any cytochromes in its reaction with ferricyanide, optimal assay conditions may not be present at such a high cyanide concentration.

Cyanide and DPN combine to form a compound with absorbance at 340 mu. The rate of formation of this compound was found to involve an increase in optical density at 340 mu of about 0.01 per 30 seconds for a mixture of 200 ug. DPN and 30 umoles of cyanide in 2.7 ml. (the proportions used in the assay system). In the initial stages of the DPNH dehydrogenase reaction, only very small amounts of DPN would be present. As a result the formation of this DPN-cyanide compound would only interfere after DPNH

oxidation had progressed considerably. The precaution was therefore taken of calculating dehydrogenase activity from the initial rate of decrease in optical density.

As the solubilized preparation was found to be devoid of DPNH oxidase activity, cyanide was omitted from the reaction mixture when assaying DPNH dehydrogenase in this preparation. It was found that the solubilized preparation was capable of oxidizing 0.54 umoles DPNH per minute per mg. of protein. This specific activity was several times higher than that of the cell residue compared with a twentyfold purification of the formic dehydrogenase activity. However, although purified some two or three times, the total DPNH dehydrogenase activity was only extracted to the extent of about 10% of the total enzyme present in the cell sediment.

Nitrate reductase.

Iida and Taniguchi (1959) have described nitrate reductase as being present in the particulate material of E.coli in quite high activity. This enzyme catalyses the reduction of nitrate to nitrite in the presence of an artificial electron donor. In the intact particulate fraction, formate can act as electron donor by means of the formate-nitrate reductase system which appears to involve formic dehydrogenase and nitrate reductase together with several co-factors. In view of its particulate nature, and its association with formic dehydrogenase, it

was of interest to investigate the presence of nitrate reductase in the particulate and solubilized preparations.

The assays of the nitrate reductase and of the formate-nitrate reductase system were carried out as outlined by Iida and Taniguchi (1959). Unfortunately, neither in this or in other papers of these authors or their associates are the details of the assay procedure reported. It was therefore necessary to develop a suitable procedure. The assay procedure adopted for the formate-nitrate reductase system consisted essentially of estimating nitrite production after incubation of the enzyme preparation with formate and nitrate in an oxygen-free atmosphere. When assaying nitrate reductase activity an artificial electron donor was substituted for formate in the above procedure.

Iida and Taniguchi (1959) used reduced methylene blue as artificial electron donor when assaying nitrate reductase. In these present studies this reagent proved unsatisfactory because the oxidized form of the dye absorbed strongly at the wavelength used for the nitrite assay. Various procedures were therefore examined for the removal of methylene blue after it had served its purpose in the enzymatic reaction. Trichloroacetic acid added to a final concentration of concentration of 5% precipitated the dye but the precipitation was not always complete or reproducible. Quantitative adsorption of methylene blue was achieved by mixing with charcoal, but potassium nitrite

TABLE 13. Enzyme activities of cell residue and solubilized preparation.

Enzyme.	Cell Residue.		Solubilized Preparation.	
	Specific activity	Total activity	Specific activity	Total activity
succinic oxidase	0.08	380	0.0	0
succinic dehydrogenase	0.11	520	0.0	0
DPNH oxidase	0.15	710	0.0	0
DPNH dehydrogenase	0.21	1,000	0.54	130
Nitrate reductase	0.25	1,190	0.08	19
Formate-nitrate reductase	0.12	570	0.0	0
Formate-methylene blue-nitrate reductase	-	-	0.06	14

The solubilized preparation (240 mg.) was prepared from 4,750 mg. of cell residue. For all enzymes, specific activity was expressed as umoles of substrate reacted per minute per mg. protein. Total activity is the product of specific activity and total protein.

The assay procedures for the enzymes are described in the text.

was also adsorbed by this procedure.

It seemed, therefore, that a different dye was required for the assay procedure. After testing a number of reagents, benzyl viologen was chosen for use in the reaction. The reduced form of this dye is blue, but is highly auto-oxidizable, yielding the oxidized form which is colourless. Because the reduced form of the dye is so rapidly reoxidized by oxygen, the manner of addition of the reduced dye to the reaction mixture presented a difficult problem. A number of systems were tested for reducing ability; catalytic reduction with molecular hydrogen in presence of palladium; reduction with nascent hydrogen generated by electrolysis of water; reduction by ascorbate, potassium borohydride or sodium hydrosulphite.

Sodium hydrosulphite was found to be the most suitable of these reductants, although its use was attended by a number of difficulties. So that the assay should proceed under optimal conditions the following points of procedure were adhered to:

(i) As hydrosulphite is unstable in solution, the salt was dissolved just before addition to the Thunberg tubes.

(ii) By placing the dye in the side arm and the reductant in the main tube the dye was not reduced until the reaction was initiated by tipping the side arm contents into the main tube.

(iii) It was found that hydrosulphite interfered with colour development in the nitrite assay. Excess hydrosulphite was therefore removed after the enzymic reaction by passing a stream of air through the solution. Reoxidation was furthermore accelerated by heating for several minutes in a boiling water bath. On checking the effect of hydrosulphite on the stability of nitrite, it was found that hydrosulphite (1.5 mg. per ml.) did not cause significant decomposition of nitrite (20 mu moles per ml.), even after five minutes' boiling.

(iv) So that the reaction should proceed under optimal conditions, it was important to include sufficient hydrosulphite to keep the dye reduced for the full period of the incubation. On the other hand, a large excess of hydrosulphite was to be avoided, as it had to be removed before nitrite determination. It was found experimentally that 1.5 mg. hydrosulphite in the reaction mixture supported maximum enzyme activity.

In early experiments, trichloroacetic acid, added to a final concentration of 5% was used to stop the enzyme reaction. It was found however, that this precipitant caused the slow decomposition of nitrite, amounting to a 15% decrease in nitrite concentration after contact for one

hour at room temperature. It was found better to stop the reaction by heating the tubes in a boiling water bath for two minutes, as this treatment did not cause any nitrite decomposition.

The details of the final assay procedures incorporating the modification discussed above are described in the Methods Section.

The cell residue had quite good nitrate reductase activity, being capable of reducing 0.25 umole nitrate per minute per mg. protein (Table 13). Only about 2% of this total activity was extracted into the solubilized preparation which had a specific activity of one third that of the cell residue. The ability to couple nitrate reduction to formate oxidation was completely lost in the soluble fraction but was present in the cell residue at a specific activity of 0.12, about half that observed with the reduced dye. However, formate-nitrate reductase activity can be restored in the solubilized preparation by the addition of methylene blue. It is therefore apparent that the required enzymes are present, but are not in communication, either due to a structural displacement or the loss of a mediating factor.

Conclusion.

Several classically insoluble enzymes have been examined in the cell residue and solubilized preparation. The activities of these enzymes are tabulated in Table 13.

Succinic oxidase and dehydrogenase, DPNH oxidase, and formate-nitrate reductase were all completely inactive in the solubilized preparation, although active in the cell residue. DPNH dehydrogenase was present in the solubilized preparation at several times higher activity than in the cell residue. However only about 10% of the total DPNH dehydrogenase activity was extracted from the cell residue. The solubilized preparation contained nitrate reductase at one third of the level of the cell residue specific activity but at only about 2% of the cell residue total activity. These experiments showed that there was largely a preferential extraction of formic dehydrogenase from the cell residue by the solubilization procedure.

2. NATURE OF THE COLOURED MATERIAL IN THE SOLUBILIZED PREPARATION.

A most interesting characteristic of the solubilized preparation was its deep amber colour. It was considered likely that this colour was due to the presence of cytochrome or flavin compounds. In order to further investigate the nature of the coloured material, the solubilized preparation was examined in the recording spectrophotometer. The absorption spectrum of the preparation closely resembled that reported for cytochrome $\underline{b}_1$.

Characterization of cytochrome $\underline{b}_1$.

Cytochrome $\underline{b}_1$ has been described by Smith (1954,a) as occurring in *E.coli* together with cytochromes $\underline{a}_1$ and $\underline{a}_2$. The difference spectrum of *E.coli* whole cells is shown in Figure 4. The absorption peaks of cytochrome $\underline{b}_1$ appear in this spectrum at 560, 533 and 432 mu. The difference spectrum of the solubilized preparation (Figure 11 ◊ shows α-, β- and γ- peaks at 560, 532 and 429 mu (Table 14). The positions of the α- and β-peaks correspond to those reported by Smith for whole cells. In comparing the Soret (γ-) peaks it should be noted that in whole cells, the cytochrome $\underline{b}_1$ Soret peak is modified by the Soret peak of cytochrome $\underline{a}_1$ which lies nearby. As the solubilized preparation appears to be devoid of cytochromes $\underline{a}_1$ and $\underline{a}_2$, it is not surprising to find the γ- peak at 429 mu, several mu removed from the position of the cytochrome

FIGURE 11. Difference spectrum (hydrosulphite reduced minus oxidized) of the solubilized formic dehydrogenase preparation (dialysed overnight). The 4 cm. light path cuvette contained 1.6 mg. protein per ml. of 0.01 M KCl.

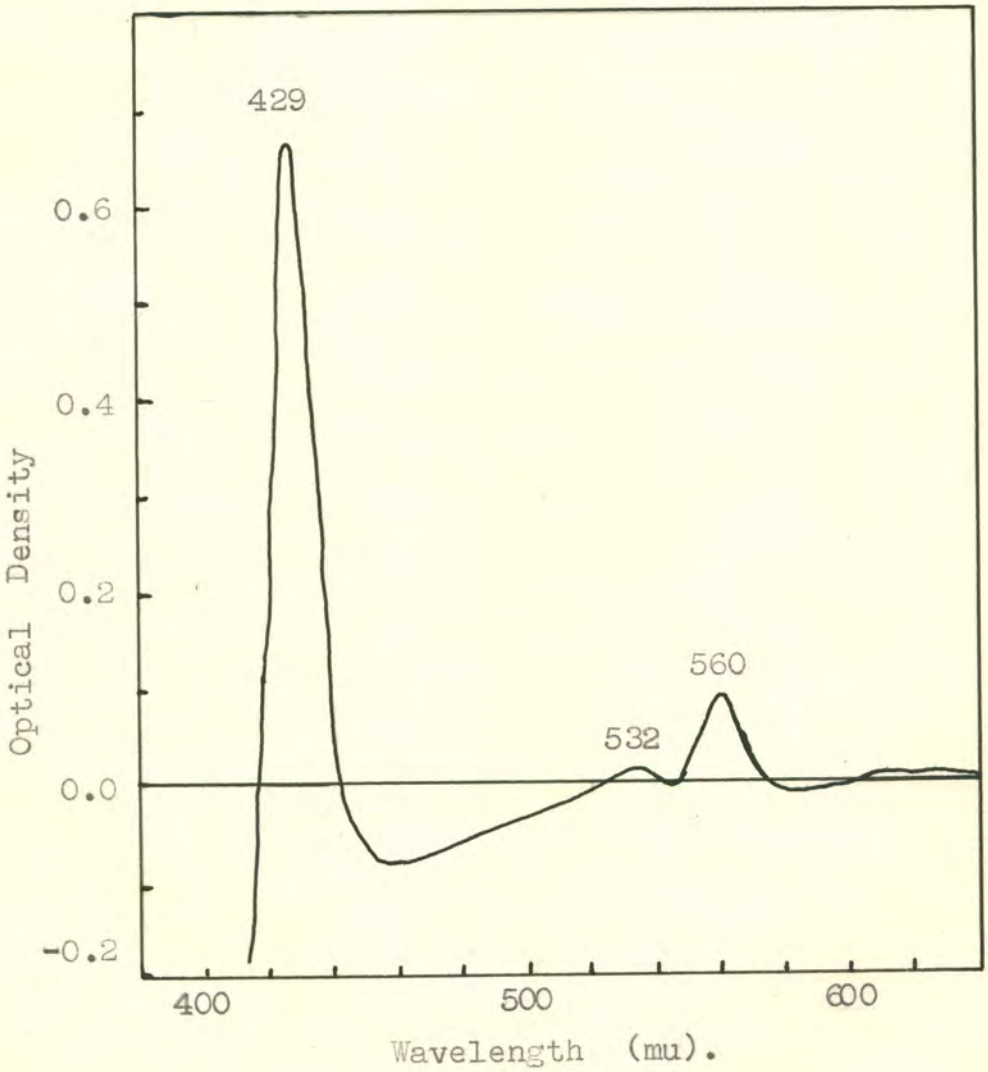


TABLE 14. Positions of absorption peaks (mu) of the solubilized preparation and of several b cytochromes reported in the literature.

	Difference spectrum		
Solubilized preparation	560	532	429
Cytochrome b_1, E.coli whole cells (1)	560	533	432

	Direct Spectrum			
	Oxidized		Reduced	
Solubilized preparation	412	560	530	428
Cytochrome b_1, E.coli whole cells (2)	415	560	528	428
Cytochrome b_1, E.coli particulate material (3)	415	560	530	430
Cytochrome b_2, yeast crystalline preparation (4)	413	557	528	424
Cytochrome b, heart muscle Mitochondria (2)	416	564	530	431

The values are obtained from the following references:

(1) Smith (1954,a)

(2) Morton (1958)

(3) Gest (1952)

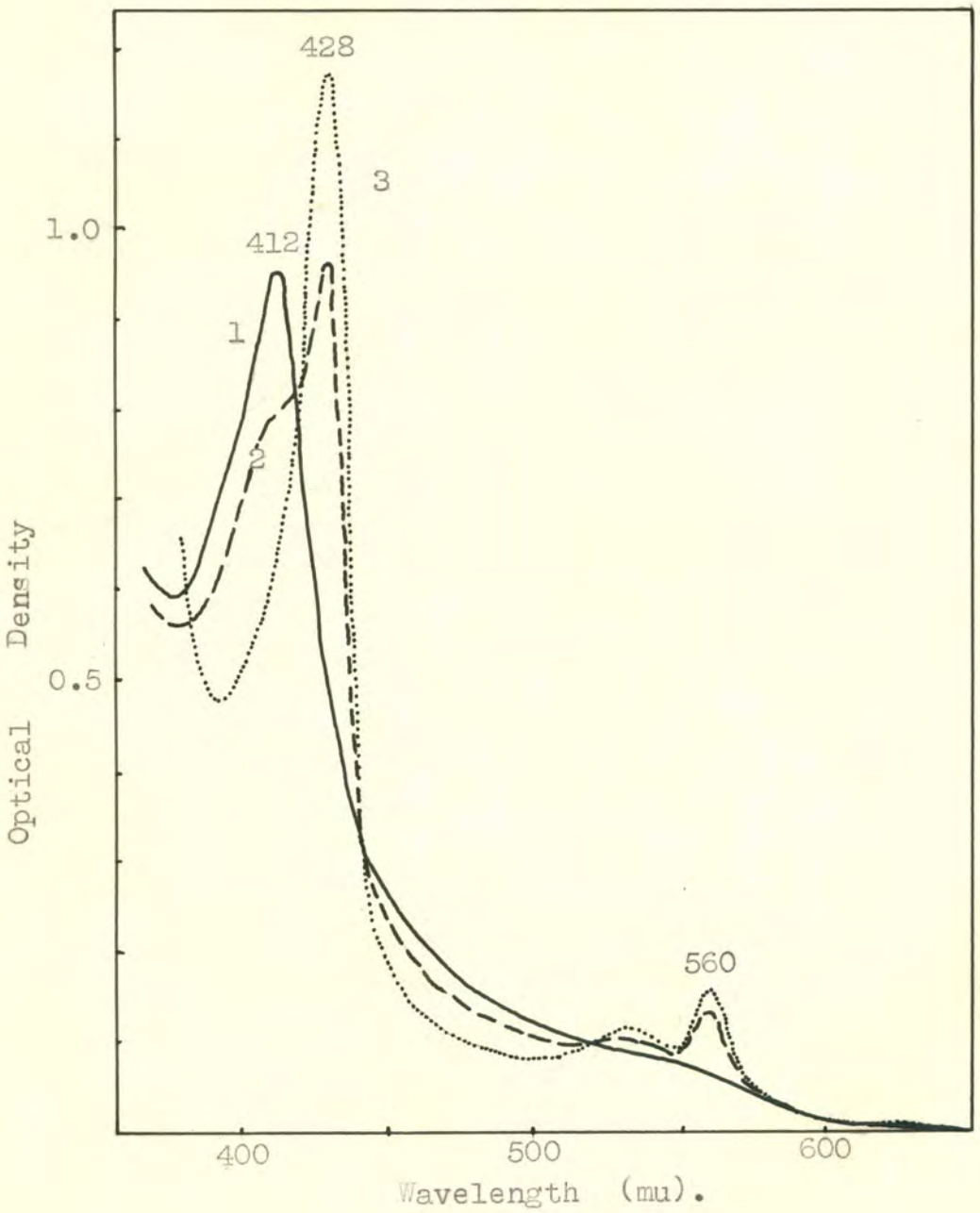
(4) Appleby and Morton (1959).

b_1 γ - peak for whole cells.

Further evidence for the solubilized preparation containing cytochrome b_1 was provided by the direct absorption spectrum. Table 14 shows the positions of the direct spectral bands of cytochrome b_1 reported in whole cells by Morton in a review (1958) and as found by Gest (1952) for particulate material suspended in deoxycholate solution. The direct spectrum of the solubilized preparation shows a strong Soret peak at 412 mu. After reduction with hydrosulphite, peaks appear at 560, 530 and 428 mu (Figure 12). The positions of the peaks coincide very closely with the positions reported in the literature for cytochrome b_1, further identifying the spectrum as that of cytochrome b_1. The absorption peaks of cytochrome b and b_2 are also shown in Table 14. Comparison shows these values to be well removed from those found for the solubilized preparation. It should be emphasized that more accurate positions for the spectral peaks can be obtained with this solubilized preparation than has previously been possible in particulate and whole cell suspensions because of turbidity difficulties in these preparations.

Another standard method for identifying cytochromes is to compare the relative heights of the absorption peaks, such as the ratio between the absorbancies of the α- and γ - peaks of the reduced compound. Unfortunately, data of

FIGURE 12. Direct absorption spectrum of the solubilized formic dehydrogenase preparation. The 4 cm. light path cuvette contained 1.6 mg. protein per ml. of 0.01M KCl.
1. oxidized spectrum;
2. preparation reduced by formate;
3. dithionite-reduced preparation;



this kind are very meagre. Smith (1954,a) has reported the ratio between the α- and γ- peaks for the difference spectrum of cytochrome $\underline{b}_1$ in whole cells but as the Soret peak of cytochrome $\underline{a}_1$ lies so close to that of cytochrome $\underline{b}_1$, comparison of this ratio with that of the solubilized preparation is of doubtful value. However, the relative heights of the α- and β- peaks of cytochrome $\underline{b}_1$ shown in the difference spectrum of Smith (1954,a) appear to be similar as those shown in the present preparation, but the small extinction of the β - peak makes comparison difficult.

Close examination of both direct and difference spectra indicate that the solubilized formic dehydrogenase-cytochrome $\underline{b}_1$ preparation is essentially free from other cytochromes. This is evidenced by the symmetry of the cytochrome $\underline{b}_1$ peaks and the absence of any other peaks between 400 and 650 mu. It should be noted, however, that in some preparations, a broad band between 620 and 640 mu was observed having an extinction of only about 5% of the α- peak of cytochrome $\underline{b}_1$. This may represent cytochrome $\underline{a}_2$ which has its α - absorption peak at 630 mu (Smith, 1954,a). As cytochrome $\underline{a}_2$ has no β - peak and very little Soret absorption, the absorption spectrum could give no further evidence of its presence.

There was no trace whatsoever of cytochrome $\underline{a}_1$ in any of the absorption spectra observed.

Quantitation of cytochrome b_1.

As cytochrome b_1 has not previously been isolated in soluble form, no extinction coefficients are available for the direct quantitation of this compound. As a result indirect methods had to be used to determine its concentration. These consisted of using extinction coefficients for mammalian and yeast b cytochromes in quantitating the difference and direct spectra of this bacterial b cytochrome and also of preparing pyridine haemochromogen which has the same extinction coefficient for all b cytochromes. The procedures for these estimations are described in the Methods Section.

1. Assuming the extinction coefficient for the α- peak of the cytochrome b_1 difference spectrum to be the same as that for mammalian cytochrome b (Chance, 1952), a cytochrome b_1 content of 0.55 mu moles per mg. of protein was estimated for the solubilized preparation after overnight dialysis. After a further five days' dialysis, the cytochrome b_1 content of the material remaining in solution increased to a value of about 0.7 mu moles per mg. protein. The fact that no cytochrome b_1 is precipitated during this dialysis provides good evidence that the cytochrome has been extracted in true solution.

2. When the concentration of cytochrome b_1 was estimated using the direct extinction coefficient for the α - peak of

yeast cytochrome $\underline{b}_2$, similar values were obtained to those calculated on the basis of mammalian cytochrome $\underline{b}$.

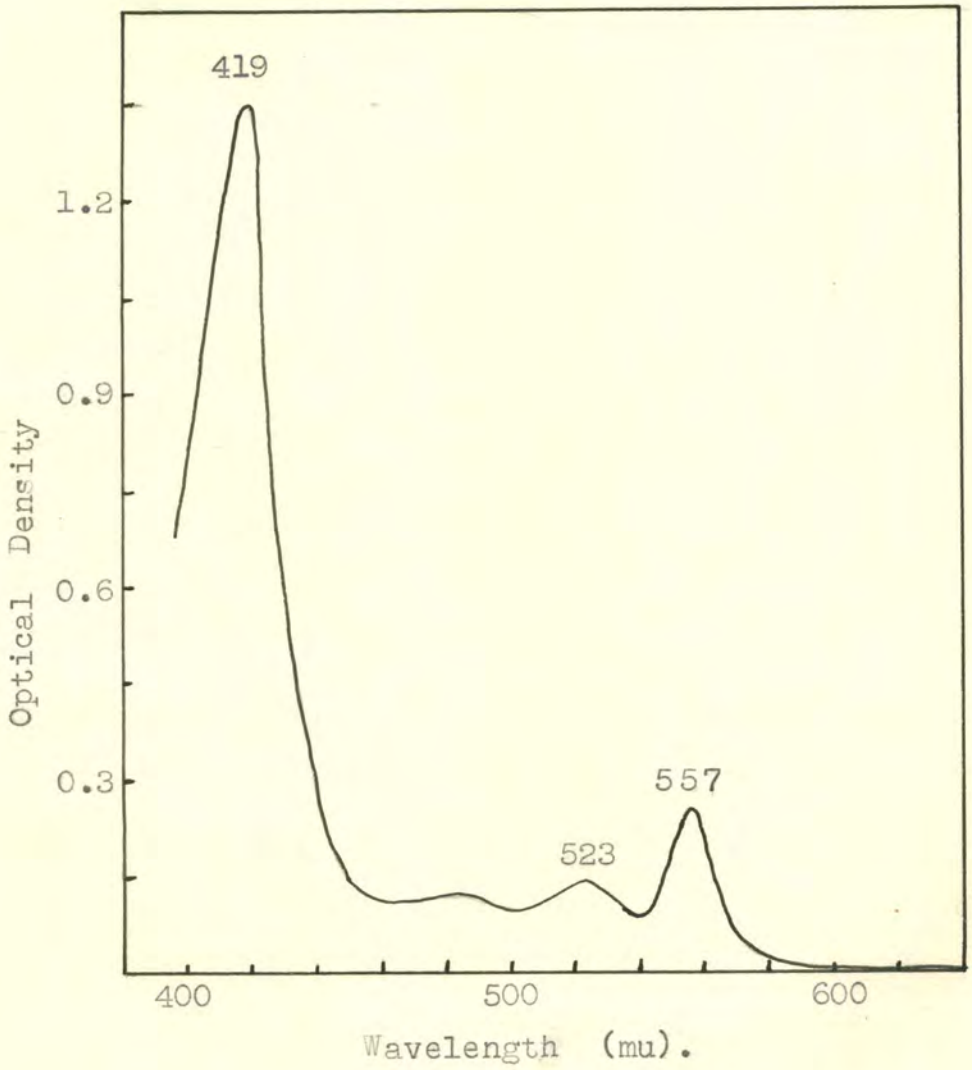
3. Treatment of any of the $\underline{b}$ cytochromes with acid acetone leads to the dissociation of the protohaem prosthetic group from the protein moiety. The protohaem remaining after evaporation of the acid acetone extract can be coupled to pyridine in alkaline solution to form the corresponding pyridine haemochromogen.

The pyridine haemchromogen isolated in this manner from the solubilized preparation, showed after reduction with hydrosulphite a direct absorption spectrum with α-, β- and γ- bands at 557, 523 and 419 mu respectively. The positions and relative heights of the absorption peaks corresponded closely to the values reported by Morton (1958) for the pyridine haemochromogen formed with protohaem.

The cytochrome $\underline{b}_1$ content of the solubilized preparation dialysed overnight was determined by this method to be 0.53 mumoles per mg. protein (Figure 13). There is therefore good agreement between the value estimated by this method and that calculated from the difference spectrum of the solubilized preparation.

On the other hand, estimation of the cytochrome $\underline{b}_1$ content of the cell residue by direct spectroscopy proved rather more difficult. A variety of techniques were applied in order to record the absorption spectrum of the cell residue. The material was suspended in various proportions

FIGURE 13. Direct absorption spectrum of the hydrosulphite reduced pyridine haemochromogen prepared from the solubilized formic dehydrogenase preparation (42 mg. protein, dialysed overnight). 1 cm. light path cuvette.



and combinations of deoxycholate, sucrose and glycerol, in some cases also sonicating the material. However, on no occasion could a satisfactory absorption spectrum be obtained on a cell residue suspension of sufficiently high concentration as to allow definite absorption peaks to be distinguished.

As neither of the first two methods for cytochrome $\underline{b_1}$ estimation described above could be applied to the cell residue, the pyridine haemochromogen of cytochrome $\underline{b_1}$ was prepared from this material. Examination of the absorption spectrum of the reduced pyridine haemochromogen showed that the cell residue contained 0.19 mumoles of cytochrome $\underline{b_1}$ per mg. protein (Table 15). Comparison with the cytochrome $\underline{b_1}$ content of the solubilized preparation indicated that the cytochrome was purified threefold in the solubilized preparation and that 14% of the cell residue cytochrome $\underline{b_1}$ had been extracted by the solubilization procedure, assuming that 5% of the cell residue total protein was extracted.

Substrate reduction of cytochrome $\underline{b_1}$.

An important property of the soluble formic dehydrogenase-cytochrome $\underline{b_1}$ preparation is that the cytochrome $\underline{b_1}$ is rapidly reducible by sodium formate. The extent of reduction by formate varied according to the particular preparation and its age. Figure 12 illustrates the direct spectrum of a typical preparation in which cytochrome $\underline{b_1}$ reduction by formate was about 70% complete compared with

TABLE 15. Cytochrome b_1 and flavin content of the particulate and solubilized preparations.

	Cell residue.	Solubilized preparation.	Proportion solubilized.
Cytochrome b_1 (difference spectrum)	-	0.55	-
Cytochrome b_1 (pyridine haemochromogen)	0.19	0.53	14%
Flavin (acid extractable)	0.15	0.39	13%
Flavin (total)	0.18	0.58	16%

The method of estimation of each compound is stated in brackets above. All concentrations are expressed as umoles per mg. protein.

The solubilized preparation referred to was dialysed overnight after extraction from the cell residue. The proportion solubilized from the cell residue for each compound was calculated on the basis of a 5% extraction of cell residue total protein into soluble form.

that obtained with hydrosulphite. In fresh preparations, the cytochrome was reduced by formate as fully, or almost as fully, as it was by hydrosulphite. After several days' storage, however, the extent of reduction by formate fell to about 60% of full reduction or even lower. On the other hand, the ability of the preparations to reduce ferricyanide did not vary greatly during storage for a similar period, so that it appeared that communication between formic dehydrogenase and cytochrome b_1 is not necessary for ferricyanide reduction. The reduction of cytochrome b_1 by formate may indicate that the soluble preparation is a macromolecular complex of the enzyme and the cytochrome, still associated as in the whole cell. On the other hand, the two components

may be reacting together as two separate soluble proteins. However, a chemical link of some kind between formic dehydrogenase and cytochrome $\underline{b}_1$ is strongly suggested by the fact that the extent of cytochrome $\underline{b}_1$ reduction by formate decreases with age. During this aging, the formic dehydrogenase activity does not decrease and cytochrome $\underline{b}_1$ remains soluble. Another possibility is that some unknown or unrecognized labile factor is necessary for communication between the two components.

The absorption spectrum of the reduced, solubilized preparation showed no definite evidence for the presence of cytochrome $\underline{a}_1$ and $\underline{a}_2$, which are thought to act as terminal electron acceptors in the complete aerobic oxidation of formate to carbon dioxide and water. As was noted in the previous section, however, the solubilized preparation can still carry out aerobic oxidation at a significant rate (0.2 umoles formate oxidized per minute per mg. protein). As cytochrome $\underline{b}_1$ is known to be slightly auto-oxidizable, it seems quite possible that this residual formic oxidase activity is not due to the natural cytochrome oxidase system, but rather is a result of auto-oxidation through cytochrome $\underline{b}_1$.

Neither DPNH nor succinate reduced cytochrome $\underline{b}_1$ in the solubilized preparation, irrespective of its age. However, the hydrosulphite-reduced preparation was slowly reoxidized by nitrate. A 3 ml. solution of the soluble preparation (1 mg. protein per ml.) in a 4 cm. light path cell, was fully reduced with a minimum of fresh hydrosulphite solution (containing 0.4 mg. sodium hydrosulphite). Reoxidation after the addition of 5 mg. of solid potassium

nitrate was rather slow, but after about ten minutes, the cytochrome was about 80% reoxidized, calculating reduced cytochrome b_1 by the method of Chance (1952). This cycle of reduction and reoxidation could be repeated several times until the solution became too cloudy for spectrophometric measurements.

On the other hand, when the solubilized preparation was reduced with a solution containing 4 umoles sodium formate under similar conditions, the cytochrome was not reoxidized by potassium nitrate even after thirty minutes' incubation. This latter result was consistent with the finding reported in the previous section that the formate-nitrate reductase system was inactive in the solubilized preparation.. However, there was no explanation forthcoming for the apparent inconsistency that cyto chrome b_1 reduced by hydrosulphite is reoxidized by nitrate, but that the formate-reduced cytochrome is not reoxidizable by nitrate.

Flavin content.

Examination of the difference or direct spectrum of the reduced solubilized preparation (Figures 11 and 12) showed that a trough appeared at about 450 mu on reduction with either dithionite or formate. This trough has been shown to be due to the presence of flavin in the preparation. The flavin was reduced by formate, but never to the same extent as by dithionite, irrespective of the age of the preparation. As the cytochrome b_1 Soret peak interfered

with the flavin trough at 450 mu, quantitation of flavin content directly from the absorption spectra is not possible.

It was therefore necessary to extract flavin from the protein with dilute perchloric acid and to estimate the extracted flavin by its decrease in absorbancy at 450 mu following the procedure described in the Methods Section. Digestion with trypsin before acid treatment allowed the extraction of a larger amount of flavin. This was termed "total flavin" to distinguish it from the "acid extractable flavin", which was extracted without trypsin digestion.

The levels of acid extractable and total flavin present in the cell residue were 0.15 and 0.18 mumoles per mg. protein and for the solubilized preparation, 0.39 and 0.58 mumoles per mg. protein, respectively (Table 15). It is interesting to note that further purification with calcium phosphate gel, led to the elimination of the flavin that is extracted only after trypsin treatment. This finding may indicate that only the acid extractable flavin is involved in the dehydrogenation of formate. This preliminary finding, however, awaits further documentation.

Conclusion.

Examination of the absorption spectrum of the solubilized preparation has shown its colour to be mainly due to cytochrome b_1 and flavin. The components were present in several times higher concentration than in the cell residue. About one-sixth of each of these compounds was

extracted from the cell residue in soluble form.

The fact that both cytochrome $\underline{b_1}$ and flavin were reducible with formate may indicate that the solubilized preparation is a flavoprotein-cytochrome $\underline{b_1}$ complex representing the first portion of the formate. oxidation chain. As was noted earlier the refractoriness of the solubilized enzyme to purification further suggested that the preparation is a macromolecular complex. Another interesting observation arising out of the attempts to further purify the solubilized enzyme, was that the coloured material was always purified together with the enzyme activity. This also supported the concept that in the solubilized preparation there has been isolated an integrated fragment of the formate oxidation chain as it occurs in the intact particulate material.

COENZYME Q AND VITAMIN K.

Introduction.

Having established cytochrome b_1 as the only cytochrome present in the purified soluble preparation, investigations turned to other compounds likely to be involved. Recently interest has been directed towards two quinones which are probably involved in the terminal respiratory chain - coenzyme Q and vitamin K. The presence of both these compounds has been reported in *E.coli* whole cells, so their presence in the particulate and soluble fractions was investigated.

In 1955, Festenstein *et al.* purified from rat liver, a compound absorbing strongly at 275 mu and forming crystals melting between 33° and 41°. In 1957, Crane *et al.* isolated a quinone from mitochondria by iso-octane extraction. This compound, which later received the name of coenzyme Q absorbed strongly at 275 mu and was capable of undergoing reversible oxidation and reduction. The absorption spectra of the oxidized and reduced forms are shown in Figure 14 The structure of the quinone was later established to be that shown in Figure 16 The number (n) of isoprenoid units attached to the quinone is dependent upon the source of the compound (Wolf *et al.*, 1958). Mammalian tissues in general contain coenzyme Q_{10}. The homologue with the shortest isoprenoid side chain so far isolated is coenzyme Q_6, from

FIGURE 14. Absorption spectrum of Coenzyme Q in absolute alcohol. The solid line represents the oxidized form and the dotted line represents the spectrum obtained after shaking with a few grains of Potassium borohydride. Concentrations used for 1 cm. path in mg./ml.: Ultraviolet range, 0.0425; visible range, 0.75.

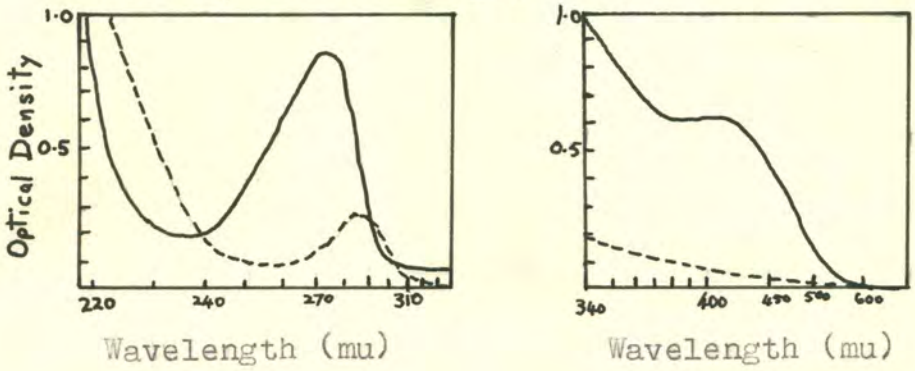
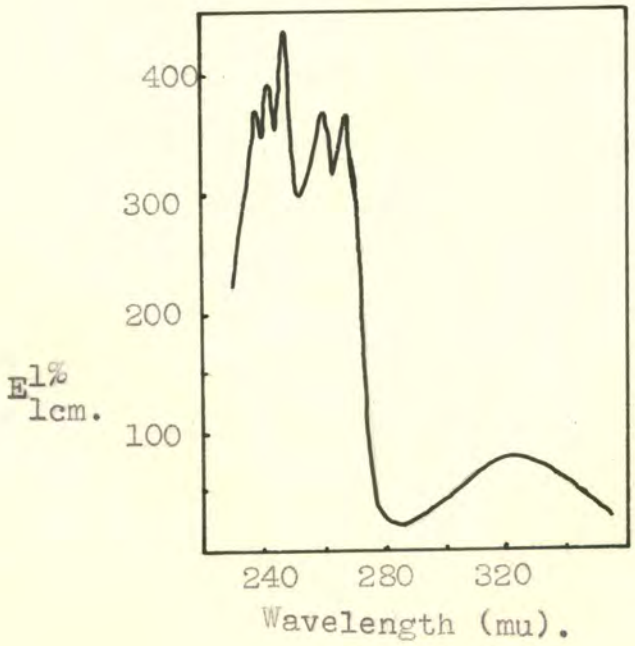


FIGURE 15. Ultraviolet absorption spectrum of synthetic Vitamin K_1.
$E^{1\%}_{1cm.}$ represents the extinction of a 1% solution with a 1cm. light path.
(Reproduced from Ewing et al., 1943.)



baker's yeast. The homologue reported to be present in E.coli is coenzyme Q_8 (Lester et al., 1959).

A considerable amount of experimental evidence has a accumulated for assigning coenzyme function to Q (Green and Lester, 1959). Firstly, it is a normal component of a wide variety of cells - animal, plant and microbial (Lester et al., 1959). Furthermore, in studies with mammalian tissues, it has been found that when coenzyme Q is extracted from mitochondria, enzymatic activity is lost, and this activity can be specifically restored by the addition of coenzyme Q. Coenzyme Q can also be shown to undergo alternate oxidation and reduction within the mitochondrion at a rate comparable with a key role in the direct chain of the electron transport system (Hatefi et al., 1959). Furthermore, inhibitors of electron transport affect the oxidoreduction state of bound coenzyme Q in the manner anticipated (Hatefi et al., 1959). This evidence, in the main, refers to systems of mammalian origin, but similar situations may exist in some bacterial cells.

In general bacteria contain coenzyme Q. The mycobacteria, however, contain no coenzyme Q but are rich in vitamin K. There are some organisms, such as E.coli and Azotobacter vinelandii which contain both Q and K. (Lester and Crane, 1959).

FIGURE 16.

The structure of coenzyme Q_n.

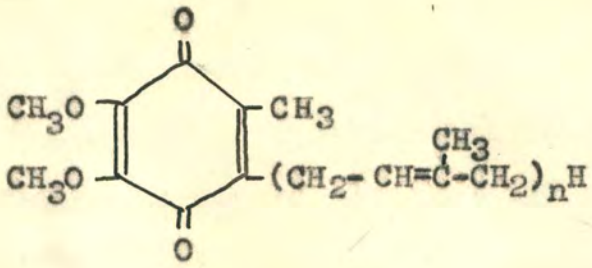
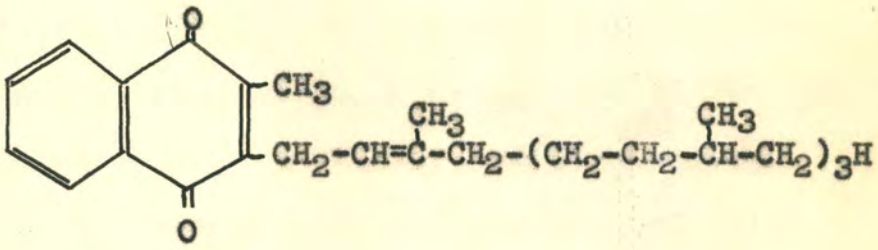


FIGURE 17.

The structure of vitamin K_1.



Vitamin K and its homologues, like coenzyme Q, are quinones, capable of undergoing reversible oxidation and reduction. The formula of vitamin K is shown in Figure 17 The K vitamins are characterized by a multibanded ultraviolet absorption. Figure 15 shows the absorption spectrum of vitamin K, which has peaks at 243, 249, 260 and 270 mu and a broad area of absorption between 310 and 340 mu. Several groups of workers have reported evidence for the involvement of vitamin K homologues in mammalian phosphorylative and respiratory systems. (Martius, 1954; Anderson and Dallam, 1959; and Colpa Boonstra and Slater, 1957). However, the relation of vitamin K to mammalian respiration is as yet uncertain. More convincing evidence for the involvement of vitamin K homologues in electron

transport and phosphorylation has come from the study of a bacterial system - subcellular particles of Mycobacterium phlei (Brodie et al., 1957; Brodie and Ballantine, 1960). These particles contain a vitamin K_1 derivative that is destroyed by irradiation with light of wave length 360 mu. After irradiation, the particles cannot aerobically oxidize succinate or couple phosphorylation to oxidation. These activities are fully restored by the addition of vitamin K_1. Furthermore, there have been reports that vitamin K analogues are involved in the nitrate reductase system of E.coli. (Medina and De Heredia, 1958; Itagaki and Taniguchi, 1959).

With these properties of coenzyme Q and vitamin K in mind, the presence of these compounds was investigated in the several E.coli preparations at present being studied.

Isolation of coenzyme Q and vitamin K.

Coenzyme Q and vitamin K were extracted from E.coli whole cells by direct extraction with iso-octane as described by Lester and Crane (1959). Three procedures were tested for ability to extract these compounds:

1. Freeze-dried cells were extracted with iso-octane.
2. The cell suspension was saponified by the method of Crane et al. (1959) before iso-octane extraction.
3. E.coli cells were dried in the oven at 100° before extracting with iso-octane as described in the Methods Section.

It was found that much less coenzyme Q was extracted from the freeze-dried cells with iso-octane than was extracted by either of the other two procedures. The reason for this was assumed to be that coenzyme Q could only be extracted after denaturation of the protein material. Iso-octane extraction of the freeze-dried protein probably did not denature any protein as water was absent during this extraction. On the other hand, drying at 100° or saponification ensured complete protein denaturation before extraction and as a result allowed quantitative extraction of coenzyme Q. Although iso-octane treatment of the saponified cell suspension led to a good coenzyme Q extraction, the procedure apparently caused some modification of the extracted quinones. This was evidenced by the broad region of absorption from about 275 mu to 250 mu for the iso-octane extract of the saponified cell suspension (Figure 18), instead of the well-defined absorption peak at 275 mu that characterizes coenzyme Q. A more characteristic spectrum was exhibited by the material extracted by iso-octane from the heat-dried cells. The absorption spectrum of an alcohol solution of this material showed a well defined peak at about 273 mu, which disappeared on reduction with potassium borohydride to give place to a smaller peak at about 284 mu (Figure19).

The coenzyme Q content of these cells as measured by the decrease in absorbancy at 273 mu was calculated to be 0.33 umoles per mg. dry weight of cells. This value lies

FIGURE 18. Absorption spectrum of an iso-octane extract (30 ml. total volume) of saponified, freeze-dried cells (0.42 g.). (1 cm. light path cuvette.)

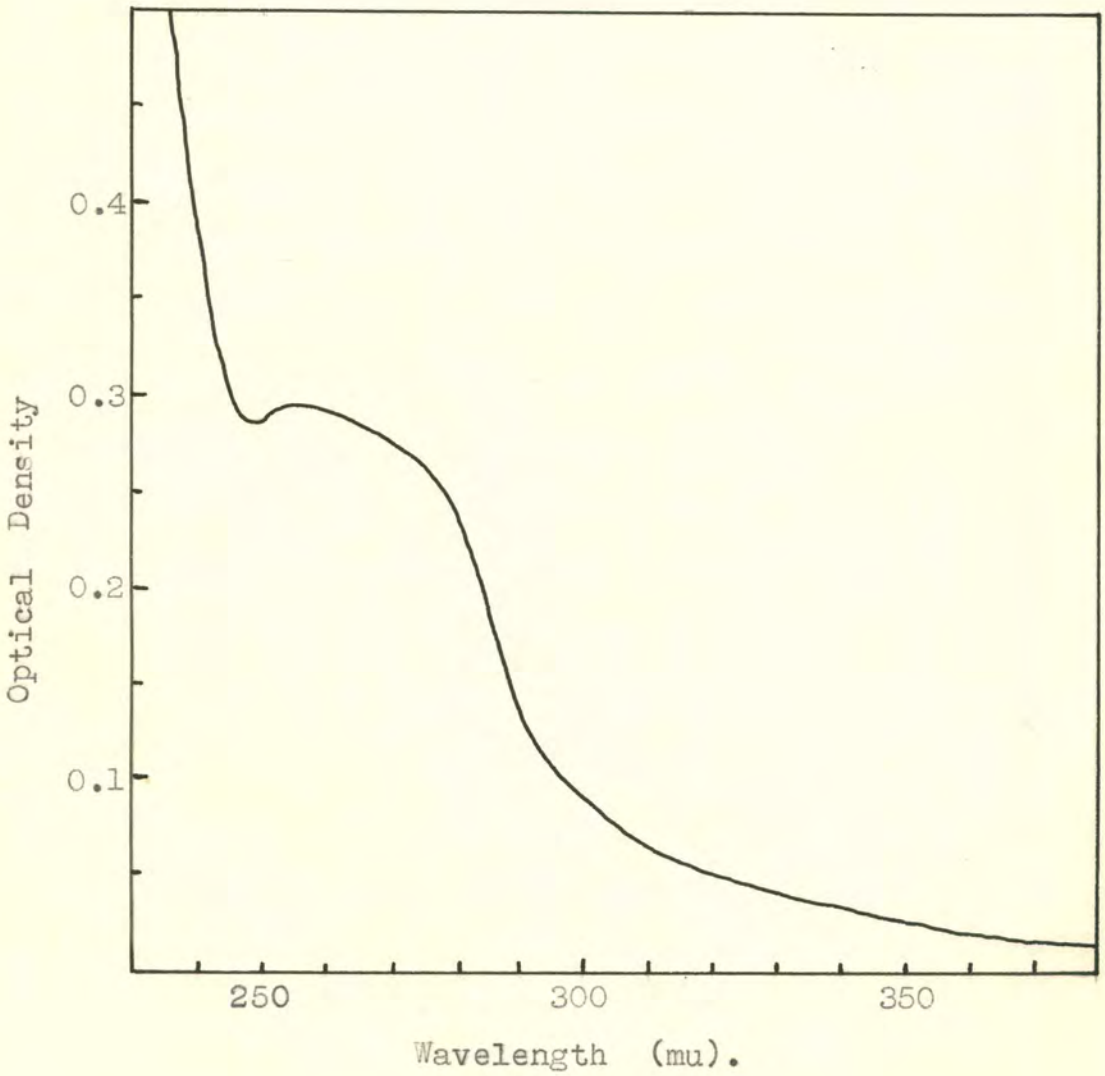
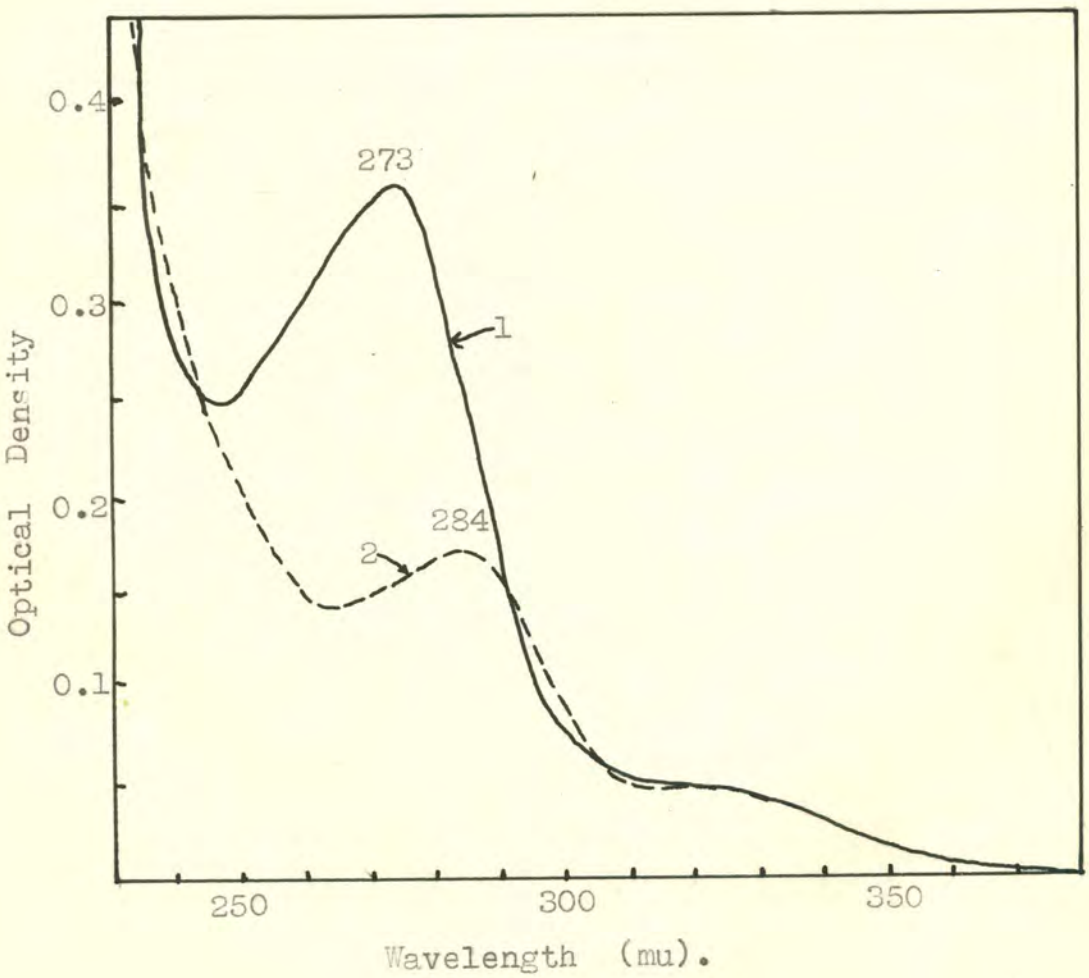


FIGURE 19. Absorption spectrum of material extracted by iso-octane from E. coli cells dried at 100°.

1. oxidized;
2. borohydride reduced.

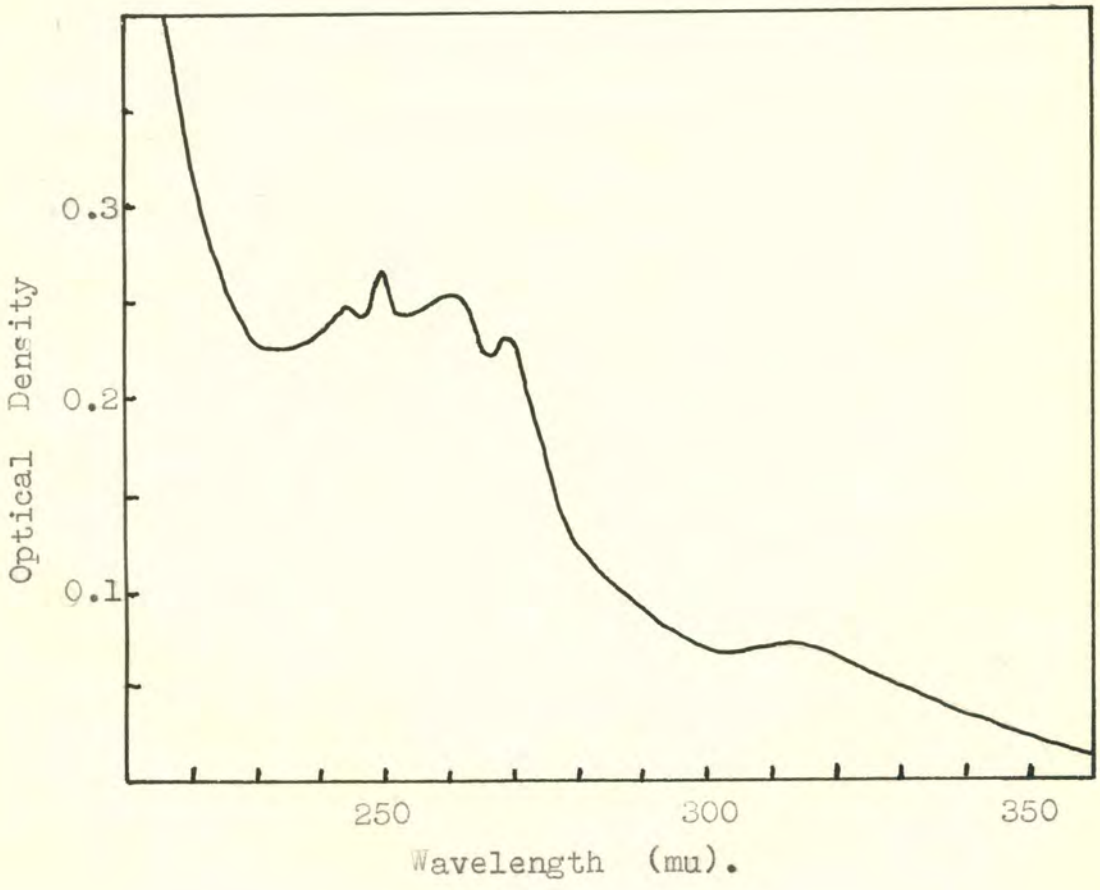
Solvent, absolute alcohol. 1 cm. light path. Each ml. of the alcohol solution corresponded to 27 mg. of the original dried cells extracted.



close to the values of 0.24 umoles per g. dry weight of cells estimated by Lester and Crane (1959) for E.coli whole cells.

Apparently vitamin K was not present in sufficiently high concentration as to interfere with the assay of coenzyme Q. However, the small absorption band between 310 and 340 mu appearing in the spectrum of the material extracted from the heat-dried cells probably is due to the presence of vitamin K, as a similar band is seen in the absorption spectrum of synthetic vitamin K_1 (Figure 15). Positive evidence of the presence of vitamin K was only possible after the removal of coenzyme Q by Decalso chromatography following the procedure described in the Methods Section. The material resulting from iso-octane extraction of the heat-dried cells was applied to the column in iso-octane and the column was eluted firstly with 5% ether in iso-octane. The absorption spectrum of the second fraction eluted by this solvent had peaks at 243, 249, 260 and 269 mu (Figure 20), identifying it as vitamin K. ~~The amount of vitamin K in this fraction was calculated to be 0.15 umole (calculated as vitamin K_1).~~ No significant amount of vitamin K was found in any other fraction. Assuming all the vitamin K to be concentrated in this fraction, this corresponded to a level of 0.3 umole vitamin K per g. dry weight of whole cells, which is of the same order as that found for coenzyme Q. It should be noted, however, that because vitamin K is slowly decomposed

FIGURE 20. Absorption spectrum of vitamin K isolated in the second 5% ether-iso-octane fraction resulting from chromatography on Decalso of an iso-octane extract of heat-dried E. coli cells (0.5 g. dry weight).

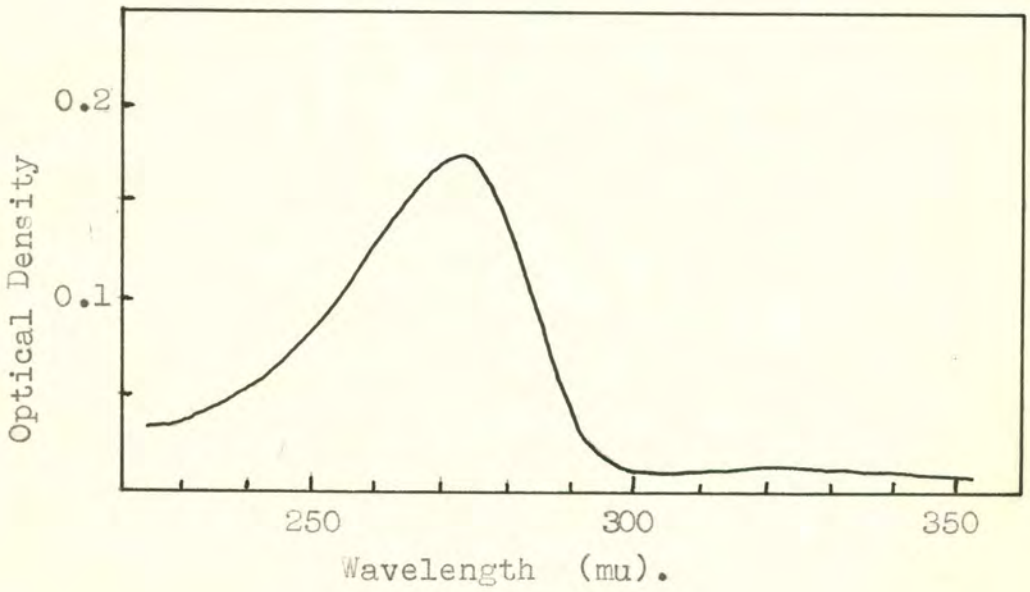


by light, this figure may be subject to some error.

The bulk of the coenzyme Q was eluted in the last two of the 5% ether fractions and in the first of the following 10% ether fractions. As in the unfractionated iso-octane extract, the spectrum of the purified quinone exhibited a well-defined peak at about 273 mu (see Figure 21) but lacked the 310-340 mu band and the high absorption in the 230-240 mu region. These absorbancies were presumably due to the presence of vitamin K in the crude iso-octane extract. None of the other fractions contained any ultraviolet-absorbing material. The relative optical densities and volumes of the initial extract and resulting fractions, indicated that good recovery was obtained. The concentration of coenzyme Q determined by this method was of the same order as that established for coenzyme Q by direct readings.

Having established coenzyme Q and vitamin K to be present in whole cells the solubilized preparation was examined using the extraction procedure found to be successful with whole cells. After drying the solubilized preparation at 100° repeated extraction with iso-octane failed to extract any ultraviolet-absorbing material, indicating that neither coenzyme Q nor vitamin K were solubilized by the deoxycholate-ammonium sulphate treatment. Preliminary experiments with the cell residue indicated the presence of coenzyme Q and vitamin K at concentrations

FIGURE 21. Absorption spectrum of coenzyme Q isolated in the first 10% ether - iso-octane fraction resulting from chromatography on Decalso of an iso-octane extract of heat-dried E. coli cells (0.5 g. dry weight).



several times higher than in whole cells. As neither of the quinones was found to be present in the soluble preparation, it was decided to move from the investigation of these specific lipids to the examination of the total lipid content of the particulate and solubilized preparations.

4. LIPID.

The concentration of lipid in the solubilized and particulate preparations was determined by isolating and weighing the material that was soluble in chloroform, following the procedure described in the Methods Section (see also Figure 22). The preparation was first extracted with a chloroform-methanol mixture (1:1). The residue remaining after evaporation of this extract to dryness was re-extracted with chloroform and the material extracted was weighed to determine the lipid content.

It was found that the cell residue had a lipid content of approximately 25 mg. per 100 mg. protein. The solubilized preparation on the other hand, contained a very low protein of lipid, amounting to only about 4 mg. per 100 mg. protein. Although quite a large amount of material was extracted by the chloroform-methanol mixture only a small proportion of this material proved to be soluble in chloroform. The chloroform-insoluble residue was later shown to be mainly composed of polyribonucleotide material, and will subsequently be referred to as such.

Earlier lipid determinations were conducted using absolute ethanol as extracting solvent by a modification of the method of Green et al. (1955). When estimating the lipid content of the solubilized preparation it was not found possible to obtain reproducible results by this method,

so it was abandoned in favour of the chloroform extraction method. The subsequent discovery of the polyribonucleotide material made it evident that this lack of reproducibility was due to the interference of this material which was apparently soluble in aqueous ethanol but not in absolute ethanol.

As well as revealing the lipid content of the solubilized preparation, the chloroform extraction procedure also showed that the total dry weight of the solubilized preparation was made up as follows:

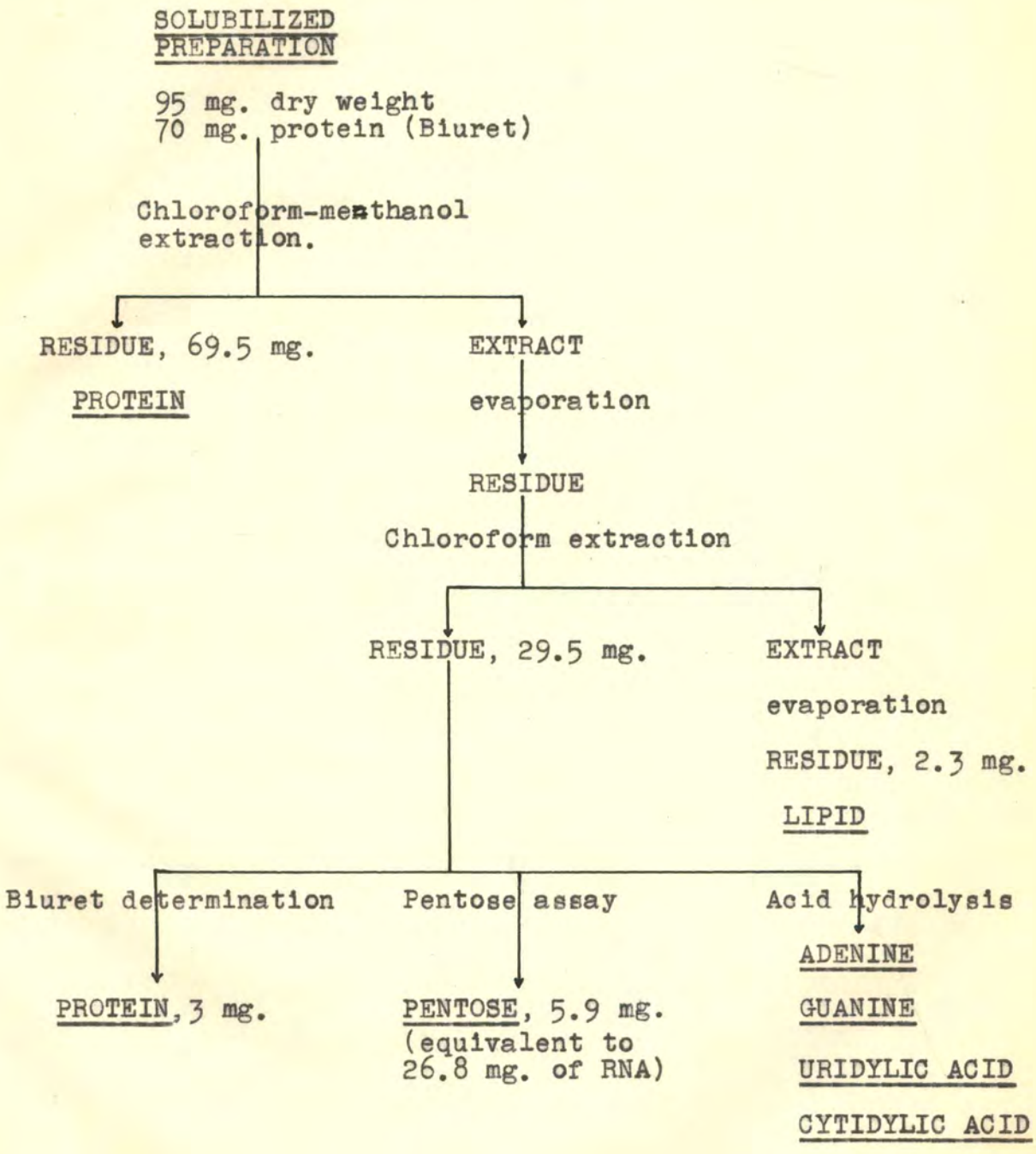
68% protein,

29% polyribnucleotide,

2.5% lipid.

The low lipid level was very significant because it provided further evidence that the solubilized enzyme is truly soluble. The deoxycholate-ammonium sulphate treatment apparently dissociated the lipoprotein material of the cell residue, releasing the protein into solution almost completely free from lipid.

FIGURE 22. Procedure for determination of the composition of the solubilized preparation. The protein and dry weight values obtained in a typical experiment are included.



5. POLYRIBONUCLEOTIDE.

An interesting aspect of the lipid determination conducted on the solubilized preparation was the material that was found to be extracted by chloroform-methanol (1:1) but insoluble in chloroform. This chloroform-insoluble residue was further investigated in order to establish its composition.

Most of the material was soluble in distilled water and all of it dissolved in dilute acid or alkali. The aqueous solution was a faint yellow. The absorption spectrum of the solution showed a high ultraviolet absorption at wavelengths below 285 mu.

It was argued that this chloroform-insoluble residue must be composed of material belonging to one or more of the four major classes of compound present in living matter: protein, lipid, polysaccharide, nucleic acid. The material was obviously not lipid as it was insoluble in chloroform. It seemed unlikely that material soluble in chloroform-methanol would be polysaccharide. A biuret determination was carried out to test for protein. This test was positive and the material was found to contain 0.1 mg. of protein per dry weight. This finding, however, left the bulk of the material unaccounted for.

In view of the high ultraviolet absorption of the chloroform insoluble residue, it was considered likely that

the residue contained nucleic acid. This was further supported by a positive pentose assay. Using the assay procedure described in the Methods Section, the chloroform-insoluble residue was found to contain 0.2 mg. pentose per mg. dry weight. This corresponded to a ribose nucleic acid (RNA) content of 0.9 mg. per mg. dry weight.

The chloroform-insoluble residue was definitely identified as being either RNA or polyribonucleotide by acid hyrolysis. Following the procedures set out in the Methods Section, the material was hydrolysed in acid, and the components of the hydrolysate were identified by paper chromatography.

TABLE 16. R_F values of standard solutions and of components of the acid hydrolysate of the chloroform-insoluble residue.

Standard solutions.		Hydrolysate components.	
Identity	R_F	R_F	Probable identity
adenine	0.27	0.29	adenine
guanine	0.17	0.17	guanine
uridylic acid	0.75	0.77	Uridylic acid
cytidylic acid	0.50	0.50	cytidylic acid
		0.63	0.63 another base

Comparison of the chromatographed hydrolysate with suitable standards under ultraviolet light, showed the presence of adenine, guanine, uridylic acid and cytidylic acid. These

are the four compounds normally liberated by acid hydrolysis of RNA. Deoxyribonucleic aced (DNA) was considered to be absent as no thymine was identified in the hydrolysate. The R_F values of the standards and hydrolysate components are tabulated in Table 16. A fifth ultraviolet-quenching spot with an R_F of 0.63 was also detected. This compound has not yet been definitely identified, but is thought to be another nitrogenous base.

The evidence presented above showed that the chloroform-insoluble material contained polyribonucleotide. It was not known whether the molecular weight of this component was sufficiently high as to establish its identity as RNA, so the name polyribonucleotide was retained. In retrospect, the presence of this material in the solubilized preparation explained some of the difficulties experienced in the earlier stages of these studies.

1. When using the method of Layne (1957) for the determination of protein concentration by the absorbancies at 280 mu and 260 mu, some difficulty was experienced as a result of an unexpectedly high absorbancy at 260 mu. This was probably due to the polyribonucleotide in the preparation.

2. It was found necessary to include several additional purification steps in the estimation of deoxycholate because a large amount of ultaviolet-absorbing material was obtained in the initial alcohol extract. In

the light of the findings above, this interfering material was clearly polyribonucleotide.

3. It is quite possible that the presence of the polyribonucleotide material caused some of the difficulty encountered in further purifying the solubilized enzyme. Korkes et al. (1951) have reported the failure of protein purification experiments as a result of the presence of nucleic acid. After removal of the nucleic acid material, the protein preparation could be fractionated successfully. In view of this report, further attempts to purify the solubilized formic dehydrogenase preparation may be much more successful after removal of the polyribonucleotide material.

It is not yet known what role the polyribonucleotide plays in the solubilized preparation, but several alternatives are possible. The material may be bound to formic dehydrogenase or to one of the other enzymes present in the solubilized material, or on the other hand it may be free in solution, unassociated with any other material. In either of these cases, the polyribonucleotide could be involved in enzyme action, but as yet this has not been investigated. The fact that the polyribonucleotide component occurs at a constant level in a number of preparations may indicate that it is a part of the enzyme system.

It is interesting to note that several workers have found polyribonucleotides associated with other enzyme

systems. Appleby and Morton (1960) have found a deoxyribopolynucleotide component in the yeast lactic dehydrogenase-cytochrome b_2 complex which they have crystallized. It was found that this component was not essential for enzyme activity, but was required for the crystallization of the enzyme. It would therefore appear to have structural function in this system.

Pinchot (1957) found that a polyribonucleotide component was essential for oxidative phosphorylation catalysed by intracellular particles from Alcaligenes faecalis. ~~Appleby and Morton (1960) found that in their system the polyribonucleotide served no such function as this.~~

Therefore, if the formic dehydrogenase-cytochrome b_1 preparation can be compared to the yeast lactic dehydrogenase-cytochrome b_2 system, one would expect the polyribonucleotide to have a structural rather than enzymic function in the formic dehydrogenase system. In any case the discovery of the polyribonucleotide adds another intriguing aspect to this already interesting preparation which opens up wide scope for the further study of the bacterial terminal respiration system.

SUMMARY

The enzyme formic dehydrogenase has been investigated in E.coli. It was confirmed that this enzyme is firmly bound to the particulate material of the cell in close association with cytochrome b_1. Cell growth and disintegration methods have been devised for the isolation of the particulate enzyme. Furthermore, a procedure was developed which allowed the extraction of the enzyme and its associated cytochrome from the particulate material into soluble form. The solubilization procedure involved fractionation of the particulate material with ammonium sulphate in presence of a relatively high concentration of sodium deoxycholate. This treatment resulted in quantitative extraction of the formic dehydrogenase and a twenty-fold purification of the enzyme. Only small amounts of the other insoluble enzymes were released by this procedure. The extraction procedure led to the break-up of the formic dehydrogenase aerobic respiration chain and no cytochromes, other than cytochrome b_1 , were extracted. Examination of the preparation showed it to contain considerable amounts of flavin and polyribonucleotide and a small amount of lipid. This soluble preparation appeared to be a flavohaemoprotein complex, representing the first portion of the formate oxidation chain of E.coli.

REFERENCES.

Anderson, W. W. and Dallam, R. D., J. Biol. Chem. 234, 409 (1959).

Appleby, C. A. and Morton, R. K., Biochem. J. 73, 539 (1959).

Appleby, C. A. and Morton, R. K., Biochem. J. 75, 258 (1960).

Basford, R. E., Tisdale, H. D., Glenn, J. L., and Green, D. E., Biochim. Biophys. Acta 24, 107 (1957).

Bomstein, R., Goldberger, R. and Tisdale, H., Biochem. Biophys. Research Communications 2, 234 (1960).

Brodie, A. F., Weber, M. M. and Gray, C. T., Biochim. Biophys. Acta 25, 448 (1957).

Brodie, A. F. and Ballantine, J., J. Biol. Chem. 235, 226 and 232 (1960).

Bunting, M. I., J. Bact., 40, 57 (1940).

Chance, B., Nature (London) 169, 215 (1952).

Colpa-Boonstra, J. P. and Slater, E. C., Biochim. Biophys. Acta 23, 222 (1957).

Crane, F. L. and Glenn, J. L., Biochim. Biophys. Acta 24, 100 (1957).

Crane, F. L., Hatefi, Y., Lester, R. L., and Widmer, C., Biochim. Biophys. Acta 25, 220 (1957).

Crane, F. L., Lester, R. L., Widmer, C. and Hatefi, Y., Biochim. Biophys. Acta 32, 73)1959).

DeBernard, B., Biochem. Biophys. Acta 23, 510 (1957).

Drury, H. F., Archiv. Biochem. 19, 455 (1948).

Duysens, L. N. M., Nature (London) 173, 692 (1954).

Edelhoch, H., Hayaishi, O. and Teply, L. J., J. Biol. Chem. 197, 97 (1952).

Elsden, S. R., Kamen, M. D. and Vernon, L. P., J. Amer. Chem. Soc. 75, 6347 (1953).

Ewing, D. T., Tomkins, F. S. and Kamm, O., J. Biol. Chem. 147, 233 (1943).

Festenstein, G. N., Heaton, F. W., Lowe, J. S. and Morton, R. A., Biochem. J. 59, 558 (1955).

Fruton, J. S., and Simonds, S., General Biochemistry, Second Edition, page 307, (1958) John Wiley and Sons, New York.

Gale, E. F., Biochem. J. 33, 1012 (1939).

Gale, E. F. and Epps, H. M. R., Biochem. J. 36, 600 (1942).

Gest, H., in Phosphorus Metabolism, McElroy, W. D. and Glass, B. (eds), Vol. II,page 522 (1952) Johns Hopkins Press, Baltimore, Md.

Gornall, A. G., Bardawill, C. J. and David, M. M., J. Biol. Chem. 177, 751 (1949).

Green, D. E., Harvey Lectures, Series 52, 177 (1956-57).

Green, D. E., Adv. in Enzymology, 21, 73 (1959).

Green, D. E. and Lester, R. L., Fed. Proc. 18, 987 (1959).

Green, D. E., Mii, S. and Kohout, P. M., J. Biol. Chem. 217, 551 (1955).

Green, D. E., Järnefelt, J. and Tisdale, H. D., Biochim. Biophys. Acta 31, 34 (1959).

Hatefi, Y., Lester, R.L., Crane, F. L. and Widmer, C., Biochim. Biophys. Acta 31, 490 (1959).

Hugo, W. B., Bact. Reviews 18, 87 (1954).

Iida, K. and Taniguchi, S., J. Biochem. (Japan) 46, 1041 (1959).

Itagaki, E. and Taniguchi, S., J. Biochem. (Japan) 46, 1419 (1959).

Kalnitsky, G. and Werkman, C. H., Archiv. Biochem. 2,113 (1943).

Kamen, M. D., in a Symposium by Clark, W. M., Kaplan, N. O. Kamen, M. D., Bact. Reviews 19, 234 (1955).

Keilin, D., Proc. Roy. Soc. (London) B98, 312 (1925).

Keilin, D., Ergeb. Enzymforsch., 2, 239 (1933).

Keilin, D. and Harpley, C. H., Biochem. J. 35, 688 (1941).

Keilin, D. and Hartree, E. F., Proc. Roy. Soc. (London) B124, 397 (1938).

Keilin, D., and Hartree, E. F., Proc. Roy. Soc. (London) B127, 167 (1939).

Keilin, D. and Hartree, E. F., Nature 164, 254 (1949).

Keilin, D. and Hartree, E. F., Nature 176, 200 (1955).

Keilin, D. and Slater, E. C., Brit. Med. Bull. 9, 89 (1953).

Korkes, S., del Campillo, A., Gunsalus, I. C., and Ochoa, S., J. Biol. Chem. 193, 721 (1951).

Layne, E., in Methods in Enzymology, Colowick, S. P. and Kaplan, N. O. (eds) Vol. III, page 447 (1957) Academic Press Inc., New York.

Lemberg, R., in a lecture entitled Bacterial Cytochromes to the N.S.W. Branch of the Australian Microbiological Society, 11th July, 1960.

Lester, R. L. and Crane, F. L., J. Biol. Chem. 234, 2169 (1959).

Lindstrom, E. S., Tove, S. R. and Wilson, P. W., Science 112, 197 (1950).

Mackler, B. and Penn, B., Biochim. Biophys. Acta. 24, 294 (1957).

MacMunn, C. A., Phil. Trans. Roy. Soc. (London) 176, 641 (1885).

MacMunn, C. A., Phil. Trans. Roy. Soc. (London) 177, 267 (1886).

Markham, R. and Smith, J. D., Biochem. J. 49, 401 (1951).

Martius, C., Biochem. Z. 326, 26 (1954).

Medina, A. and DeHeredia, C. F., Biochim. Biophys. Acta 28, 452 (1958).

Mickle, H., J. Roy. Microscop. Soc. 68, 10 (1948).

Morton, R. K., Reviews of Pure and Applied Chemistry 8, 161, (1958).

Mosbach, E. H., Kalinsky, H. J., Halpern, E., Kendall, F. E., Archiv. Biochem. Biophys 51, 402 (1954).

Nossal, P. M., Aust. J. Exptl. Biol. Med. Sci. 31, 583 (1953).

Peterson, E. A. and Sober, H. A., J. Am. Chem. Soc. 78, 751 (1956).

Pinchot, G. B., Biochim. Biophys. Acta 23, 660 (1957).

Pinsent, J., Biochem. J. 57, 10 (1954).

Postgate, J. R., J. Gen. Microbiol. 14, 545 (1956).

Quastel, J. H. and Whetham, M. D., Biochem. J. 19, 520 (1925).

Rendina, G. and Singer, T. P., Biochim. Biophys. Acta 30, 441 (1958).

Ringler, R. L. and Singer, T. P., Biochim. Biophys. Acta 29, 661 (1958).

Sato, R. and Egami, F., Bull. Chem. Soc. Japan 22, 137 (1949).

Singer, T. P. and Kearney, E. B., in Methods of Biochemical Analysis, Glick, D. (ed.) Vol. IV, page 307 (1957) Interscience Publishers Inc., New york.

Slater, E. C., Adv. in Enzymology 20, 147 (1958).

Smith, L., Archiv. Biochem. Biophys. 50, 299 (1954,a).

Smith, L., Bact. Rev. 18, 106 (1954b).

Snell, F. D. and Snell, C., Colourimetric Methods of Analysis, Third edition, Vol. II, page 802, D. Van Nostrand and Co., New York. (1949).

Sorby, H. C., Quart. J. Micro. Sci. 16, 76 (1876).

Taniguchi, S., Asano, A., Iida, K., Kono, M., Omachi, K. and Egami, F., Proc. International Symposium on Enzyme Chemistry, Japan, page 238 (1958).

Vernon, L. P. and Kamen, M. D., Archiv. Biochem. Biophys. 44, 298 (1953).

Williams, F. R. and Hagar, L. P., Biochim. Biophys. Acta 38, 556 (1960).

Weibull, C., J. Bacteriol. 66, 688 and 696 (1953).

Wolf, D. E., Hoffman, C. H., Trenner, N. R., Arison, B. H., Shunk, C. H., Linn, B. O., McPherson, J. F., and Folkers, K., J. Am. Chem. Soc. 80, 4752 (1958).

Wolin, M. J. and Lichstein, H. C., J. Bact. 72, 762 (1956).

Wrigley, C. W., Report of Practical Work Carried Out During Science IV, 1958.

Yakushiji, E., and Okunuki, K., Proc. Imp. Acad. (Tokyo) 16, 299 (1940).