ENDOGENOUS RESPIRATION

OF

Ochromonas malhamensis

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SUMMARY.

Dark-grown Ochromonas malhamensis was examined for intracellular carbon reserve materials which enabled the organism to sustain a constant and moderate rate of respiration for twenty hours in the absence of any exogenous carbon or energy sources. The organism was cultured in a medium which supplied uniformly-radioactive glucose as a major source of carbon. The cell material of radioactive cells harvested from such a culture was separated chemically into four fractions essentially consisting of soluble intermediates, lipid, polysaccharide and protein. It was established these four fractions had approximately the same specific radioactivity per atom of carbon.

Intact radioactive cells were allowed to respire in the absence of externally furnished organic substrates. The total and specific radioactivities of the carbon dioxide liberated and the material in the four fractions prepared from these cells were determined at intervals. It was clearly apparent that a water-soluble polysaccharide was the sole endogenous carbon substrate during the initial ten hours of endogenous respiration. Furthermore, carbon dioxide was the sole product of the respiration of this polysaccharide.

The water-soluble polysaccharide reserve material was purified by precipitation of the 1- hexadecyl pyridinium-borate complex on a cellulose column and elution of the column with a buffer gradient of decreasing pH. The reserve

material was separated into two components (polysaccharides 1 and 2) by these procedures.

Both polysaccharide 1 and 2 were found to be composed solely of glucose within the limits of detection of the methods employed. An infrared spectrum of polysaccharide 2 indicated that it was a beta-linked glucose polymer. A homologous series of oligosaccharides were products of the partial hydrolysis of polysaccharide 2 catalysed by an endohydrolytic beta- D- 1,3 glucanase. A disaccharide was isolated from the products of this reaction and was shown to be a beta- 1,3 linked diglucoside. Polysaccharide 1 appeared to have a similar structure to polysaccharide 2, but it was not possible to examine its chemical properties in any detail.

It was concluded that the intracellular reserve materials sustaining endogenous respiration of Ochromonas malhamensis were essentially beta- 1,3 linked glucose polymers.

Contrary to expectation the R.Q. was found to be less than 1.0 during endogenous respiration of Ochromonas. It was established that Ochromonas fixed carbon dioxide during endogenous respiration and this fixation may have been the cause of the low respiratory quotient. Carbon dioxide was found to be incorporated largely into soluble intermediates and protein. The first stable radioactive product of carbon

dioxide fixation by intact cells was aspartic acid. At least two primary carboxylation reactions were demonstrated using an Ochromonas broken cell preparation. One of these reactions appeared to be catalysed by L- malate : NADP oxidoreductase, decarboxylating (E.C. 1.1.1.40.). The other reaction was undefined.

STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in any other University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except when due reference is made to such material in the text.

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ABBREVIATIONS

NAD and $NADH_2$	Oxidised and reduced forms respectively of nicotinamide adenine dinucleotide.
NADP and $NADPH_2$	Oxidised and reduced forms respectively of nicotinamide adenine dinucleotide phosphate.
ADP and ATP	Adenosine 5' di-and tri-phosphates respectively.
GDP	Guanosine diphosphate.
iP	Inorganic orthophosphate.
GSH	Reduced Glutathione.
CoA	Coenzyme A.
p-CMB	p-chloro-mercuribenzoate.
tris	2-amino-2-hydroxymethyl-propane-1:3-diol.

Chapter 1

INTRODUCTION

Ochromonas malhamensis was isolated in pure culture by Pringsheim (1952). Hamilton, Hutner and Provasoli (1952) then showed that certain chrysomonads, among them Ochromonas, required added vitamin B_{12} for growth. At this time there was a need for a method which would specifically assay cyanocobalamin. Certain natural materials contained compounds structurally related to vitamin B_{12} which were acting as the vitamin for a number of micro-organisms while they had no vitamin activity for animals. Because of the broad specificity of micro-organisms for B_{12}-like compounds, vitamin B_{12} assay methods employing micro-organisms were of limited value where the material assayed was being considered as a source of the vitamin for animals. In search of a micro-organism with a narrow vitamin B_{12} specificity Ford (1953) examined Ochromonas specifity for B_{12}-like compounds. He found that this organism had a high order of specificity for cyanocobalamin and that the growth of the organism in the dark was related to the vitamin B_{12} concentration in the medium. Ford described a method for assaying vitamin B_{12} with this organism. Prior to the adoption of this assay technique it had been the practice to

extract B_{12} compounds from natural materials by steaming them with 1 per cent sodium acetate at pH 5.0. However it was found that the presence of more than 0.1 per cent sodium acetate in the assay medium markedly inhibited the growth of Ochromonas in the dark.

The work reported in this thesis was originally intended to be a study of this phenomenon of acetate inhibition. Initially it was found that acetate inhibited the endogenous respiration of Ochromonas which had been grown in the dark. Inhibition did not occur with acetate concentrations less than 6.0×10^{-3} M, while maximum inhibition occurred with concentrations greater than 1.1×10^{-2} M. During maximum inhibition the respiration rate was reduced to less than 10 per cent of the normal rate. Acetate inhibition was apparently pH dependent e.g. at pH 7.0 1.5×10^{-1} M acetate reduced the respiration rate by 54 per cent, while 1.1×10^{-2} M acetate was without effect, however at pH 5.5 1.1×10^{-2} M acetate reduced the respiration rate by more than 90 per cent. Formate and propionate were also found to inhibit the endogenous respiration of Ochromonas; propionate reduced the respiration rate by 95 per cent at concentrations less than 3×10^{-3} M.

At this stage there seemed to be little point in pursuing this line of investigation without first learning something about the endogenous respiration which was subject

to acetate inhibition. It was hoped that some insight into the nature of the materials acting as the substrate for endogenous respiration would suggest more profitable ways of investigating the problem.

Pringsheim (1952) described Ochromonas malhamensis as being "full of reserve substances-leucosin, oil, volutin and granules of unknown nature ----. In older cultures these drops disappeared, presumably being used up in the course of metabolism." This observation, that Ochromonas appeared to have considerable stores of reserve material, was borne out by the manometric experiments of Reazin (1954) and Johnson, Holdsworth, Porter and Kon (1957) who showed the organism was capable of sustaining a constant and moderately high rate of respiration for over 18 hours in the absence of any exogenous organic carbon source.

Reserve compounds probably occur widely in higher plants and animals e.g. starch, glycogen, triglyceride, etc. While similar types of material have been demonstrated in a large number of micro-organisms, too often it has been assumed that these materials must function as energy-storage compounds. Tetrahymena pyriformis was an example where such an assumption would have been false. Under certain conditions Tetrahymena synthesised an intracellular glycogen-type polysaccharide which accounted for 22 per cent of the dry weight of cell material (Ryley 1951).

However in the absence of extracellular nutrient the aerobic metabolism of the organism was shown to be dependent on processes other than the breakdown of the polysaccharide (Ryley 1952).

A variety of materials have been shown to be substrates for endogenous respiration of micro-organisms. Only in a few instances have there been definite demonstrations that the consumption of oxygen and the liberation of carbon dioxide, which are the yardsticks of endogenous respiration, were the direct result of the catabolism of a specific compound. Most of the earlier reports of quantitative variation of endogenous reserve materials in micro-organisms have involved the use of cytochemical techniques. Changes in the quantity of polysaccharide in a cell were noted by observing the intensity of the periodate-Schiff or iodine staining reactions. Variation in the lipid content was assessed from the size and intensity of the lipid granules after the micro-organism had been stained with fat-soluble dyes such as Sudan black. Volutin granules, which were generally believed to consist of polyphosphates, were detected by means of their intense basophilic metachromatic staining. Generally these methods were semi-quantitative and rather non-specific; indeed the association of polyposphates with volutin granules was recently queried (Martinez 1963).

Frequently the nature of an endogenous reserve material was inferred from the value of the respiratory quotient (R.Q.) which was determined manometrically. However as unsupported evidence little reliance can be placed in such inferences. Where the R.Q. was determined by the Warburg direct method, often it was not established whether the respiratory rate was altered in the absence of carbon dioxide. R.Q. values may give a false indication of the nature of the reserve material if there are other terminal products of metabolism in addition to carbon dioxide. There have been instances where the gas exchange was the result of processes other than endogenous respiration e.g. in order to explain R.Q. values which varied between 1.9 and 2.5, Chester (1959a) suggested that both respiration and fermentation of yeast endogenous reserves took place at the same time.

An early observation of the utilization of endogenous reserves by micro-organisms was reported by Meissner (1900). He studied the appearance and disappearance of glycogen from yeast by staining the cells with iodine. Cells filled with glycogen produced more than the theoretical amount of carbon dioxide during the fermentation of a sugar substrate. Meissner called the glycogen a temporary reserve material and pointed out the survival value of such material to the cell. Harden and Young (1902) showed by chemical analysis

that glycogen was the principal reserve carbohydrate in yeast. It was generally held, but never actually demonstrated, that this glycogen was the ultimate substrate of endogenous respiration in yeast, e.g. Harden (1932). Stier and Stannard (1936) produced the first tangible evidence that glycogen might be the endogenous substrate for respiration. They reported that during endogenous respiration of yeast the R.Q. was 1.0 and using an iodine staining technique they observed a progressive decrease in the quantity of a red-brown staining material during prolonged endogenous respiration. Endogenous fermentation rates of yeast have been related to the content of an alkali-insoluble carbohydrate (Fales 1951) and to a decrease in cellular carbohydrate content (Chester 1959b). However it was not until 1960 that Eaton (1960) definitely demonstrated by chemical analysis that only glycogen was utilized by yeast during endogenous respiration. The quantity of glycogen which disappeared from the yeast cells was shown to be equivalent to the amount of carbon dioxide evolved. Eaton (1961) went on further to demonstrate that there were two distinct glycogen components that acted as endogenous substrates for aerobic respiration by yeast. Although it was found that there were two substrates for endogenous fermentation only one of these was a substrate for aerobic respiration. The other fermentation substrate was shown to be trehalose.

Palmstierna (1956) reported that an alkali-stable polyglucose accumulated intracellularly in Escherichia coli.

Although they did not show that this glycogen-like polysaccharide was the substrate of endogenous respiration in the absence of exogenous substrates, Holme and Palmstierna (1956a) demonstrated that the polysaccharide carbon was utilized by E. coli for the synthesis of protein in a carbon-deficient, nitrogen-containing medium. From chemical analyses of the carbohydrate and glycogen content of E. coli during endogenous respiration, Dawes and Ribbons (1962a) concluded that glycogen was the primary endogenous substrate in this organism.

During a study of the survival of Aerobacter aerogenes, Strange, Dark and Ness (1961) showed that a glycogen-type polysaccharide was degraded and oxidized during endogenous metabolism of cells which had been grown in a medium which allowed the accumulation of this polysaccharide. A glycogen-like polysaccharide has also been isolated from avian Mycobacterium tuberculosis although there was no evidence that the polysaccharide was an endogenous substrate for the organism (Chargaff and Moore 1944). It had been shown that Rhodospirillum rubrum incorporated carbon from succinate into a polysaccharide, but the role that this polysaccharide might have played in the endogenous metabolism of the organism was not

investigated (Stanier, Doudoroff, Kunisawa and Contopolou 1959).

A number of polysaccharide reserve materials have been reported to occur in protozoa and algae. Some of these polysaccharides have been isolated and extensively characterized. Clarke and Stone (1960) isolated and characterized a beta-1,3- linked glucoside from Euglena gracilis. This polysaccharide was identified with the paramylon granules which could be observed in the organism microscopically. These granules have been observed to decrease in size and number during starvation of Euglena (Padilla and Buetow 1959). Danforth and Wilson (1961) found that Euglena respiring endogenously had a R.Q. of 1.0 and they suggested the endogenous reserves might be carbohydrate in nature.

As mentioned earlier, a glycogen-type polysaccharide was found to accumulate in Tetrahymena pyriformis (Ryley 1951). Paramylon granules in Peranema trichophorum were shown to contain a polysaccharide with beta-1,3-linked glucose residues (Cunningham, Manners and Ryley 1962). Archibald, Manners and Ryley (1958) isolated and characterized a water soluble beta-1,3-glucoside from Ochromonas malhamensis. Chilomonas paramecium was shown to form a polymer which was similar to beta-amylose (Hutchens 1948). Earlier, Mast and Pace (1934) had observed that the quantity of carbohydrate reserve

material in Chilomonas decreased under conditions of starvation. Although it was probable that in the majority of these instances the protozoal and algal polysaccharides would probably serve as the substrates for endogenous respiration of the organisms, in no case was this definitely demonstrated. As was found in the case of Tetrahymena pyriformis (Ryley 1952), the assumption of such a role for these polysaccharide materials may be incorrect.

Stephenson and Whetham (1922) probably made the first observation that lipid material could be an endogenous reserve for micro-organisms. They recorded that the non-phosphatide lipid content of Mycobacterium phlei began to decrease rapidly after the organism had passed into the stationary phase. In the absence of exogenous nutrient, the R.Q. of suspensions of Mycobacterium tuberculosis were observed to decrease from 0.85 to 0.72 (Andrejew 1948).

The situation concerning lipid reserves in yeast is not clear. Stier and Stannard (1936) and Spiegelman and Nozawa (1945) reported that several species of yeast had R.Q. values of 1.0 during endogenous respiration. Eaton (1960) definitely demonstrated that glycogen was a substrate of endogenous respiration in yeast. However, Meyerhof (1925), who was the first to determine the R.Q.

of yeast respiring in the absence of added substrate, found R.Q. was 0.85. Stickland (1956) also found that the R.Q. of yeast in the absence of exogenous substrates was 0.85. Furthermore, Stickland found that the polysaccharide content of the yeast cells, which was as much as 30 per cent of the dry weight, did not decrease during endogenous respiration. The nature of the non-carbohydrate substrate of endogenous respiration was not investigated, but Lindegren (1945) suggested that this reserve material was lipid and Reiner, Gest and Kamen (1949) reported that Saccharomyces cerevisiae assimilated radioactivity into the lipid fraction of the cells by fermentation of ($^{14}C_6$) glucose.

Bagley and Johnson (1953) reported that lipid accumulated in Escherichia coli when the organism was grown in a medium where acetate was the prime source of carbon. It was not shown whether this lipid might serve as a reserve material, but this report will be discussed later in relation to the effect of environment on the nature of the reserve materials formed.

The ciliate Tetrahymena pyriformis was found to contain 15 to 20 per cent dry weight of lipid (McKee, Dutcher, Groupe and Moore 1947). Ryley (1952) showed that this organism did not utilize an intracellular store of polysaccharide during endogenous respiration and it was established that the R.Q. of endogenous respiration was 0.85.

It was suggested that the organism was oxidizing fat or protein rather than carbohydrate. However Pace and Lyman (1947) were unable to observe microscopically any utilization of fat during respiration of T. pyriformis. The quantity of reserve lipid in Paramecium caudatum was reported to undergo a rapid decrease when the organism was deprived of external substrates (Grobicka and Wasilewska 1925).

From the foregoing it was apparent that the status of lipid reserves as substrates for endogenous respiration was not definitely established. However a specific lipid-type compound definitely has been shown to be a substrate of endogenous respiration in micro-organisms. A polymer of beta-hydroxy butyric acid was shown by Lemoigne (1927) to be a major constituent of the cell material of Bacillus megaterium. Recently this polymer was shown to occur quite widely among bacteria (Forsyth, Hayward and Roberts 1958). Under certain conditions this polymer accounted for 45 per cent of the dry weight of the cell material. Both Bacillus cereus and B. megaterium utilized stored poly-beta-hydroxybutyrate when washed suspensions of the cells were shaken in the presence of oxygen (Macrae and Wilkinson 1958a). Rhodospirillum rubrum photo-assimilated acetate, butyrate and beta-hydroxybutyrate into poly-beta-hydroxybutyrate (Doudoroff and Stanier 1959). Using (^{14}C) acetate R. rubrum incorporated radioactivity into the

polymer with little dilution of the carbon. When the washed labelled cells were illuminated in the presence of ammonium chloride 90 percent of the radioactive polymer disappeared and much of the (^{14}C) became distributed among the other cell constituents. The fate of the radioactive polymer when the cells were allowed to respire in the absence of any substrate was not investigated. Doudoroff and Stanier (1959) also established that poly-beta-hydroxybutyrate acted as the substrate for the endogenous respiration of Pseudomonas saccharophila. Three species of Hydrogenomonas were found to be rich in poly-beta-hydroxybutyrate (Schlegel, Gottschalk and Von Bartha 1961). Although the amount of polymer in the cells was found to decrease during endogenous respiration, the decrease was not correlated with the amount of oxygen taken up and it was suggested that there was another endogenous substrate in addition to the polymer.

Several observations have been made implicating protein or free amino acid pools as substrates for endogenous respiration of micro-organisms. When washed cell suspensions of Sarcina lutea, which had been grown on a peptone medium, were aerated ammonia was released and the concentration of the free amino acid pool was reduced while the lipid and carbohydrate content of the cells remained constant (Dawes and Holms 1958). If the organism was grown on a glucose-peptone medium there was a reduction in the carbohydrate

content of the cells during aeration of a washed suspension. These glucose-peptone grown cells still utilized the amino acid pool and released ammonia at a rate which was less than that of the peptone grown cells. Thus the carbohydrate was not the sole substrate of endogenous respiration in glucose-peptone grown cells.

The role of intracellular glycogen as a substrate for the endogenous respiration of Escherichia coli has already been mentioned. Dawes and Ribbons (1926a) found that when the glycogen reserves of this organism were exhausted by endogenous respiration the cells began to release ammonia. It was not definitely established whether protein or the amino acid pool was the source of this ammonia, although the concentration of the amino acid pool was found to remain constant during the period of ammonia release. Since the release of ammonia was subsequent to and not concomitant with the depletion of the polysaccharide reserves of the organism, it was not clear whether the material releasing nitrogen was a true intracellular reserve. The release of ammonia in this instance may have been a reflection of a situation where the cell had exhausted all its reserve material and was forced to oxidize some of its normal cellular constituents. A reserve material should be present in excess of the quantity necessary for the survival of the cell. If the cells remained viable during the period when ammonia was released, then it might be

inferred that the source of ammonia was a reserve material. It would appear that a compound such as glycogen could act only as a reserve material. However Wilkinson (1959) has indicated that some substances may be produced as an attempt to detoxicate toxic end-products of metabolism. Protein is an essential constituent of all cells so that the distinction between essential protein and reserve protein may not be easily determined.

When washed suspensions of Aerobacter aerogenes were aerated the quantity of protein in the cells was steadily reduced (Strange, Dark and Ness 1961). This loss of protein was accompanied by an evolution of ammonia. The utilization of protein was concomitant with the utilization of the carbohydrate reserves of this organism. As much as 25 per cent of the cell protein was lost without affecting the viability of the cells and thus it appeared that the protein was acting in the capacity of a reserve material.

Pseudomonas aeruginosa was another organism that liberated ammonia in the course of endogenous respiration (Warren, Ells and Campbell 1960). During endogenous respiration there was a reduction in the size of the amino-acid pool, but this reduction was accounted for by the quantity of amino acids released from the cells into the medium. In the same report it was stated that no changes were observed in any other cell constituents. However, it was subsequently reported (Campbell, Gronlund

and Duncan 1963) that Pseudomonas aeruginosa liberated material, which had an absorption maximum at 260 millimicrons, into the medium during endogenous respiration. It was found that this process was accompanied by a loss of ribonucleic acid and an increase of inorganic phosphate in the cells. The production of ammonia and the consumption of oxygen were shown to be primarily associated with the ribosomal fraction of the cell. It was concluded that the data implicated ribonucleic acid as the major endogenous substrate in Pseudomonas aeruginosa. Presumably the ribonucleic acid was degraded as a source of readily oxidized pentose residues and the bases were excreted into the medium. Since the release of material absorbing at 260 millimicrons has been observed during the starvation of a number of micro-organisms (Holden 1958, Higuchi and Uemura 1959, Stephenson and Moyle 1949, Strange 1961), it might transpire that ribonucleic acid is a general reserve material in micro-organisms.

From the foregoing discussion of the different types of reserve materials it was apparent that some micro-organisms may possess more than one endogenous source of carbon and energy e.g. Sarcina lutea and Aerobacter aerogenes. It was also evident that the composition of the growth medium could influence the nature and quantity of the reserve material synthesised by the cell. The relation between

bacterial composition and environment has been the subject of recent reviews (Wilkinson and Duguid 1960, Duguid and Wilkinson 1961, Herbert 1961). There was sufficient evidence to support the general statement that the high lipid and high polysaccharide content of micro-organisms was usually associated with growth in media where nitrogen was the factor limiting growth (Herbert 1961). This relationship has been mentioned before in the case of Sarcina lutea (Dawes and Holms 1958). When E. coli was grown in a medium where glucose was limiting, little glycogen was formed; when ammonia was limiting the glycogen content of the cells was high in the final stages of growth (Holme and Palmstierna 1956b). Analogous observations were reported by Ribbons and Dawes (1963). When E. coli was grown in glucose-ammonium or glucose-tryptone media, the organism utilized carbohydrate reserves before the release of ammonia was observed during endogenous respiration. If the organism was grown in a tryptone medium the carbohydrate content of the cells was low and a release of ammonia was detected at the earliest times after the washed cells were transferred to a phosphate-buffer medium. Similar results have been reported for Aerobacter aerogenes (Strange, Dark and Ness 1961) and Torula utilis (Herbert and Tempest 1961).

The same relationship was evident in micro-organisms

which formed poly-beta-hydroxybutyrate as a reserve material. When Bacillus megaterium was grown in a medium where carbon was limiting, little polymer was formed; when nitrogen was limiting growth high levels of the polymer were synthesised (Macrae and Wilkinson 1958b).

There may be another type of relationship between the degree of oxidation-reduction of a carbon substrate and the formation of lipid or polysaccharide reserve materials. When an acetate medium was supplemented with graded amounts of glucose Dagley and Johnson (1953) found that the amount of polysaccharide synthesised by E. coli increased with the increasing concentration of glucose in the medium. The increase in polysaccharide content was accompanied by a decrease in the content of lipid. Macrae and Wilkinson (1958b) reported that the quantity of poly-beta-hydroxybutyrate synthesised by Bacillus megaterium depended greatly on the presence of acetate in the medium.

It is apparent from the previous reports that careful attention must be given to the constitution of the growth medium, the conditions of growth and the composition of the environment in which the cells respire endogenously, before making comparisons of reserve materials among micro-organisms. By itself, the knowledge of the chemical constitution of a micro-organism has little significance unless it can be related to the factors mentioned.

Aaronson and Baker (1961) reported that lipid accounted

for approximately 33 per cent by weight of the cell material of dark-grown Ochromonas malhamensis. Archibald, Manners and Ryley (1958) isolated from Ochromonas a water soluble polysaccharide which they presumed was the material that constituted the leucosin deposits observed by Pringsheim (1952). This polysaccharide was characterized and found to be primarily a linear beta-1,3- linked glucan containing a small percentage of mannose. Ford and Goulden (1959) estimated the total carbohydrate in dark-grown Ochromonas to be approximately 50 per cent of the dry weight of cell material; the organism was grown in a glucose-citrate-casein hydrolysate medium. Thus there were at least two materials, lipid and carbohydrate, that were potential substrates of endogenous respiration in Ochromonas.

Reazin (1954) found that Ochromonas, which had been grown on a glucose-citrate-amino acid medium, respired endogenously at a constant rate for 20 hours. After 20 hours of endogenous respiration the respiratory rate began to decline. From a kinetic analysis of this decline Reazin concluded that only one endogenous substrate became rate limiting. Through all stages of endogenous respiration the R.Q. remained constant at a value of 0.8 and it was suggested, without other experimental evidence, that some compound other than carbohydrate acted as the endogenous substrate. The first part of this thesis was concerned with ascertaining whether one or both of the two reserve materials

previously detected or some other material formed by dark-grown Ochromonas was the substrate for endogenous respiration of this organism.

Within more recent times, most of the attention given to the problems of endogenous metabolism has been directed toward assessing the magnitude of endogenous respiration during respiration of exogenous substrates. Some micro-organisms have a relatively high rate of endogenous respiration compared with their rate of respiration in the presence of added substrates e.g. Penicillium chrysogenum (Blumenthal, Koffler and Heath 1957) and Sarcina lutea (Dawes and Holms 1958). In order to obtain a quantitative measure of the respiration of the external substrates it was necessary to make some correction for the endogenous respiration.

Barker (1936) introduced a manometric method to correct the total respiration rate for the endogenous respiration. However the validity of this technique was often questionable since the carbon of some substrates was oxidatively assimilated and it was not always possible to test the extent of this assimilation. Also it was found in some instances that the oxidation of the external substrate altered the rate of endogenous respiration (Blumenthal et al. 1957).

An isotopic technique for assessing endogenous respiration was suggested by Burris (1949). In this

technique the organism was grown in a medium so that it became uniformly labelled with carbon-14. Then determination of the radioactivity of the respired carbon dioxide would give a measure of the endogenous respiration rate during the oxidation of an unlabelled substrate. Blumenthal et al. (1957) and Blumenthal (1963) have reviewed the literature concerned with the use of both the manometric and isotopic techniques and they have criticised some of the methods employed.

As a logical extension of the technique where labelled cells were used to assess the rate of endogenous respiration, it would have been possible at the same time to ascertain the nature of the reserve material by following the disappearance of radioactivity from the components of the cell material. Blumenthal, Koffler and Goldschmidt (1952) have pointed out that the release of (^{14}C) carbon dioxide from cells which were not uniformly-labelled would not be representative of the total endogenous turnover. Therefore the cells used to assess endogenous respiration by this isotopic technique should be uniformly labelled. As a corollary to this proposition it follows that the disappearance of radioactivity from the constituents of non-uniformly-labelled cells may not represent the disappearance of carbon from an endogenous substrate of respiration. Thus it might be difficult to assess the results obtained from any but uniformly labelled cells.

The methods described in this thesis, which were employed to detect the substrate of endogenous respiration in Ochromonas, were derived from the foregoing considerations.

Another aspect of the problem of endogenous respiration, hitherto largely ignored, but recently raised by Dawes and Ribbons (1962b), was the role played by the inorganic components of the milieu such as water, oxygen, carbon dioxide, nitrogen and phosphate. These factors may influence the pattern of endogenous respiration. A significant observation in the present context was made by Palmstierna(1956) who found that E. coli could synthesise carbohydrate material from carbon dioxide in the absence of other exogenous carbon and nitrogen sources. The second part of this thesis was concerned with the participation of carbon dioxide in the endogenous metabolism of Ochromonas. As an extension of the findings made with carbon dioxide, carboxylation reactions in Ochromonas were briefly investigated.

For many years it was thought that the ability to utilize atmospheric carbon dioxide was confined to the higher plants, algae, photosynthetic bacteria and chemosynthetic bacteria. Then Wood and Werkman (1935) presented the first experimental evidence that heterotrophic non-photosynthetic bacteria assimilated carbon dioxide. It was ten years after this observation before the first primary carboxylation reaction was described by Ochoa (1945).

Including this discovery, to date a total of twelve primary carbon dioxide fixation reactions have been described.

Ochoa (1945) was the first to show that the reductive carboxylation of alpha-oxoglutarate to form isocitrate was catalysed by an enzyme obtained from pig heart:

alpha-oxoglutarate + CO_2 + $NADPH_2$ $\rightleftharpoons$ isocitrate + NADP.

Isocitrate dehydrogenase (L-isocitrate:NADP oxidoreductase decarboxylating E.C. 1.1.1.42) from pig heart was NADP specific. Both a NADP- and a NAD-specific enzyme have been demonstrated in yeast (Kornberg and Pricer 1951). It was originally thought that the NADP- specific enzyme was located in the cytoplasm and the NAD-specific enzyme was in the mitochondria, but some doubt has been expressed concerning the validity of this (Ziegler and Lindane 1958). There have been no reports of carbon dioxide fixation via this reaction in bacteria.

The next discovery of a primary carbon dioxide fixation reaction also occurred in Ochoa's laboratory (Ochoa, Mehler and Kornberg 1947). An enzyme obtained from a liver extract catalysed the reversible reductive synthesis of malate from pyruvate and carbon dioxide in the presence of $NADPH_2$:

pyruvate + CO_2 + $NADPH_2$ $\rightleftharpoons$ malate + NADP.

This enzyme, which was found to be widely distributed in

animal tissues, bacteria and plants (Ochoa 1951a), was NADP-specific (Ochoa 1955). "Malic" enzyme (L-malate:NADP oxidoreductase decarboxylating E.C.1.1.1.40) was found to have a low affinity for carbon dioxide (Utter 1959). A "malic" enzyme found in Lactobacillus arabinosus appeared to be different to those found in animal and plant tissues since it had only been shown to catalyse the decarboxylation of malate and the product of this decarboxylation was lactate rather than pyruvate (Korkes, del Campillo and Ochoa 1950). Furthermore, decarboxylation catalysed by this enzyme was NAD-dependent. It was not possible to demonstrate carbon dioxide fixation with the enzyme from L. arabinosus.

Warburg and Christian (1936) found that yeast extracts could oxidize 6 phospho-gluconate in the presence of NADP. Horecker and Smyrniotis (1952) were able to demonstrate the reversibility of this reaction and thus established another primary carboxylation reaction :

$$\text{ribulose-5-phosphate} + CO_2 + NADPH_2 \rightleftharpoons 6\ \text{phospho-gluconate} + NADP$$

The reaction appeared to be analogous to those already mentioned in that reduction was linked to NADP and a keto-acid was involved. The enzyme catalysing this reaction (6 phospho-D-gluconate:NADP oxidoreductase decarboxylating E.C.1.1.1.44) was found to be widespread among plants,

animals and micro-organisms.

A different type of carboxylation reaction, requiring no external energy source, is catalysed by the enzyme phosphopyruvate carboxylase (orthophosphate:oxaloacetate carboxy-lyase phosphorylating E.C. 4.1.1.31) (Bandurski and Greiner 1953) :

phosphopyruvate + CO_2 $\rightleftharpoons$ oxaloacetate + orthophosphate

The reaction is accompanied by a large decrease in free energy and is generally regarded as irreversible. The enzyme has a high affinity for carbon dioxide and phosphopyruvate (Walker and Brown 1957, Walker 1957) and its presence has been demonstrated only in plants and autotrophic bacteria.

An enzyme (ITP: oxaloacetate carboxy-lyase transphosphorylating E.C. 4.1.1.32) purified from chicken liver was shown to catalyse the reversible reaction -

phosphopyruvate + CO_2 + IDP $\rightleftharpoons$ ITP + oxaloacetate

(Utter and Kurahashi 1954). This enzyme was also active with GDP but not with ADP (Kurahashi, Pennington and Utter 1957). The specifity of the avian enzyme for GDP and IDP did not extend to an enzyme isolated from plants. The plant enzyme required ADP and was inactive with either IDP or GDP (Mazelis and Vennesland 1956). The affinity of the avian enzyme for

carbon dioxide was found to be slightly greater than that of "malic" enzyme (Stickland 1959).

In order to explain the labelling pattern observed in phosphoglycerate, which was the first stable radioactive product detected after exposure of Chlorella to (^{14}C) carbon dioxide, it was suggested that ribulose diphosphate might be the primary acceptor of carbon dioxide (Bassham, Benson, Kay, Harris, Wilson and Calvin 1954). This prediction was confirmed in the reports of Quayle, Fuller, Benson and Calvin (1954) and Weissbach, Smyrniotis and Horecker (1954) who demonstrated that the enzyme ribulose diphosphate carboxylase catalysed a reaction which was believed to proceed as follows :

ribulose-1,5-diphosphate + CO_2 ⟶ 3-phospho glycerate.

Attempts to reverse this reaction were not successful; the enzyme had a very low affinity for carbon dioxide (Weissbach, Horecker and Hurwitz 1956). Kinetic studies of the incorporation of (^{14}C) carbon dioxide by the photosynthetic bacterium Rhodopseudomonas spheriodes indicated that the ribulose diphosphate carboxylase catalysed reaction was operative in this organism (Stoppani, Fuller and Calvin 1955). This enzyme has been detected in nearly all the chemosynthetic bacteria investigated (Vishniac, Horecker and Ochoa 1957). There have been only two reports of the

enzyme in heterotrophic bacteria (Fuller 1956, Quayle and Keech 1959).

The condensation of carbon dioxide with propionyl CoA, according to the reaction -

$$\text{Propionyl CoA} + \text{ATP} + CO_2 \rightleftharpoons \text{methylmalonyl CoA} + \text{ADP} + \text{iP.,}$$

was demonstrated with an extract of pig heart by Flavin, Ortiz and Ochoa (1955). There has been one report of this reaction in a micro-organism (Gibson and Knight 1961). The activity of theenzyme (propionyl CoA:CO_2 ligase, ADP E.C.6.4.1.3.) catalysing this reaction was found to be inhibited by avidin and the purified enzyme was shown to contain biotin (Kaziro, Ochoa, Warner and Chen 1961).

Bachhawat, Robinson and Coon (1956) established that carbon dioxide was fixed by rat liver slices during the breakdown of leucine to acetoacetate. Subsequently Knappe and Lynen (1958) found that the reaction responsible for the uptake of carbon dioxide could be formulated as follows :

$$\text{beta-methyl crotonyl CoA} + \text{ATP} + CO_2 \rightleftharpoons \text{beta-methyl glutaconyl CoA} + \text{ADP} + \text{iP.}$$

The activity of the enzyme (beta-methyl crotonoyl CoA: CO_2 ligase, ADP E.C.6.4.1.4.) catalysing this reaction was also inhibited by avidin (Lynen, Knappe, Lorch, Jutting and Ringelman 1959).

Following the demonstration of a requirement for carbon dioxide during fatty acid synthesis, Wakil (1958) purified an enzyme (acetyl CoA: CO_2 ligase, ADP E.C. 6.4.1.2.) which catalysed the reversible carboxylation of acetyl CoA-

acetyl CoA + ATP + CO_2 $\rightleftharpoons$ malonyl CoA + ADP + iP.

This enzyme also apparently required biotin for activity since it was inhibited by avidin (Wakil, Titchener and Gibson 1958).

An enzyme, amino imidazole ribonucleotide carboxylase, obtained from chicken liver was found to catalyse the incorporation of carbon dioxide into the 6-position of the purine ring during the biosynthesis of purine nucleotides (Lukens and Buchanan 1959). The reaction has been demonstrated to proceed according to the equation -

5-amino-imidazole ribonucleotide + CO_2 $\longrightarrow$ 5-amino-4-imidazole carboxylic acid ribonucleotide.

There have been no reports of this enzyme in plants or micro-organisms.

An enzyme that catalyses the formation of oxaloacetate by direct condensation of pyruvate and carbon dioxide was recently discovered in pigeon liver mitochondria (Utter and

Keech 1960). This enzyme (pyruvate:CO_2 ligase, ADP E.C. 6.4.1.1.) required catalytic amounts of acetyl CoA for activity and the reaction was shown to proceed as follows -

$$\text{pyruvate} + \text{ATP} + CO_2 \xrightleftharpoons{\text{acetyl CoA}} \text{oxaloacetate} + \text{ADP} + \text{iP}.$$

The enzyme was apparently biotin-dependent since the reaction was inhibited by avidin. The enzyme was shown to be specific for ATP and had a high affinity for carbon dioxide (Utter 1961).

The most recently discovered carboxylation reaction was another where phosphopyruvate was converted to oxaloacetate (Siu, Wood and Stjernholm 1961). The reversible reaction was shown to proceed according to the equation -

$$\text{Phosphopyruvate} + CO_2 + \text{iP} \rightleftharpoons \text{oxaloacetate} + \text{pyrophosphate}.$$

This reaction was notable in that it appeared to be the first example of pyrophosphate serving as a source of high energy phosphate. The enzyme (phosphopyruvate carboxytransphosphorylase) catalysing this reaction was shown to have a high affinity for carbon dioxide (Siu and Wood 1962). The enzyme was not biotin-dependent since its activity was not inhibited by avidin.

The twelve foregoing reactions have been discussed extensively in a number of reviews (Ochoa 1951b, Utter

1959, Utter 1961, Walker 1962, Wood and Stjernholm 1962).

There appears to be only a single report of carbon dioxide fixation by a heterotrophic organism in the absence of exogenous carbon sources (Holme and Palmstierna 1956b). There was no evidence that this fixation was peculiar to the endogenous metabolism of E. coli or if the fixation was a function necessary for respiration by the cell, be this with endogenous or exogenous substrates. Outwardly this carbon dioxide fixation would appear to be autotrophic. However, the endogenous metabolism of an organism such as E. coli would be expected to proceed by the usual heterotrophic pathways and an autotrophic mechanism of fixation would probably be unlikely under these conditions.

The ability to incorporate carbon dioxide during endogenous respiration would spare the carbon reserves of the organism, but in a heterotrophic organism there would be no net gain in energy from this process. It would appear more likely that the function of carbon dioxide fixation was to replenish dicarboxylic acid intermediates which had been drained from the tricarboxylic acid cycle for the syntheais of amino acids and other essential materials. A special case may obtain where an organism forms purely energy reserve materials such as polymetaphosphate. Carbon dioxide fixation then may be a means of obtaining carbon compounds, during endogenous respiration for syntheses essential to the survival of the cell, these syntheses being

possible because of the reserve of energy.

The work reported in this thesis indicated that Ochromonas malhamensis incorporated carbon dioxide primarily into amino acids and intermediates of the tricarboxylic acid cycle during endogenous respiration.

Table 1

The materials listed across were dissolved in de-ionised water and the pH of the solution was adjusted to 5.5. The volume of the solution was adjusted to 1 litre and the resultant solution was five times the concentration required for growth. This solution was stored in bulk at -10°. The growth medium was prepared from this solution by diluting it one in five with deionised water and adding vitamin B_{12} so that the final concentration was 0.4 millimicrogram per ml.

Table 1

Growth medium for *Ochrobactrum melitensis*.

Casein Hydrolysate	25g.
Glucose	50g.
Ammonium citrate	4g.
KH_2PO_4	1g.
$CaCl_2$	750mg.
$Na_2MoO_4 . 2H_2O$	250mg.
Metals Solution*	100ml.
DL-Tryptophan	500mg.
DL-Methionine	1g.
L-Cystine	500mg.
Choline chloride	10mg.
Inositol	50mg.
p-Amino-benzoic acid	5mg.
Thiamine hydrochloride	10mg.
Biotin	50micrograms
Tween 80	5ml.

*The metals Solution contained the following amounts of material in one litre.

$MnSO_4 . H_2O$	6.15g.
$ZnSO_4 . 7H_2O$	11.0g.
$FeSO_4 . 7H_2O$	1.0g.
$CoSO_4 . 7H_2O$	300.0mg.
$CuSO_4 . 5H_2O$	40.0mg.
H_3BO_3	60.0mg.
KI	1.0mg.
Ethylene diamine tetraacetic acid	5.0g.

Chapter 2

GENERAL METHODS

Maintenance and growth of the organism.

The original culture of Ochromonas malhamensis was obtained from the culture collection of algae and protozoa at the Botany School, Cambridge, England. The organism was maintained in axenic culture by weekly transfer in a medium described in Table 1. This medium was essentially the same as the devised by Ford (1953). The cultures were grown at room temperature in daylight.

Experimental cultures were obtained by subculturing 5 ml. of a week old maintenance culture into 300 ml. amounts of medium in 2 litre conical flasks. These flasks were agitated on a reciprocating shaker which made 95 shakes with a 2.5 inch stroke every minute. In order to obtain dark grown cells the shaker was situated inside a light proof incubator maintained at 28°. These cultures were usually grown for 3 days.

Larger cultures up to 8 litres volume were grown in 10 litre glass carboys at 28° in a light-proof incubator. These cultures were agitated by a stream of sterile air which was blown into the medium at the bottom of the carboy through a looped, perforated, polyethylene tube. The air

was sterilised by pumping it through a large cotton-packed filter and then into a 65 cm. stainless steel tube packed with 120 mesh carborundum and maintained at 600° in an electric furnace. A long sterile rubber tube connected the sterile air supply to the carboy inside the incubator. After passing through this long tube the sterile air had cooled sufficiently so that it did not raise the temperature of the medium higher than 28°. A thin layer of sterile, 20 centistoke silicone fluid (Dow Corning Corporation, Midland, Mich., U.S.A.) was spread on the surface of the medium to prevent frothing. These large cultures were also grown for 3 days.

All cells were harvested at 4° by centrifuging at 900g. for 10 minutes. Usually these cells were washed twice, by resuspending and centrifuging them in water, before they were finally suspended in the medium chosen for the particular experiment.

Dry weight determinations.

At the outset a curve relating the dry weight of cell material per ml. of cell suspension and the extinction of the cell suspension at 580 millimicrons was determined by experiment. The values for this curve were obtained by making a series of dilutions in water, of an Ochromonas suspension and then measuring the extinction of these dilutions at 580 millimicrons. Subsequently aliquots of

the series of dilutions were delivered on to tared aluminium planchets. The planchets were dried initially at 90° and then left at 100° for 15 hours. Then the planchets were reweighed and the dry weight was determined by difference. Subsequently it was only necessary to measure the extinction of the cell suspension and the corresponding dry weight was read from the curve.

Assay of radioactivity.

Chemicals isotopically labelled with carbon-14 were purchased from The Radiochemical Centre, Amersham, England. Whenever possible radioactivity of materials was assayed by liquid scintillation techniques. The installation for the assay of radioactivity consisted of a type N664 A scintillation detector and a type N530 F scaler (Ekco Electronics Ltd.) coupled with a type N102 pulse analyser (Dynatron Radio Ltd., Maidenhead, Berks.). The photomultiplier was operated at 1100 volts and the pulse analyser was set at a threshold of 10 volts with a channel width of 30 volts. The scintillation detector was maintained at 2° to reduce electronic "noise".

To estimate radioactivity liquid samples up to a maximum volume of 1 ml. were added to 5 ml. of a water-tolerant liquid scintillation system devised by Bray (1960). The measurements were corrected for quenching due to the sample by using an internal standard. This standard was a solution

of (4-^{14}C) cholesterol in toluene. Quantities of the standard solution, known to give approximately ten times the counting rate of the sample, were added to the source container after the radioactivity of the sample had been assayed. Then the counting rate of the mixture was determined. The sample counting rate was multiplied by the factor

$$\frac{\text{counting rate of standard}}{(\text{counting rate of sample + standard}) - (\text{counting rate of sample})}$$

to obtain a value corrected for quenching. Then the quench-corrected value was corrected for background radiation. The background counting rate was determined prior to the addition of the sample, when only the liquid scintillator was in the source container. Under the conditions described this system assayed the standard with an efficiency of 59 per cent.

Occasionally radioactive samples were prepared for assay in the form of barium (^{14}C) carbonate. Whenever there was sufficient material the barium carbonate was assayed as an infinitely thick layer; in excess of 30 mg. of barium carbonate was compressed into a 1 cm^2 planchet. The radioactivity of the barium carbonate on the planchet was determined using a type N620 shielded end-window Geiger-Muller tube (Ekco Electronics Ltd.). Counting rates so

determined were corrected for detector resolving time and then for background radiation. The corrected counting rates were converted to absolute radioactivities using a factor determined empirically with barium carbonate of known specific radioactivity. A saturation curve was plotted from values determined with the same barium carbonate. The counting rate of barium carbonate samples which were less than infinitely thick were converted to absolute radioactivities using the saturation curve.

Paper Chromatography.

Chromatography was performed in all-glass tanks that were lined with sheets of Whatman 3MM paper. The lower edge of the paper linings was immersed in the equilibration solution at the bottom of the tank. The chromotography papers were equilibrated with the atmosphere inside the tank for at least 8 hours and wherever possible overnight (approximately 15 hours). The effective length of paper for a one-dimensional analysis was 17 inches. If a radioautograph was to be made from a chromatogram which had been developed in two dimensions, then the development area of the chromatogram was limited to 12x15 inches. If development required the solvent to run off the end of the paper, the lower edge of the paper was serrated with pinking shears to facilitate drop formation. When the development was complete the papers were suspended in a fume hood and

allowed to dry in an air draught at room temperature.

Paper electrophoresis.

Paper electrophoresis was performed in an enclosed apparatus which was constructed from perspex sheet. Whatman 3MM papers were used exclusively. The length of paper between the anode and cathode was 46 c.m. The paper was supported by a web of nylon thread arranged so that the midpoint of the paper was 5 cm. higher than the ends. Each end of the paper dipped into a trough of electrolyte which was connected to a second trough of the same electrolyte by a bridge of Whatman 3MM paper. A potential difference was applied through platinum wire electrodes which traversed the length of the second trough. Using a small brush, the paper was moistened with electrolyte to a point 2 cm. each side of the origin line, which was midway between the anode and cathode and at the apex of the paper. The electrolyte fronts were allowed to meet at the origin line by capillary action. Thirty minutes were allowed for the electrolyte to become uniformly distributed through the paper before the voltage was applied.

Detection of radioactivity on chromatogram and electrophoresis papers.

One-dimensional chromatograms and electrophoresis papers were cut down the line of development into strips 1.5 inches

wide. Radioactive areas were detected on these strips by passing them through an Actigraph 2 radioactive paper-strip scanner mounted with a type C47 windowless gas flow detector (Nuclear Chicago Corporation). A reference mark was made on the paper with a drop of a solution containing a non-volatile (^{14}C) compound.

Radioactive areas were detected on two-dimensional chromatograms by radioautography. Reference marks were made in three corners of the chromatogram with radioactive ink. Kodirex no-screen X-ray film (Kodak (Australasia) Pty. Ltd., Melbourne) was exposed to the chromatogram in lead-lined exposure holders. Exposure varied from two to four weeks. Exposed films were developed with Kodak D19 developer. After superimposing the developed film on the chromatogram so that the reference marks matched, the radioactive areas were outlined on the chromatograms with a pencil.

Chapter 3

ASSIMILATION OF ($^{14}C_6$) GLUCOSE BY OCHROMONAS GROWN IN THE DARK.

Cytochemical methods have been commonly employed to detect the loss of endogenous reserve materials from micro-organisms. These techniques are being supplanted by more specific chemical analyses which have been developed in recent times. Thus it is possible to assay various cell constituents and detect any variation in the quantity of these constituents during endogenous respiration of an organism. Because of the diversity of materials which have been found to act as reserve materials in micro-organisms, it is not always possible to assay the cell material for all the possible reserve substances at the one time. This may be a disadvantage since the interpretation of the results could rest on the demonstration of a relationship between the various cell constituents.

Another approach stems from a technique which has been used extensively to assay endogenous respiration in the presence of exogenous substrates (reviewed by Dawes and Ribbons 1962 b). When this technique is employed the micro-organism is made radioactive by growth on a uniformly-labelled (^{14}C) carbon source. Then the rate at which these cells liberate (^{14}C) carbon dioxide is a measure of

the endogenous respiration. It should be possible to detect the endogenous reserve material in these radioactive cells by correlating the evolution of (^{14}C) carbon dioxide with the disappearance of radioactivity from any of the cell constituents.

Methods based on this idea have been used to detect the endogenous reserve materials in Ochromonas. In this chapter two methods of obtaining radioactive cells are described since there was some doubt whether cells, which had been grown by the first method, would give valid results. However, it has been found that the stringent criteria, which have to be met when preparing radioactive cells for assaying endogenous respiration during the respiration of exogenous substrates, need not be strictly adhered to for the present purpose, providing all variables are measured.

In addition a procedure for the separation of the cell material of Ochromonas into the broad categories of soluble intermediates, lipid, polysaccharide and protein is described in this chapter. This procedure evolved from the following considerations. Ethanol and diethyl ether extraction would be expected to separate lipid and soluble intermediates from the rest of the cell material. It was known that lipid could be prepared free of non-lipid materials by partition between the phases of a chloroform, methanol and water mixture. (Folch, Lees and Sloane-Stanley

1957). Therefore the soluble intermediates in the ethanol-ether extracts could be separated from the lipid. Archibald et al. (1958) had found that a polysaccharide could be extracted from Ochromonas with hot water. If this hot water extraction was performed after the ethanol and ether extractions then the polysaccharide would be relatively free of soluble intermediates. The material remaining after these extractions would contain any other water-insoluble polysaccharide, nucleic acid and protien.

Materials and Methods.

Ochromonas was labelled with carbon from ($^{14}C_6$) glucose by two methods. The procedure for Method 1 was as follows. A 200 ml. culture of Ochromonas was grown in a light-proof shaker incubator for 48 hours, using a modified form of the medium described in Table 1. The medium was modified by replacing one per cent of glucose with one per cent of sucrose. When the 48 hours had elapsed, 10 microcuries and 2 micromoles of ($^{14}C_6$) glucose were added to the culture which was shaken in the incubator a further 3 hours.

In Method 2 a 300 ml. culture of Ochromonas was grown in the dark in the shaker incubator for 3 days using the medium described in Table 1. This medium contained 30 microcuries of ($^{14}C_6$) glucose and the specific radioactivity of this glucose was initially 10 microcuries per gm.

Cells grown by either method were harvested and washed twice by centrifuging with cold water. The cell paste was freeze-dried and the dry material was weighed.

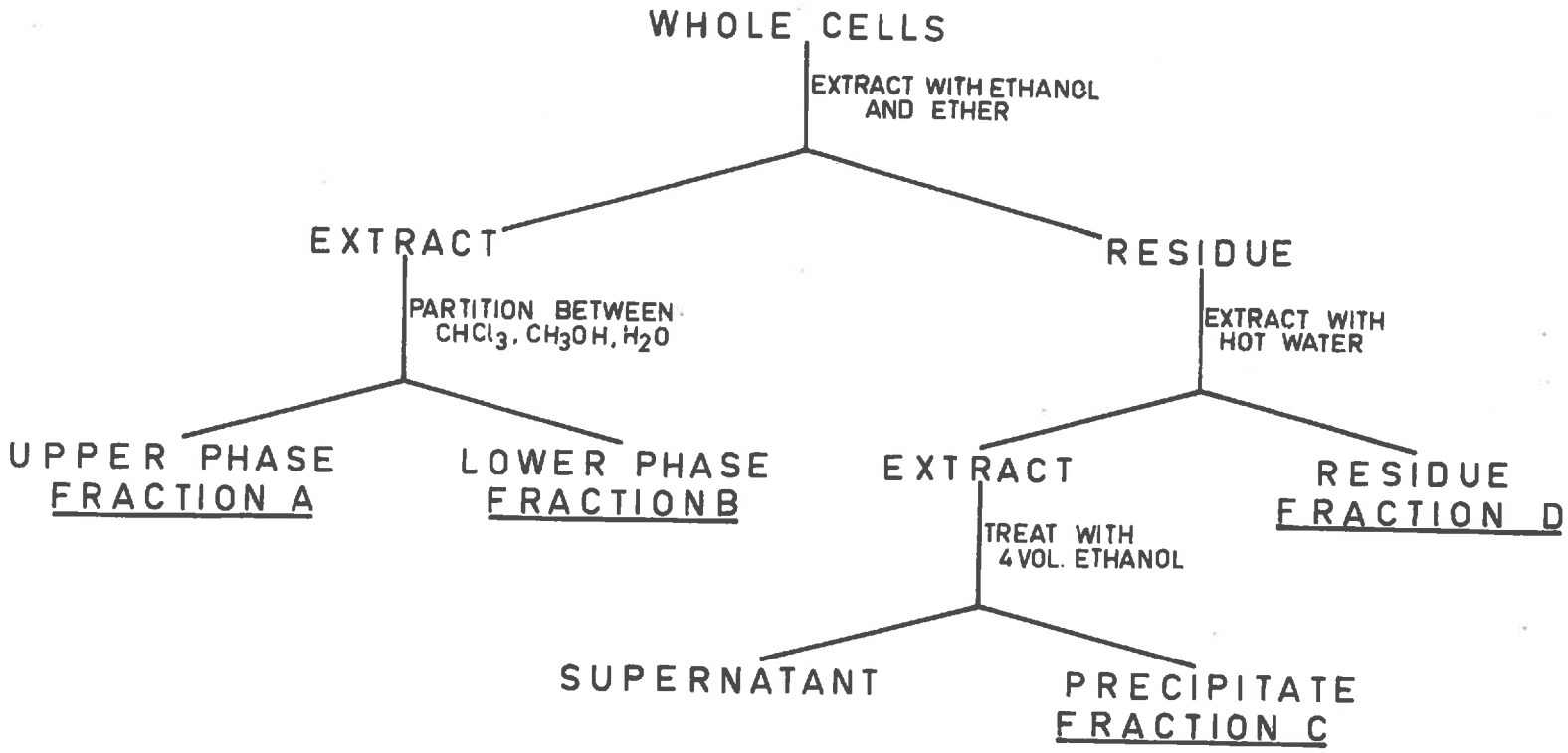
The dry material was separated into fractions by the following methods. The solid material was suspended in 95 per cent ethanol in the ratio of 100 mg. of solid per 10 ml. of solvent. The solid was extracted with the solvent for 10 minutes at 70° in a water bath. Undissolved solid was recovered from the cooled ethanolic extract by centrifuging. The solid was re-extracted with ethanol-diethyl ether (3:1 by volume) for 15 minutes at 30° using the same ratio of solvent to solid. Finally the solid was extracted twice more with diethyl ether at room temperature (12-26°). The combined ethanol-ether extracts were evaporated to dryness at less than 30° in a rotary evaporator. The brown semi-solid residue was emulsified with 3.0 ml. of water and the emulsion was shaken thoroughly with 15.0 ml. of a mixture of chloroform and methanol (2:1 by volume) in a stoppered centrifuge tube. The two phases of the biphasic mixture were separated by centrifuging. The upper phase was removed with a teat pipette and the interface was washed carefully with 7.0 ml. of a mixture with the same solvent composition as the original upper phase (Folch et al. 1957). The upper phase and washings were combined and both this and the lower phase were evaporated to dryness under a stream of nitrogen. These upper and lower phases are hereafter referred to as

fractions A and B respectively. Fraction A was dissolved in water and fraction B was dissolved in a mixture of toluene and methanol (1:1 by volume). Samples of both solutions were assayed for radioactivity by the liquid scintillation technique and additional samples were dried on tared aluminium planchets which were weighed.

The dry solid remaining after the ethanol-ether extractions was extracted twice for 15 minutes at 80° with 10 ml. of water and then extracted once with water at room temperature. Undissolved material was recovered by centrifuging. The combined aqueous extracts were treated with 4 volumes of 95 per cent ethanol and the mixture was allowed to stand for 15 hours at 4°. A white precipitate was recovered from the mixture by centrifuging. This precipitate was washed twice with ethanol, twice with diethyl ether and allowed to air dry. The mother liquors and washings were combined and concentrated to approximately 5 ml. in a rotary evaporator. The concentrated solution was treated with 4 volumes of ethanol at 4°. A small amount of precipitate was washed and dried as described before and added to the original precipitate. The white solid was called fraction C. The mother liquors and washings from the second precipitation were combined. Fraction C was dissolved in water and samples of the solution were taken for dry weight and radioactivity estimations.

The residue left after the water extractions was washed

FIGURE 1.


WHOLE CELLS
EXTRACT WITH ETHANOL AND ETHER
EXTRACT
RESIDUE
PARTITION BETWEEN $CHCl_3$, CH_3OH, H_2O
EXTRACT WITH HOT WATER
UPPER PHASE FRACTION A
LOWER PHASE FRACTION B
EXTRACT
RESIDUE FRACTION D
TREAT WITH 4 VOL. ETHANOL
SUPERNATANT
PRECIPITATE FRACTION C

twice with ethanol, twice with diethyl ether and dried. The dry material called fraction D was weighed. A weighed sample of this material was moistened with a drop of ethanol to reduce interfacial tension and then dissolved in formamide (1.0 ml. per 2.5 mg. of solid) by heating the suspension at 140° in an oven for 3 hours. The clear straw-coloured solution obtained after this treatment was allowed to cool to room temperature before being made up to a known volume with formamide. A sample of this solution was assayed for radioactivity by the liquid scintillation technique.

The washings of fraction D were combined with the mother liquors and washings of fraction C. This solution was assayed for radioactivity by the liquid scintillation technique and a sample was taken for an estimation of the dry weight.

A flow-sheet of the preceding separation is depicted in Figure 1.

Results and Discussion.

Initially radioactive Ochromonas cells were obtained by adding ($^{14}C_6$) glucose to a culture in the late-logarithmic phase of growth and the cells were harvested after a short period of incubation with the radioactive glucose (Method 1). The culture was grown in a medium with sucrose replacing glucose as the major source of carbon to prevent dilution

Table 2

Distribution of radioactivity among fractions prepared from Ochromonas cells grown by Method 1.

Ochromonas was cultured in 200 ml. of medium in the dark for 48 hours. Then 10 microcuries of ($^{14}C_6$) glucose with a specific radioactivity of 5 microcuries per micromole were added to the culture. Growth was allowed to proceed a further 2 hours in the dark before half of the culture was removed. The cells from this half of the culture were harvested and washed with water by centrifuging and frozen immediately in a solid carbon dioxide-ethanol freezing mixture. Three hours after the addition of the radioactive glucose the cells from the other half of the culture were harvested washed and frozen. Both samples of frozen cells were freeze dried and the cell material was separated into fractions according to the procedure outlined in Figure 1. The radioactivity in the fractions was assayed by the liquid scintillation technique. Column A shows the per cent of the total radioactivity in each fraction. Column B shows the radioactivity (in microcuries) of each fraction.

Table 2

Time of incorporation of ($^{14}C_6$) glucose in hours	2		3		
Total radioactivity in the cell material in microcuries	0.90		1.17		
	A	B	A	B	Increment (microcuries) 2-3 hours
Fraction A	18.8	0.169	15.9	0.186	+ 0.017
Fraction B	24.4	0.220	17.6	0.206	- 0.014
Fraction C	31.8	0.286	38.2	0.447	+ 0.161
Fraction D	15.9	0.143	15.9	0.186	+ 0.043
Supernatant + washings	9.2	0.083	12.5	0.146	+ 0.063

of the high specific radioactivity of the added glucose. This also ensured a rapid uptake of radioactivity.

The distribution of radioactivity among the fractions prepared from the radioactive cells is shown in Table 2. Fraction C had acquired the largest proportion of the radioactivity assimilated by the cells. The remainder of the radioactivity incorporated by the cells was distributed evenly among the other fractions. The amount of radioactivity recovered in the washings and mother liquors from fraction C and D was found to be variable. It was apparent that fraction C was incorporating radioactivity at a greater rate than any other fraction; the increase of radioactivity in fraction C accounted for 60 per cent of the increment in total radioactivity of the cells between 2 and 3 hours of incubation.

Methods, similar to Method 1, have been used by various investigators to obtain radioactive cells for the assessment of endogenous respiration during the respiration of exogenous substrates. These methods have been criticised (Blumenthal et. al. 1957) on the grounds that some reserve materials might not become radioactive or might become only slightly radioactive when these methods were employed. This criticism appeared to be equally applicable to the problem of determining the nature of the endogenous reserve materials using radioactive cells. Consequently radioactive Ochromonas cells were obtained by an alternate method where

Table 3

Distribution of radioactivity among fractions prepared from Ochromonas cells grown by method 2.

Ochromonas was cultured in the dark for 72 hours in 300 ml. of medium containing 30 microcuries of ($^{14}C_6$) glucose with a specific radioactivity of 10 millimicrocuries per mg. The cells were harvested from the culture and washed with water by centrifuging. The cell material was separated into fractions according to the procedure outlined in Figure 1. The radioactivity in the fractions was assayed by the liquid scintillation technique. Specific radioactivities are expressed as millimicrocuries per mg. of material. Experiments 1 and 2 are duplicates.

Table 3

Experiment		1			2
Total radioactivity recovered in the cell material (microcuries)		2.78			3.46
	weight of material in fractions (mg.)	% of total radioactivity incorporated	specific radioactivity	% of total radioactivity incorporated	specific radio-activity
Fraction A	47.3	10.3	6.1	11.1	5.9
Fraction B	58.7	23.2	11.0	24.2	11.3
Fraction C	113.8	32.2	7.9	33.1	7.8
Fraction D	93.8	27.2	8.1	28.4	8.3
Supernatant + washings	22.3	6.9	8.1	3.3	-
Weight recovered	335.9				
Initial weight	341.6				
Recovery (%)	98				

the culture was grown from a small inoculum in a medium containing ($^{14}C_6$) glucose (Method 2). Table 3 shows the specific radioactivities of the fractions and the distribution of radioactivity among the fractions prepared from cells grown by Method 2.

Again the largest proportion of the total radioactivity assimilated by the cell was present in fraction C. From a comparison of the distribution of radioactivity among the constituents of the cells produced by both methods, it was apparent that fractions B and D had acquired proportionately more of the total radioactivity assimilated by the cells labelled by Method 2 than the corresponding fractions obtained from cells grown by Method 1. In the experiment with cells grown by Method 1 the proportion of the total radioactivity in fractions B and D was decreasing with time of incubation. It seemed that the material in fraction C of cells produced by Method 1 was still being actively synthesised, while the synthesis of the substances in the other fractions was either near completion or very much slower than synthesis of the material in fraction C. Therefore it seemed that Method 1 could produce cells in which the reserve materials would not have the same specific radioactivity.

In the experiment with cells grown by Method 2, approximately 10 per cent of the total radioactivity originally added to the medium was recovered in the cell material. A

further 53 per cent remained in the medium after the cells were harvested and it was presumed that the missing 37 per cent had been metabolised to volatile products.

An examination of the specific radioactivities (units of radioactivity per unit weight) of the fractions prepared from cells grown by Method 2, showed that there was little difference between the fractions, with the exception of fraction B. Fraction B had a higher specific radioactivity than the other fractions and the glucose originally in the growth medium. This situation could occur only if the material in fraction B contained a higher proportion of carbon per unit weight than glucose, e.g. if fraction B consisted of lipid. The small differences in specific radioactivity that were observed between the materials in the other fractions also could reflect differences in the proportion of carbon per unit weight. Although these fractions had approximately the same specific radioactivities the glucose carbon assimilated by the cells must have been diluted by carbon incorporated from other non-radioactive substrates in the medium, since the specific radioactivities were less than that of the glucose in the medium at the start of growth.

The weight of materials recovered in the separated fractions accounted for more than 98 per cent of the weight of the starting material (Table 3).

It was not possible to grow Ochromonas in a medium containing a single uniformly-radioactive carbon source, but the cells were grown in a medium where the major carbon source was uniformly-labelled glucose. Therefore it was necessary to determine the specific radioactivities of the constituents of cells grown under the latter conditions in order to establish whether these cells were uniformly radioactive or sufficiently near to this condition so that they might be used to detect the endogenous reserve materials. Specific radioactivities of the separated fractions expressed in terms of radioactivity per unit weight were adequate for the purpose of observing the utilisation of radioactive materials in the fractions. However the materials in these fractions were likely to have different proportions of carbon per unit weight. In order to compare these fractions it was necessary to determine the specific radioactivity per atom of carbon and this required some knowledge of the nature of the materials in the fractions.

Chapter 4

ANALYSIS OF RADIOACTIVE MATERIALS IN FRACTIONS PREPARED FROM OCHROMONAS GROWN WITH ($^{14}C_6$) GLUCOSE.

Chapter 3 described how cells, which had been labelled by oxidative assimilation of ($^{14}C_6$) glucose and alternatively by growth on ($^{14}C_6$) glucose, were separated chemically into four fractions. This chapter is concerned with an examination of the nature of the radioactive materials in the four fractions.

Materials and Methods.

In order to obtain larger amounts of cell material for analysis, a 2.5 litre culture of Ochromonas was "labelled" by growing it in a medium containing 50 microcuries of ($^{14}C_6$) glucose (Method 2). The cell material was separated into fractions exactly as described in the previous chapter and these fractions were examined as follows.

Fraction A. This fraction contained ethanol-soluble, non-lipid cell constituents. The material in this fraction was separated into three sub-fractions by treatment with ion-exchange resins.

Fraction A was dissolved in 1.0 ml. of water and the

solution was added to a column (50 x 5 mm.) of the strong cation-exchange resin Dowex 50 in the acid form (8 per cent cross-linked, 100-200 mesh). The solution was allowed to run in level with the top of the resin and then the column was washed with 12 ml. of water to remove compounds that were not exchanged on to the resin. The water eluate was added to another 50 x 5 mm. column containing the anion-exchange resin Dowex 1 in the acetate form (4 per cent cross-linked 50-100 mesh). Compounds that were not exchanged by this resin were washed from the column with 15 ml. of water. This water eluate, hereafter called the neutral fraction, was distilled to dryness in a rotary evaporator at less than 35°.

The Dowex 50 column was eluted with 20 ml. of N-ammonium hydroxide solution to displace the components of fraction A which had been exchanged by the resin. The ammonium hydroxide eluate was concentrated to dryness in a rotary evaporator. The dry residue was called the basic fraction. The Dowex 1 column was eluted with 20 ml. of 6N-formic acid. The formic acid eluate was concentrated to dryness and henceforth the dry material was called the acidic fraction.

The three sub-fractions were dissolved and made up to a known volume with water. Samples of these solutions were assayed for radioactivity by the liquid scintillation technique.

Samples of the neutral fraction were analysed by chromatography on Whatman number 1 paper with the solvent ethyl acetate-pyridine-water (12:5:4 by volume) (Smith 1960). Reducing sugars were detected on the dry chromatograms by spraying them with a freshly prepared 0.1M solution of oxanilic acid in water and then heating the papers at 110° for 10 minutes. The sprayed chomatograms were examined under an ultraviolet lamp giving light essentially at 253 millimicrons (Horrocks and Manning 1949, Charalampous and Mueller 1953). Non-reducing carbohydrates were detected by dipping the chromatogram in a solution of 0.3 per cent (w/v) lead tetraacetate in chloroform and allowing it to air dry before dipping in a solution of 0.03 per cent (w/v) rosaniline base in 10 per cent (v/v) acetic acid in acetone (Sampson, Schild and Wicker 1961). Using the Actigraph 2 radioactive paper-strip scanner, radioactive areas were located on an unsprayed strip taken from the same chromatogram. The radioactive areas were eluted from the chromatogram using the techniques described in Chapter 8.

A radioactive compound with an Rg of 0.73 (Rg is the ratio of the distance the compound has moved to the distance glucose has moved), which was detected on the chromatogram of the neutral fraction, was hydrolysed with 2N-sulphuric acid by heating the solution in a sealed tube at 100° for 8 hours. The cooled hydrolysate was neutralised with barium carbonate. The precipitate of barium sulphate

and excess barium carbonate was removed by centrifuging. A sample of the supernatant solution was analysed by electrophoresis on Whatman 3MM paper with an electrolyte consisting of 0.2M sodium arsenite, pH 9.6 (Frahn and Mills 1956). A potential difference of 400 volts was applied for 16 hours. Reducing sugars were detected by drying the paper at 60°; a coloured product was formed as the result of a reaction between arsenite and the reducing sugars. Other carbohydrates were detected by using the lead tetraacetate-rosaniline reagent (Sampson et al. 1961). Caffeine was used as a non-migrating marker during electrophoresis with arsenite so that the distances migrated by the sugars could be corrected for electro-osmosis. Radioactive components were detected on unheated strips of the electrophoresis paper using the Actigraph 2 radioactive paper-strip scanner. The radioactive sugar-arsenite complexes were split and eluted from the paper with 0.015N-hydrochloric acid (Thorn and Busch 1960). Ions were removed from the eluate by allowing it to pass through a column of the anion-exchange resin Amberlite CG400 in the formate form (100-200 mesh) and a column of the cation-exchange resin Dowex 50 in the acid form, respectively. The effluent from the columns was concentrated to a small volume in a rotary evaporator and analysed by chromatography on paper with the solvent ethyl acetate-pyridine-water (12:5:4 by volume).

The basic and acidic fractions were analysed by two-dimensional chromatography with the solvent systems phenol-water and butan-1-ol-acetic acid-water. Radioautographs were made from three chromatograms of each fraction. Then a separate chromatogram of each fraction was sprayed with either ninhydrin or bromcresol green or acid molybdate (see Chapter 8 for the details of these procedures).

Fraction B. This fraction contained the cell lipids virtually free of other cell constituents (Folch et al. 1957). The material left after the removal of the chloroform-methanol solvent was contaminated with a small quantity of water. This water was removed by dissolving the solid in a mixture of 99.5 per cent ethanol and anhydrous ether (1:1 by volume). The ether and water-ethanol azeotropic mixture were distilled off at room temperature under reduced pressure.

This lipid material was separated into 8 classes of lipid by column chromatography on silicic acid using the techniques devised by Hirsch and Ahrens (1958). A 300 mg. sample of the anhydrous lipid was extracted several times with petroleum ether (boiling range 60-80°). The extract was concentrated to approximately 15 ml. in a rotary evaporator. The petroleum ether-insoluble residue was dissolved in the smallest possible volume of methanol. The methanolic solution was transferred in drops to three Whatman 3MM filter

paper discs which were cut so that their diameter was the same as the internal diameter of the silicic acid column. Each drop of solution was allowed to dry on the paper before the addition of the next drop and in this manner all of the methanol solution was transferred to the discs.

The silicic acid column was essentially the same as that described by Hirsch and Ahrens (1958). The silicic acid (325 mesh) was a commercial product specially prepared for lipid chromatography (Bio-Rad Laboratories, Richmond, Calif., U.S.A.) and it was used without further treatment. The silicic acid column was given a series of dehydrating washes as described by Hirsch and Ahrens and then the paper discs impregnated with the petroleum ether-insoluble lipid were placed on top of the silicic acid. Next, the 15 ml. of petroleum ether-soluble lipid was added to the column and the column was eluted with the solvents petroleum ether (60-80^{0} boiling range), diethyl ether and methanol in a stepped gradient as described by Hirsch and Ahrens (1958).

The separated fractions were concentrated to dryness in a rotary evaporator. The dry fractions were dissolved in toluene, with the exception of fraction 8 which was dissolved in methanol, and samples of these solutions were used to determine the radioactivity by the liquid scintillation technique and the dry weight of the fractions.

The lipid fractions were analysed by ascending chromatography on silicic acid impregnated papers using the techniques described by Marinetti (1962) and the solvents n-heptane-2,6 dimethyl-4-heptanone (96:6 by volume) and 2,6 dimethyl-4-heptanone-acetic acid-water (40:20:3 by volume) (Marinetti, Erbland and Kochen 1957). Rhodamine 6G was used as a general lipid stain, amino-phosphatides were detected with ninhydrin and plasmalogens were detected with 2,4 dinitro phenyl-hydrazine (Marinetti 1962). The chromatograms were treated with phosphomolybdic acid and stannous chloride to detect choline (Levine and Chargaff 1951) and phosphate was detected by the technique of Bandurski and Axelrod (1951).

Fraction C. This fraction was known to contain a water-soluble polysaccharide (Archibald et al. 1958). Since this polysaccharide was obtained by water extraction of the cell material it was likely to be accompanied by other water-soluble polysaccharides or intermediates which were not soluble in ethanol and ether. The polysaccharide was separated from most of these contaminants by selective precipitation of the polysaccharide with a long-chain paraffin quaternary nitrogen compound in the presence of borate (Barker, Stacey and Zweifel 1957).

A series of buffers were made to cover the range pH8.0 to pH 11.0 by adjusting one volume of 0.05 M sodium tetra-borate solution to the desired pH value, either with 0.4 N

sodium hydroxide solution or with 0.4N hydrochloric acid. Then the solutions were mixed with 0.1 ml. of a 1 per cent (w/v) solution of fraction C in water. The mixture was equilibrated at 30° in a water bath before it was treated with 0.1 ml. of a 0.15 M solution of hexadecyl trimethylammonium bromide which had been preheated to 30° (since the critical solution temperature of the latter in water was 26° - Adam and Pankhurst 1946). After 15 minutes the solution was cooled to below 26° then briefly centrifuged to pack the precipitate of complex and excess hexadecyl trimethylammonium bromide. Aliquots of the supernatant liquid were assayed for radioactivity by the liquid scintillation technique.

Using the optimum conditions for the precipitation of the radioactive polysaccharide determined from the previous experiment, 50 mg. of fraction C was selectively precipitated between pH values of 8.5 and 9.8 by the same technique in order to obtain sufficient material for further analysis. The precipitated complex was washed with water by centrifuging before it was dissolved in a small volume of 1N-acetic acid. Cations were removed from the acetic acid solution by allowing it to flow slowly through a 1.5 x 20 cm. column of the cation-exchange resin Zeokarb 225 in the acid form (50 mesh). The column effluent was distilled to dryness under reduced pressure at less than 40°. Boric acid was removed from the dry residue by dissolving the residue in

150 ml. of methanol and then distilling off the methanol and volatile methyl borate under reduced pressure (Hill, Khym and Cheniae 1953). The methanol distillation was repeated three more times. The residue was dissolved in a little water and then treated with four volumes of ethanol at 4° for 15 hours. The white precipitate was collected and washed by centrifuging once with ethanol, twice with diethyl ether and air dried. When the ether had evaporated the solid was dried in a vacuum desiccator over anhydrous calcium sulphate.

The dry material, as a Nujol mull, was examined with a Grubb-Parsons type S4 infrared spectrophotometer over the region 10.5 to 14 microns.

The dry polysaccharide was dissolved in 2N-sulphuric acid in the proportions of 1 mg. per ml. and the polysaccharide was hydrolysed by heating this solution in a sealed tube at 100° for 4 hours. The hydrolysate was neutralised with barium carbonate and the barium sulphate and excess barium carbonate were removed by centrifuging. The supernatant liquid was analysed by chromatography on Whatman number 1 paper using the solvent ethyl acetate-pyridine-water (12:5:4 by volume) (Smith 1960).

A partial hydrolysate of the polysaccharide was obtained by heating 10 mg. with 1 ml. of 0.5 N hydrochloric acid in a sealed tube at 100° for 20 minutes. The hydrolysate was dried in a vacuum desiccator over sodium hydroxide.

The dry residue was dissolved in water and analysed by chromatography on Whatman number 1 paper with the solvent mixtures propan-1-ol-water-ethyl acetate (7:2:1 by volume) (Albon and Gross 1952) and ethyl acetate-pyridine-water which was described previously.

Fraction D. This fraction contained the cell protein, nucleic acids and water insoluble polysaccharides which would not have been extracted by the ethanol-ether and water treatments. The amount of protein and the proportion of the total radioactivity in the protein were assayed so that any radioactivity in other materials could be obtained by difference.

The dry material was ground to a fine powder with a pestle and mortar. A sample of the powder was dissolved by allowing it to stand 10 hours in a solution of 0.1 per cent (v/v) thioglycolic acid in 2N-sodium hydroxide. The protein was precipitated from this solution by acidifying it with 10 per cent (w/v) trichloracetic acid solution. The precipitate was extracted with hot trichloracetic acid and washed with acetone, ethanol and diethyl ether essentially as described by Simkin and Work (1957). The protein content of the dry material obtained by this procedure was estimated by a modification of the method of Westley and Lambeth (1960).

Table 4

Distribution of radioactivity among the components of Fraction A separated by ion exchange.

Ochromonas was cultured in 2.5 litres of medium for 72 hours in the dark. The medium contained 50 microcuries of ($^{14}C_6$) glucose with a specific radioactivity of 2 millimicrocuries per mg. The cells were harvested from the culture and washed with water by centrifuging. The cell material was separated into fractions according to the procedure outlined in Figure 1. Fraction A was separated into 3 subfractions by passing it through a column of a cation exchange resin and then through a column of an anion exchange resin. The solution passing through both columns was called the neutral fraction. The cation exchange resin was eluted with ammonia and the eluate was called the basic fraction. The anion exchange resin was eluted with formic acid and the eluate was called the acidic fraction. The radioactivity of the fractions was assayed by the liquid scintillation technique.

	Radioactivity (millimicrocuries)
Basic fraction	101
Acidic fraction	53
Neutral fraction	343
Total in fractions	497
Total in starting material	509
Recovery (%)	97.5

Between 0.1 and 2.0 mg. of the dry protein was dissolved by shaking it mechanically with 2.0 ml. of the alkaline copper tartrate reagent. A 1.0 ml. aliquot of this solution was diluted with 1.0 ml. of water, then the solution was shaken with 200 mg. of the dry Dowex 1 resin for 5 minutes. The mixture was centrifuged briefly and the supernatant liquid was removed from the resin with a teat pipette. A 1.0 ml. aliquot of this supernatant liquid was treated with 3.0 ml. of the colour reagent and the extinction of the solution was determined at a wavelength of 446 millimicrons. Three times crystallised bovine serum albumin (Sigma Chemical Co., St. Louis, Misso., U.S.A.) was used as a protein standard in this assay. Samples of the treated material were dissolved in formamide (see Chapter 3, Methods) and assayed for radioactivity by the liquid scintillation technique.

Similar determinations of radioactivity and protein content were made with the untreated fraction D material.

Results and Discussion.

Fraction A. The distribution of radioactivity among the subfractions obtained by an ion-exchange separation of the components of Fraction A is shown in Table 4. The basic and acidic fractions were further analysed by two-dimensional chromatography and radioautography. It was apparent that

all the radioactive components of the basic fraction were either amino acids or peptides since they all produced the violet colour which was characteristic for alpha-amino acids when they were treated with ninhydrin. The radioactive components of the acid fraction failed to give the reaction with ninhydrin which was characteristic of amino acids; three components contained phosphate and most of the other radioactive components stained acid to the indicator.

Of the total radioactivity in Fraction A, 69 per cent was not exchanged by either a cation- or an anion-exchange resin. This neutral fraction was analysed by paper chromatography and seven components were revealed when the chromatograms were treated with reagents to detect reducing sugars and non-reducing carbohydrates. Of these seven components only two were radioactive and these had Rg values of 1.00 and 0.73 with the ethyl acetate-pyridine-water solvent. From a measurement of the areas under the curves traced by the Actigraph 2 radioactive paper-strip scanner, it was estimated that these two radioactive components had total radioactivities in the ratio of 1:49 respectively. The faster, less radioactive of the two components was probably glucose since it gave the characteristic yellow fluorescence of a reducing hexose when the paper was treated with the oxanilic acid reagent and viewed under ultraviolet light.

The slower (Rg 0.73) more radioactive component failed to give a positive test for a reducing hexose or a reducing pentose with the oxanilic acid reagent. However it was detected by the less sensitive lead tetraacetate-rosaniline reagent. Only one radioactive component could be detected when a neutralised acid hydrolysate of the Rg 0.73 compound was examined by paper electrophoresis with an arsenite electrolyte. This component had an Mr value (rate of migration relative to that of ribose) of 0.31. A number of authentic sugars migrated in a band with an Mr of 0.31 using an arsenite electrolyte. These were galactose, mannose, rhamnose, arabinose and glycerol. The arsenite complex of the radioactive component was split and eluted from the electrophoresis paper. The eluted material was analysed by paper chromatography with the solvent ethyl acetate-pyridine-water. Two separate radioactive components with Rg values of 0.86 and 1.79 were detected on the chromatogram. The Rg values of authentic galactose, glycerol and rhamnose were found to be 0.87, 1.79 and 1.80 respectively.

Since the radioactive Rg 1.79 component did not produce the fluorescence characteristic of reducing sugars when the chromatogram was treated with the oxanilic acid reagent and yet it produced a prominent violet colour with the less-sensitive lead tetraacetate-rosaniline reagent, it was concluded that this component was glycerol and not rhamnose.

From measurements of the areas under the curves traced by the Actigraph 2 radioactive paper-strip scanner, it was estimated that the galactose and glycerol moieties possessed total amounts of radioactivity in the ratio of 2:1.

As the materials in the fractions, prepared from cells which had been grown by Method 2, had similar specific radioactivities (see Table 3), then it might be assumed that the galactose and glycerol portions of the Rg 0.73 component also would have similar specific radioactivities. Therefore it would follow that the molar ratio of galactose to glycerol would be approximately 1:1. Thus 98 per cent of the radioactivity in the neutral fraction was present in a compound tentatively shown to consist of galactose and glycerol.

Colin and Guegen (1930) first noted the existence of a glycerol galactoside in Rhodymenia palmata. The marine red alga Irideae laminaroides was found to contain 1 to 4 per cent by weight of an alcohol-soluble glycerol galactoside (Benn, Putman, Trucco and Hassid 1953). This compound was an early radioactive product of photosynthesis from (^{14}C) carbon dioxide by the red alga. Putman and Hassid (1954) subsequently identified the radioactive compound as alpha-D-galactopyranosyl-2-glycerol. Acylated derivatives of beta-D-galactopyranosyl-1-glycerol have been found in

Table 5

Distribution of radioactivity among the components of Fraction B separated by chromatography on silicic acid.

Fraction B was prepared from Ochromonas cells which were grown as described in Table 4. Using the techniques described by Hirsch and Ahrens (1958) 300 mg. of Fraction B material (681 millimicrocuries) was analysed by chromatography on a silicic acid column.

Fraction	Composition of eluant	Volume of eluant (ml.)
1.	1% (v/v) diethyl ether in petroleum ether.	50
2.	1% ether in pet. ether.	75
3.	1% ether in pet. ether	225
	then 4% ether in pet. ether.	60
4.	4% ether in pet. ether	240
	then 8% ether in pet. ether.	200
5.	8% ether in pet. ether	450
	then 25% ether in pet. ether.	5
6.	25% ether in pet ether.	200
7.	Ether	300
8.	Methanol	400

Table 5

Fraction	Weight of material in eluate (mg.)	Radioactivity millimicrocuries	Specific radioactivity millimicrocuries / mg.
1.	-	-	-
2.	-	-	-
3.	65.9	151	2.29
4.	47.0	112	2.38
5.	1.1	3	2.73
6.	9.2	24	2.61
7.	75.6	162	2.14
8.	83.5	154	1.85
Total	282.3	606	
Recovery (%)	94	89	

Chlorella (Benson, Wiser, Ferrari and Miller 1958) and in wheat flour (Carter, McCluer and Slifer 1956). No suggestions have been made concerning the biological functions of these compounds.

In conclusion Fraction A appeared to consist mainly of intermediates of metabolism and a compound containing glycerol and galactose.

Fraction B. The lipid mixture extracted from the cell material of Ochromonas which had been grown by Method 2 was separated into 8 classes by column chromatography on silicic acid according to the procedures described by Hirsch and Ahrens (1958). The radioactivity and weight of the materials in these eight classes are shown in Table 5.

Fractions 3,4,7 and 8 contained 96 per cent by weight of the lipid recovered from the column and also accounted for 95 per cent of the total radioactivity recovered. In their procedure for the separation of a lipid mixture into eight major classes, Hirsch and Ahrens (1958) found that fraction 3 contained cholesteryl ester, fraction 4 contained triglyceride and free fatty acids, fraction 7 contained monoglyceride and fraction 8 contained phospholipids.

Fractions 3, 4 and 7 obtained from the Ochromonas lipid were examined by chromatography on silicic acid impregnated paper with the solvent mixture heptane-2,6 dimethyl-4-heptanone. It was evident that some decomposition

of the lipids in these fractions must have occurred if the separation achieved on the silicic acid column here was the same as that obtained by Hirsch and Ahrens (1958). The chromatogram of fraction 3 had one major component with an Rf of 0.81 and two slower components that were stained only weakly by rhodamine 6G. Only the Rf 0.81 component could be detected by radioautography. The paper chromatographic behaviour of this radioactive material was consistent with that of a sterol ester.

Unfortunately the specific radioactivities of all the lipid fractions were low, so that it was difficult to chromatograph sufficient material to produce a satisfactory radioautograph without causing the spots to streak.

In addition to components that had Rf values similar to those of triglyceride and fatty acids, fraction 4 contained material having a Rf value consistent with that of diglyceride material. This diglyceride could have been a product of the deacylation of the triglyceride. The radioautograph made from the chromatogram of fraction 7 showed that there were two radioactive components as well as radioactive monoglyceride. One of these radioactive components possibly was unesterified fatty acid ; the other component had an Rf identical with that of authentic cholesterol. The fatty acids were probably the products of deacylation of the monoglyceride. The presence of sterol

in Fraction 7 was not predicted. However, Ochromonas was known to contain ergosterol and other unidentified sterols (Aaronson and Baker 1961) whose disposition in this elution scheme were unknown.

The radioactive material in Fraction 8 did not move from the origin of a chromatogram which was developed with the solvent heptane-2,6 dimethyl-4-heptanone. This behaviour was consistent with the properties of phospholipids. Seven components were separated from Fraction 8 on silicic acid impregnated paper which had been developed with the solvent 2,6 dimethyl-4-heptanone-acetic acid-water. Only two of these seven components possessed sufficient radioactivity that could be detected on a radioautograph after 4 weeks exposure. These two radioactive components had Rf values of 0.55 and 0.45 and they were the only components to give a positive spot test for phosphate. These two components failed to react with ninhydrin and they produced a non-characteristic green colour when they were tested for choline. Three of the five non-radioactive components gave weak positive tests for amino groups, while a fourth component with a Rf of 0.36 was the only component on the chromatogram to produce the characteristic blue colour when it was tested for choline.

Hack, Yaeger and McCaffery (1962) reported that they were unable to detect phosphate, choline or amino-containing

compounds among Ochromonas "phosphatides". Although it was possible to detect these functions in the present instance, the compounds bearing these functions did not behave like authentic choline-or amino-phosphatides during silicic acid-paper chromatography as described by Marinetti (1962).

Ochromonas lipid appeared to consist of approximately equal amounts of sterol ester, triglyceride, monoglyceride and phospholipid.

Fraction C. It had been found that it was possible to precipitate neutral polysaccharides with a long-chain quaternary nitrogen compound if the polysaccharide was converted into a polyanion by forming a complex with borate (Barker, Stacey and Zweifel 1957). If the pH of the reaction was controlled it was possible in some cases to effect a selective precipitation of a single polysaccharide from a mixture of polysaccharides.

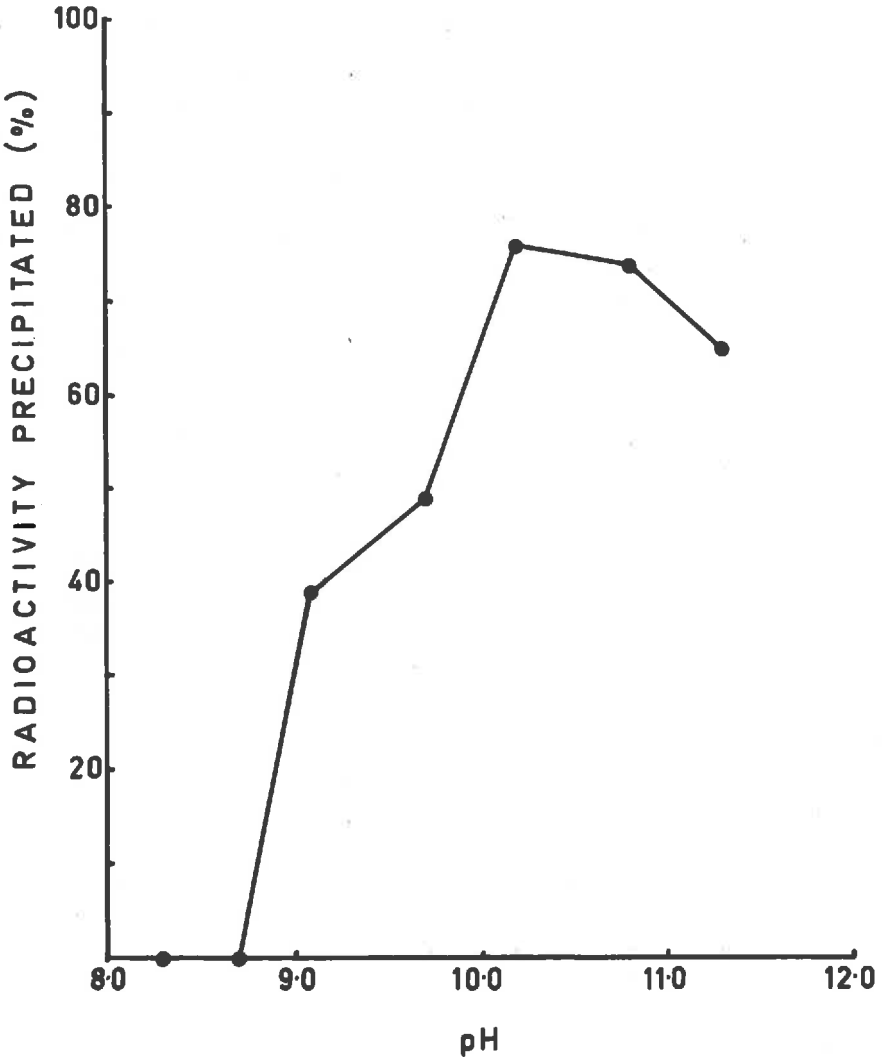
When a solution of Fraction C in borate was treated with hexadecyl trimethylammonium bromide over a range of pH values it was found that a precipitate did not form below pH 8.3. At pH values higher than 8.8 a heavy coacervation formed and at a pH of 10.2 more than 75 per cent of the total radioactivity present in the solution of Fraction C had been brought out of solution in the precipitate. By weight, 68 per cent of the original Fraction C material was recovered

Figure 2

Selective precipitation of polysaccharide in Fraction C as the hexadecyl trimethyl ammonium-borate complex.

One volume of a 1% (w/v) solution of Fraction C was added to one volume of a series of 0.05 M. tetraborate buffers spanning the range pH 8.0 to pH 11.0. The mixtures were maintained at 30°, and were treated with one volume of 0.15 M. hexadecyl trimethylammonium bromide. The mixtures were cooled to 20° and the precipitated complex and hexadecyl trimethylammonium bromide were removed by centrifuging. The radioactivity remaining in the supernatant liquid was assayed by the liquid scintillation technique.

FIGURE 2.


RADIOACTIVITY PRECIPITATED (%)
100
80
60
40
20
8·0
9·0
10·0
11·0
12·0
pH

from the precipitated complex after borate and the long-chain quaternary nitrogen compound had been removed. The nature of the other 12 per cent of material which was not precipitated was not investigated. (Figure 2.)

The material recovered from the precipitated complex was hydrolysed and the hydrolysate was examined by paper chromatography. The chromatogram was sprayed with the oxanilic acid reagent to detect reducing sugars and four components with Rg values of 1.00, 0.50, 0.39 and 0.32 were revealed. When the sprayed chromatogram was examined under an ultraviolet light (essentially 253 millimicrons) the 0.50 component was found to be absorbing radiation in this region, while the other three components all possessed the yellow fluorescence which was characteristic of reducing hexoses. The 0.39 and 0.32 components were only very weakly fluorescent while the Rg 1.00 component was prominent.

The components with Rg values of 1.00 and 0.50 were the only components of the four, previously detected with oxanilic acid, that were detected with the lead tetraacetate-rosaniline reagent. Also, these two components were the only radioactive materials on the chromatogram. It was estimated, from the areas under the curves traced by the Actigraph 2 radioactive paper-strip scanner, that the ratio of radioactivity in the Rg 1.00 and 0.50 components was approximately 34:1. The evidence obtained indicated that the Rg 1.00 material was glucose and it was also apparent

Figure 3

The relation between partition coefficient and the degree of polymerisation of the products of a partial acid hydrolysis of polysaccharide from fraction C.

Polysaccharide from fraction C was partially purified by selective precipitation of the hexadecyl pyridinium-borate complex (between pH 8.5 and 9.8). The partially purified material was hydrolysed with 0.5N-HCl, at 100° for twenty minutes. The hydrolysate was analysed by one dimensional chromatography and the components were detected on the chromatograms with the oxanilic acid and lead tetraacetate-rosaniline reagents.

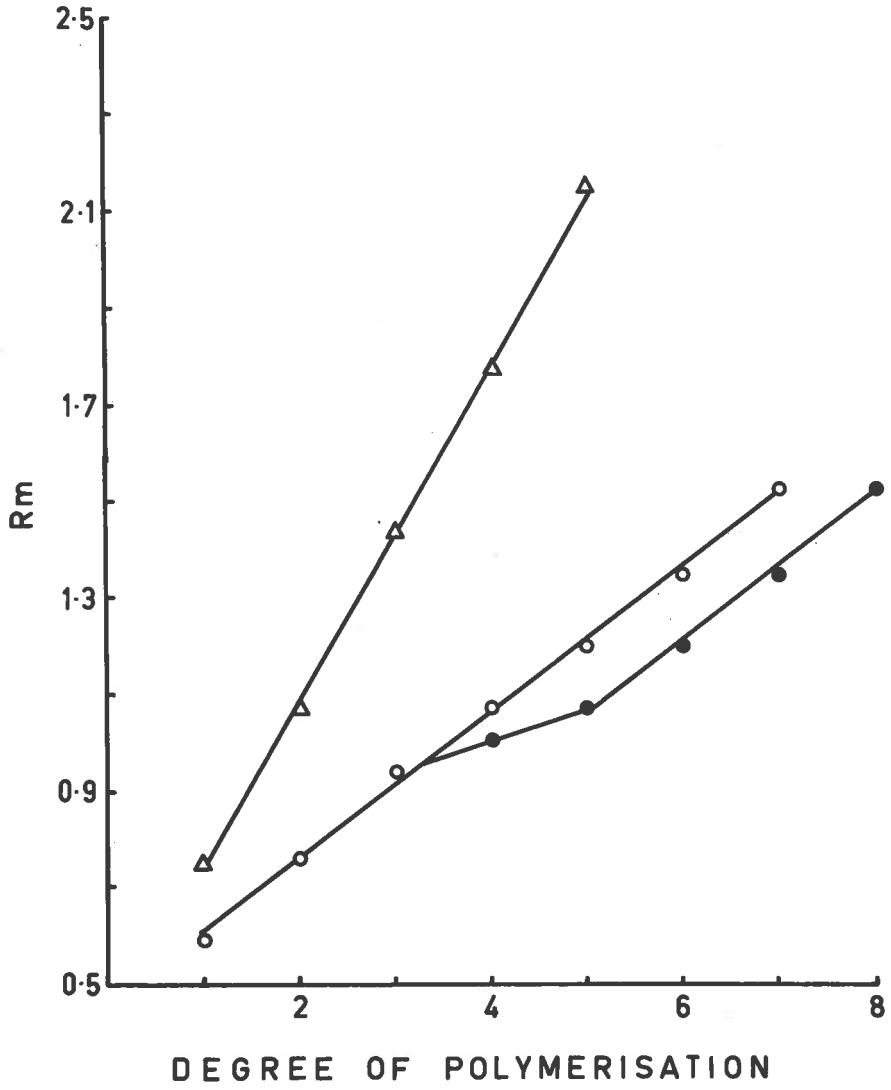
○ Values obtained from a chromatogram developed with the solvent ethyl acetate-pyridine-water (12:5:4 by volume). A component with an Rg of 0.46 was not included in the plot.

● A plot of the same values just mentioned which included the component with an Rg of 0.46.

△ Values obtained from a chromatogram developed with the solvent propan-1-ol-water-ethyl acetate (7:2:1 by volume). The component with an Rg of 0.46 was not included in the plot.

$Rm = \log \left(\frac{1-Rf}{Rf}\right)$ where Rf is the ratio of the distance moved by the component to the distance moved by the solvent front.

FIGURE 3.



that this glucose was the major component in the acid hydrolysate of the partially purified Fraction C material.

When the material recovered from the precipitated complex was partially hydrolysed with acid and the hydrolysate was examined by paper chromatography using two solvent systems, a series of radioactive components were separated on the chromatograms. The component with the greatest mobility in both solvent systems had an Rg value of 1.00. With the exception of a component with a Rg of 0.46, all the radioactive components produced a brown colour when the chromatogram was sprayed with oxanilic acid and heated. Under an ultraviolet light all the brown-coloured spots possessed the yellow fluorescence characteristic of reducing hexoses. The exceptional Rg 0.46 component absorbed rather than fluoresced when the oxanilic acid-sprayed chromatogram was examined by the light of an ultraviolet lamp. Furthermore, this component produced a grey-blue colour with the lead tetraacetate rosaniline reagent, while all the other components were characterised by a mauve colour. The Rg 0.46 component possibly was the same as the Rg 0.50 material detected in the complete acid hydrolysate.

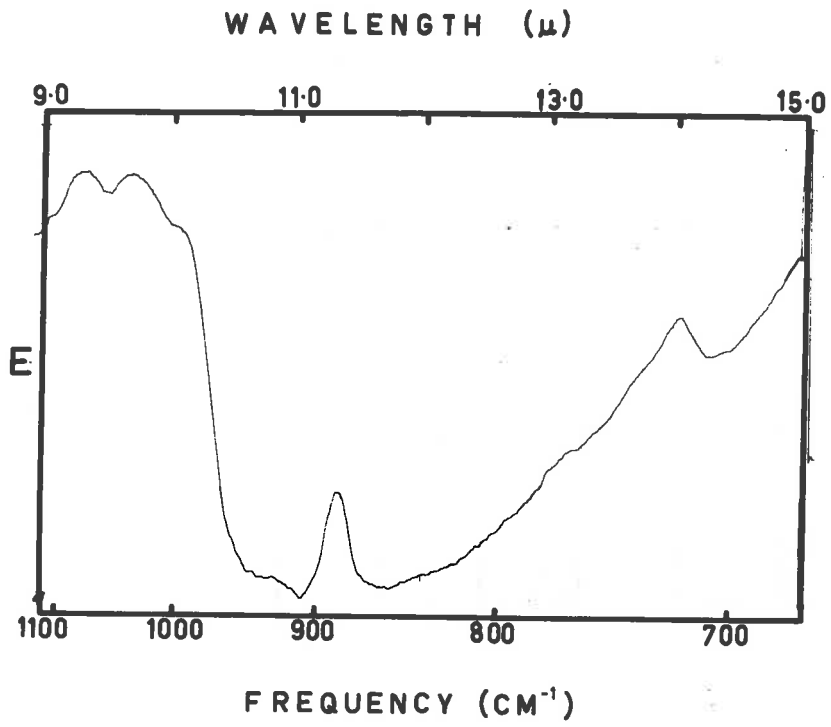
A plot of log $\frac{(1-Rf)}{Rf}$ against a predicted degree of polymerisation for each component separated from the partial hydrolysate by paper chromatography is shown in Figure 3. A straight line relationship was obtained if the component with a Rg of 0.46 was excluded from the calculation. This relation

Figure 4

Infrared spectrum of a partially purified polysaccharide from Fraction C.

The polysaccharide was partially purified by selective precipitation between pH 8.5 and 9.6 of the hexadecyl trimethylammonium-borate complex. The partially purified material was analysed as a Nujol mull at 26°. A Grubb-Parsons type S4 spectrophotometer with a sodium chloride prism was employed.

FIGURE 4.


WAVELENGTH (μ)
9·0
11·0
13·0
15·0
E
1100
1000
900
800
700
FREQUENCY (CM⁻¹)

was found to hold when the partial hydrolysate was examined by chromatography using two separate solvent systems. French and Wild (1953) have demonstrated that there was a linear relationship between the partition coefficient and the degree of polymerisation of a homologous series of oligosaccharides obtained from a number of different linear homopolymers. Omission of the component with an Rg of 0.46 appeared to be justified since its properties with the two detecting reagents were not consistent with the properties of the other components on the chromatogram. If the Rg 0.46 component was included in the plot it did not fit the line of the new series introduced when it was included in the calculations. Therefore it seemed that this component was not a member of the homologous series represented by the other components on the chromatogram.

An infrared spectrum of the material recovered from the precipitated complex prepared from Fraction C, showed a broad, weak type 1 peak at 928 $cm.^{-1}$ and a sharp, moderate type 2b peak at 887 $cm.^{-1}$. No significant absorption was evident in the type 2a region. A broad, strong peak at 720 $cm.^{-1}$, the significance of which was unknown, obscured a type 3 absorption, which appeared as a slight inflexion at approximately 765 $cm.^{-1}$. (Figure 4.)

It has been shown that absorption in the region 960 to 730 $cm.^{-1}$ of the infrared spectra of glucopyranose derivatives was correlated with certain stereochemical

features of the molecule (Barker, Bourne and Whiffen 1956). All derivatives of glucopyranose which had an alpha-glycosidic bond were characterised by absorption at or close to 844 cm.$^{-1}$ (type 2a). This absorption was not shown by compounds that were devoid of alpha-glycosidic bonds. Absorption in this region was attributed to deformation of the equatorial C_1-H bond (Barker, Bourne, Stephens and Whiffen 1954).

Inspection of the infrared spectra of derivatives of beta-glucopyranose showed that all these compounds produced an absorption band at or close to 891 cm.$^{-1}$ (type 2b). However absorption in this region was not exclusively due to the presence of beta-glycosidic links. Therefore, by itself, absorption at 891 cm.$^{-1}$ was not sufficient evidence for the presence of a beta-link.

Absorption by the alpha and beta anomers of glucopyranose at 917 and 920 cm.$^{-1}$, respectively (type 1), was thought to be due to ring vibration (Burket and Badger 1950). Absorption by the alpha anomers was strong, while that of the beta anomers was usually less intense. In the region of 766 cm.$^{-1}$, absorption (type 3) by glucopyranose was assigned to the presence of axial C-O bonds (Barker et al. 1954). Since this type of bonding did not occur in the derivatives of beta-glucopyranose, none or very weak absorption was seen at 766 cm.$^{-1}$ while the alpha anomers possessed a moderately intense absorption in that region.

Although the absorption peak observed at 887 cm.$^{-1}$ with the partially purified polysaccharide from Fraction C did not necessarily prove the presence of a beta-link, this observation coupled with the absence of a type 2a absorption and the presence of weak type 1 and type 3 absorptions provided strong circumstantial evidence for the presence of beta-linked residues.

On the basis of the foregoing results, it appeared that Fraction C was largely composed of a polysaccharide, which was tentatively assumed to be a beta-linked glucoside.

Fraction D. This fraction contained the residue of the cell material which had not been extracted by ethanol-ether or hot water. Using bovine serum albumin as a protein standard, the untreated Fraction D material was assayed for protein by a copper-complexing method. The quantity of protein estimated in this material was 102 per cent of the weight of starting material. Either the average amino acid residue weight per peptide bond of the Fraction D protein was less than that of bovine serum albumin or there were other materials in Fraction D that were interfering with the assay.

Consequently the crude Fraction D material was treated according to a procedure designed to free protein of salts, free amino acids and nucleic acids, since it had been found that these substances could interfere with the assay (Westley

and Lambeth 1960). When Fraction D had been treated in this way there was a 1 per cent loss of weight and a 3 per cent loss of protein i.e. the total weight of material could be accounted for as protein. However, the total radioactivity per unit weight of the treated Fraction D material was 10 per cent less than that of the untreated Fraction D material. Some degradation of protein must have occurred. It was concluded that Fraction D consisted almost exclusively of protein.

No allowance was made in the chemical fractionation procedure for the presence of poly-beta-hydroxybutyrate in the cell material. Therefore the cell material was examined for this polymer separately.

A quantity of non-radioactive, freeze-dried cell material was obtained from Ochromonas cells which had been grown under conditions identical to those employed to produce isotopically labelled cells. An alkaline hypochlorite extract was prepared from one gm. of this cell material by the method of Williamson and Wilkinson (1958). Alternatively the dry cell material was extracted with chloroform according to the method described by Schlegel et al. (1961). It was found, using both procedures, that Ochromonas did not contain poly-beta-hydroxybutyrate when it was grown under the conditions described here. This finding was consistent with other observations that the formation of poly-beta-hydroxybutyrate by micro-organisms was associated with growth on carbon

substrates, such as acetate, butyrate etc. (Macrae and Wilkinson 1958b and Doudoroff and Stanier 1959). The synthesis of this polymer by Ochromonas would appear to be unlikely under any conditions since the growth of the organism was inhibited by compounds such as acetate and propionate.

A number of chemical fractionation procedures for micro-organisms have been described (Roberts, Cowie, Abelson, Bolton and Britten 1955, Roodyn and Mandel 1960). Such procedures are essentially variants based on the original procedure of Schmidt and Thannhauser (1945). These variants have arisen from a desire to examine a particular cell component where it was necessary to ensure "clean" fractions, especially when isotopes were being used. In the present case the method of chemical fractionation was dictated by somewhat different needs since it was desired to examine all the major cell components which could have been potential endogenous reserve materials for the cell. Furthermore, the cell material was almost uniformly radioactive so that a small amount of cross contamination between the fractions would not greatly alter the interpretation of the results. Materials such as deoxyribonucleic acid and ribonucleic acid, which would have contained a small amount of the total radioactivity in the cell material of uniformly radioactive cells, were not singled out and their disposition among the fractions prepared from Ochromonas cell material was unknown.

Chapter 5

DISSIMILATION OF (^{14}C) RESERVE MATERIALS DURING ENDOGENOUS RESPIRATION OF OCHROMONAS MALHAMENSIS.

It was proposed (Chapter 1) that it should be possible to detect the endogenous reserve materials in a (^{14}C) labelled micro-organism by observing any loss of radioactivity from the cell constituents during endogenous respiration in the absence of exogenous substrates. When non-uniformly radioactive cells were employed to assess endogenous respiration during the respiration of exogenous substrates, Blumenthal et al. (1952) found that (^{14}C) carbon dioxide released by such cells was not representative of the total endogenous turnover. Therefore uniformly radioactive cells were a prequisite for the experiment proposed here since it was necessary to have a measure of the total endogenous turnover in order to be certain that some endogenous reserve material was not overlooked. In Chapter 3 it was shown that when Ochromonas was grown in a complete medium containing ($^{14}C_6$) glucose, the cell material was almost uniformly labelled.

With the development of a satisfactory chemical fractionation of the cell material, it was possible to follow the dissimilation of any (^{14}C) reserve materials among the cell constituents during endogenous respiration of the "uniformly" labelled cells. Non-uniformly labelled

Table 6

Suspension medium for Ochromonas

In a total volume of 5 litres this medium contained the following materials:

NaH_2PO_4	1.5g.
$MgSO_4 . 7H_2O$	3.0g.
$CaCl_2$	750.0mg.
$Na_2 MoO_4 . 2H_2O$	250.0mg.
Metals solution*	50.0ml.

The solution was made in deionised water and the pH was adjusted to 5.5.

*The metals solution was the same as that described in Table 1.

cells grown by Method 1 were observed under the same conditions as the "uniformly" labelled cells and the results obtained with both types of cells were compared.

Materials and Methods.

In media containing ($^{14}C_6$) glucose or to which ($^{14}C_6$) glucose was subsequently added, 300 or 200 ml. cultures of Ochromonas were grown in the dark by the two alternative methods described in Chapter 3. When the cultures had grown for the desired time the cells were harvested and washed in the manner described in Chapter 2. The washed cells were suspended in 30 ml. of a medium adapted from that devised by Ford (1953). The suspension medium is described in Table 6.

Endogenous respiration of cells grown by Method 1.

The suspension of radioactive cells, which had been grown by Method 1 and made radioactive by the oxidative assimilation of ($^{14}C_6$) glucose, was divided into 3.0 ml. portions. These 3.0 ml. portions of cell suspension were transferred to separate foil-wrapped 30 ml. bottles. The bottles were fitted with skirted, rubber vaccine caps.

Air, which had been pumped through cotton-packed filters, was bubbled through the suspensions at a constant rate. The air was blown into the suspensions through perforated loops of 1.5 mm. bore polythene cannula fitted over the

end of 18 gauge syringe needles which pierced the rubber vaccine caps. In order to reduce the loss in volume due to the evaporation of water from the suspensions, the gas entering the bottles was saturated with water vapour by bubbling it through a water filled gas washing bottle.

The gas escaping from the bottles, through another 18 gauge syringe needle, was bubbled through three rubber-capped centrifuge tubes containing successively, 5 ml. of 0.2N-sulphuric acid and two quantities of 5 ml. of a solution consisting of 2-ethoxy ethanol and ethanolamine (2:1 by volume) (Jeffay and Alvarez 1961). The constituents of the latter mixture were both freshly distilled, since solutions that were kept longer than a few days produced a powerful phosphorescence when they were added to Bray's phosphor solution, which was used for the liquid scintillation counting. Even freshly distilled samples of these two liquids were found to produce a small amount of phosphorescence but this could be suppressed by thoroughly cooling the source containers at 2° before the radioactivities of the contents were assayed in the scintillation detector.

By these means the cell suspensions were aerated in the dark. Radioactive carbon dioxide liberated by the cells was swept from the bottles and trapped in the 2-ethoxy ethanol-ethanolamine mixtures. The acid traps prevented any aerosol of non-volatile radioactive cell material from being carried over into the 2-ethoxy ethanol-ethanolamine traps.

The bottles were agitated gently with a mechanical shaker at room temperature (20-25°). At prescribed intervals the contents of a bottle and a 1.0 ml. water washing of that bottle were transferred to a 25 ml. centrifuge tube where the cells were killed immediately by the addition of 4 volumes of 95 per cent ethanol. The cell material was separated chemically into fractions as described in Chapter 3. The two 5 ml. quantities of 2-ethoxy ethanol-ethanolamine solution were combined and the trapped carbon dioxide was assayed for radioactivity by the liquid scintillation technique. The sulphuric acid solutions in the first trap were neutralised with barium carbonate, since acid solutions caused a high degree of quenching of the phosphor solution used for assaying radioactivity by liquid scintillation (Bray 1960). The precipitated barium sulphate was removed by centrifuging. The neutralised supernatant solutions were found to possess negligible amounts of radioactivity when they were assayed by the liquid scintillation technique.

Endogenous respiration of cells grown by Method 2.

The suspension of radioactive cells, which had been grown in a medium containing ($^{14}C_6$) glucose (Method 2), was transferred to a foil-wrapped 70 ml. bottle. This bottle had the same provision for gassing as described for the bottles used with the Method 1 cells. As a consequence of the difficulties experienced with the 2-ethoxy ethanol-

ethanolamine mixture, two tubes each containing 5 ml. of 2N-sodium hydroxide solution replaced this mixture and the radioactive carbon dioxide was trapped in the alkali. The trapped carbon dioxide was later precipitated as barium carbonate. Another reason for this change was the necessity to determine the specific radioactivity of the evolved carbon dioxide since this value was important for the interpretation of the results. It was not possible to determine the specific radioactivity of the carbon dioxide which had been trapped in 2-ethoxy ethanol-ethanolamine.

The bottle was agitated gently with a mechanical shaker at room temperature. Samples of 3.0 ml. were withdrawn, at intervals, from the radioactive cell suspension in the bottle by means of a syringe. These samples were treated with 4 volumes of ethanol and the cell material was separated into fractions as described in Chapter 3. Each time a sample was taken from the cell suspension the two tubes of sodium hydroxide solution were replaced by two tubes of fresh sodium hydroxide solution. Carbonate was precipitated from the alkaline solutions by adding 3.5 ml. of a solution 1.5 M to barium chloride and 1.0 M to ammonium chloride. The barium carbonate precipitate was collected by centrifuging and the supernantant liquid was discarded. The barium carbonate was washed once with water and twice with ethanol. The barium carbonate suspended in ethanol was collected by filtration on a disc of Whatman number 42 paper supported by a perforated

polyethylene planchet. The planchet together with the paper disc had been weighed previously. During the filtration the planchet and paper disc were held in a stainless-steel filtration device similar to that described by Overman and Clark (1960). While the planchet and paper disc were still in this apparatus the barium carbonate was washed free of ethanol with acetone and sucked dry. The planchet and paper disc plus barium carbonate were reweighed to obtain the weight of barium carbonate by difference. The barium carbonate was assayed for radioactivity with an end-window detector as described in Chapter 2.

Manometry.

Manometric measurements were made at 28° using the Warburg direct method, as outlined by Umbreit (1957a). Similar measurements were also made in an atmosphere of approximately 1.5 per cent (v/v) carbon dioxide in air using the carbon dioxide-buffer technique of Pardee (1949). Each manometer flask contained 60 micromoles of potassium phosphate buffer pH 5.5 and a washed cell suspension of Ochromonas, all in a total liquid volume of 3.0 ml. A correction was made for the retention of carbon dioxide by the phosphate buffer by using a factor that was read from a graph showing the relation between pH and the ratio (apparent solubility of carbon dioxide)/(real solubility of carbon dioxide) (Umbreit 1957b).

The respiratory rates (Qo_2) were expressed as the number of microlitres of oxygen taken up per mg. dry weight of cell material per hour.

Results and Discussion.

Uniform radioactivity of the cells.

Specific radioactivity expressed as radioactivity per unit weight was adequate for the purpose of following changes in the distribution of radioactivity among the fractions prepared from radioactive cells. However it was necessary to determine the specific radioactivities of these fractions in terms of radioactivity per atom of carbon before any conclusions could be reached concerning the uniformity of radioactive-labelling of the cell material. Approximate values of the specific radioactivity expressed in this way were calculated for the materials in the fractions prepared from cells which had been grown by Method 2 (Table 3). For the purpose of these calculations it was assumed that lipid, polysaccharide and protein represented the whole of the material in fractions B, C and D respectively. The following values were accepted as the percentage of carbon per unit weight of these materials; lipid 68 per cent, polysaccharide 44 per cent and protein 52 per cent. The value for the polysaccharide was readily calculated and the value for protein was quoted by Fruton and Simmonds (1958). The lipid value was calculated from the proportions of the various constituents

separated on the silicic acid column (Chapter 4, Table 5) by assuming that cholesteryl palmitate, triolein, monoolein and dioleyl lecithin were representative of the compounds in fractions 3, 4, 7 and 8 respectively.

The specific radioactivities calculated for the fractions prepared from Ochromonas cells which were grown by Method 2 were:

	millimicrocuries per milliatom of carbon.
Fraction B (lipid)	195
Fraction C (polysaccharide)	215
Fraction D (protein)	185
Glucose in the medium	303

Because of the heterogeneous composition of fraction A, it was not possible to calculate the specific radioactivity of this fraction. Since the materials in fraction A appeared to be mostly intermediates of metabolism, then the value of the specific radioactivity of this fraction should be similar to the values calculated for the other fractions. It was apparent from the figures given above that, in addition to glucose, there were other carbon compounds in the medium that were providing carbon for the synthesis of the materials in fractions B, C and D, since the specific radioactivity of the materials in these three fractions was less than the specific radioactivity of glucose in the growth medium. However, the non-radioactive carbon was not directed to any one fraction

Table 7

The distribution of radioactivity in the fractions during endogenous respiration of Ochromonas cells which had been grown by method 1.

Ochromonas was cultured as described in Table 2. Then 10 microcuries of ($^{14}C_6$) glucose with a specific radioactivity of 5 microcuries per mg. was added to the culture which was grown a further 3 hours. Then the cells were harvested and washed with water by centrifuging. Finally the cells were suspended in 30 ml. of the medium described in Table 6. Portions (3.0 ml.) of this suspension were allowed to respire for certain intervals of time in the dark under a stream of filtered air. Carbon dioxide was trapped in ethanolamine and at the end of the period of respiration the radioactivity was assayed by the liquid scintillation technique. At the same time the cell material was separated into fractions by the procedure outlined in Figure 1 and the radioactivity of the fractions was also assayed by liquid scintillation. Column 4 shows the percent of the total radioactivity recovered in the fraction. Column 5 shows the specific radioactivity in millimicrocuries per mg.

Table 7

Time (hours) of endogenous respiration	0		1.5		5.5		10		20	
	R	S	R	S	R	S	R	S	R	S
Fraction A (intermediates)	15.9		22.1		27.2		31.3		32.3	
Fraction B (lipid)	17.5	11.2	16.1	11.5	15.1	11.5	11.4	10.7	14.3	
Fraction C (polysaccharide)	38.2	10.5	32.5	10.1	25.8	10.0	21.0	9.8	0.2	
Fraction D (protein)	15.8		15.1		14.4		14.5		10.2	
Supernatant + washings	12.6		6.7		5.2		5.1		3.3	
Carbon dioxide	0		2.3		12.3		16.5		39.7	
Total radioactivity recovered (microcuries)	0.233		0.225		0.233		0.227		0.226	

in particular and there was in fact approximately uniform radioactive-labelling of the materials in fractions B, C and D.

The specific radioactivities that were calculated from the figures available for the fractions prepared from cells that were grown by Method 1 were:

	millimicrocuries per milliatom of carbon.
Fraction B (lipid)	198
Fraction C (polysaccharide)	287
Glucose added to the medium	839,000

It was apparent that the materials in fraction B and C did not have the same specific radioactivities. Thus, cells which had been grown by Method 1 were not uniformly radioactive. Only the cells which had been grown by Method 2 were sufficiently near to fulfilling the conditions that were required of cells which might be used to detect the reserve materials by the method described in this chapter.

Endogenous respiration of cells grown by Method 1.

When cells which had been made radioactive by Method 1 were allowed to respire in the absence of any added substrates, fractions B and C which had been prepared from these cells both lost radioactivity (column R, Table 7). After 20 hours of this endogenous respiration almost all of the radioactivity

had disappeared from fraction C while the total radioactivity of fraction B had been reduced by 18 per cent. The reduction of the total radioactivity in fractions B and C was accompanied by an increase in the total radioactivity offraction A and an increase in the total radioactivity of the evolved carbon dioxide. The magnitudes of these changes were such that it could be concluded that the radioactive material in fraction C must have contributed to the increase in the total radioactivities of both fraction A and the carbon dioxide.

The specific radioactivities (units of radioactivity per unit weight) of the materials in fractions B and C did not alter significantly during the initial 10 hours of endogenous respiration; during this time the total radioactivities were reduced by 18 and 45 per cent respectively. Either the materials within fraction B and C were uniformly radioactive or, fortuitously, the non-radioactive components were releasing non-radioactive carbon dioxide at the same rate and to the same extent as the radioactive components were releasing (^{14}C) carbon dioxide. Fraction C consisted largely of a single polysaccharide (Chapter 4) and therefore the presence of a non-radioactive carbon source in this fraction did not have to be taken into account. Thus the material in fraction C must have been uniformly radioactive, since the specific radioactivity had remained virtually constant while the total radioactivity had been reduced by 45 per cent.

Table 8

Distribution of radioactivity among subfractions of Fraction A obtained during endogenous respiration of cells grown by Method 1.

An Ochromonas culture was labelled with ($^{14}C_6$) glucose by Method 1. The washed radioactive cells were allowed to respire endogenously and the suspension was sampled at intervals. The cell material in each sample was separated into fractions (Table 7). Fraction A of each sample was in turn separated into acidic, basic and neutral subfractions as described in Table 4. The radioactivity of the subfractions was determined by the liquid scintillation technique.

Time of endogenous respiration (hours)	0	1.5	5.5	10	20
	Radioactivity (millimicrocuries)				
Basic fraction	7.4	7.9	7.8	7.5	9.5
Acidic fraction	3.9	4.6	4.9	3.0	2.0
Neutral fraction	25.0	32.1	35.4	36.5	33.0
Total in three sub-fractions	36.3	44.6	48.1	47.0	44.5
Total originally in Fraction A	37.1	52.2	63.3	71.2	73.0
Difference (Fraction A -subfractions)	0.8	7.6	15.2	24.2	28.5

On the other hand it was found (Chapter 4) that fraction B was heterogenous in composition. Therefore it was possible that a non-radioactive lipid was being catabolised at the same rate and to the same extent as the radioactive lipid so that the specific radioactivity of the lipid remaining was always constant. When the radioactive material in fraction C had been utilized the total radioactivity of fraction D began to decrease.

The materials in fraction A were analysed further in an attempt to determine the nature of the material causing the increase in total radioactivity of this fraction during endogenous respiration of cells produced by Method 1. The fraction was separated into acidic, basic and neutral sub-fractions by means of ion-exchange resins as described in Chapter 4. The distribution of the total radioactivity among the sub-fractions of fraction A is shown in Table 8. Although the radioactivity of the neutral fraction increased with the time of endogenous respiration, it was apparent from the recoveries of radioactivity in the three fractions that the material primarily responsible for the increase in the total radioactivity of fraction A had been lost during the preparation of the sub-fractions. It was possible that the missing radioactive material was the salt of a volatile acid and the free acid was lost to the atmosphere after Fraction A had been treated with the cation-exchange resin.

Endogenous respiration of cells grown by Method 2.

Radioactive cells were grown by culturing Ochromonas in a

Table 9

The distribution of radioactivity among the fractions during endogenous respiration of Ochromonas cells which had been grown by method 2.

Ochromonas was cultured in a medium containing 30 microcuries of ($^{14}C_6$) glucose with a specific radioactivity of 10 millimicrocuries per mg. as described in Table 3. The cells were harvested, washed with water by centrifuging and suspended in 30 ml. of the medium described in Table 6. The suspension of cells was allowed to respire in the dark in the absence of added substrates, in a stream of filtered air. Carbon dioxide evolved was trapped in sodium hydroxide. The carbonate was precipitated as barium carbonate and assayed for radioactivity with a Geiger-Muller detector. At intervals samples of the cell suspension were removed and the cell material was separated into fractions as outlined in Figure 1, and the radioactivities of the fractions were assayed by the liquid scintillation technique. Column R shows the percent of the total radioactivity that was present in the fraction. Column S shows the specific radioactivity in millimicrocuries per mg.

Table 9

Time (hours) of endogenous respiration	0		2		4	
	R	S	R	S	R	S
Fraction A (intermediates)	11.1	5.9	11.0	6.1	10.3	6.0
Fraction B (lipid)	24.2	11.3	27.4	11.6	28.0	12.3
Fraction C (polysaccharide)	33.1	7.8	30.9	8.6	22.2	5.6
Fraction D (protein)	28.4	8.3	23.0	6.1	28.1	8.1
Supernatant + washings	3.3	-	4.7	-	3.0	-
Carbon dioxide	0	0	3.2	4.6	8.7	4.5
Total radioactivity recovered (microcuries)	0.346		0.308		0.297	

Table 2 continued

Time (hours) of endogenous respiration	9		12		21.5	
	R	S	R	S	R	S
Fraction A (intermediates)	10.5	6.0	10.0	5.9	10.1	5.6
Fraction B (lipid)	25.4	11.6	21.8	11.9	20.9	12.3
Fraction C (polysaccharide)	14.5	6.3	10.2	-	6.6	-
Fraction D (protein)	26.9	8.2	29.2	9.1	24.8	7.8
Supernatant + washings	4.1	-	3.3	-	1.9	-
Carbon dioxide	18.5	4.8	25.6	4.7	36.9	4.1
Total radioactivity recovered (microcuries)	0.319		0.346		0.356	

medium containing uniformly-labelled glucose (Method 2). Samples were taken at intervals from a suspension of these cells which were respiring aerobically in the absence of exogenous substrates and the cell material was separated into fractions and assayed for radioactivity. The distribution of the radioactivity among the fractions is shown in Table 9. During the initial 8 hours of endogenous respiration, the amount of radioactivity in the carbon dioxide liberated by the cells could be equated solely with the disappearance of radioactivity from Fraction C. However, after 8 hours of endogenous respiration the total radioactivity in Fraction B began to decrease and it was no longer possible to equate the amount of radioactivity disappearing from Fraction C with the amount of radioactivity appearing in the carbon dioxide. No increase in the total radioactivity of Fraction A was observed during this experiment. The total radioactivity of Fraction D did not decrease significantly in the time that samples of the cell suspension were taken. However, the radioactive material in Fraction C had not been exhausted by the time the last sample of cells was taken and the breakdown of the material in Fraction D may not have occurred until the material in Fraction C was all utilized. In the experiment with the Method 1 cells all the material in Fraction C had been utilized when the decrease in total radioactivity of Fraction D was detected.

The specific radioactivities of the materials in Fractions A, B and D and the liberated carbon dioxide were all virtually constant for the duration of the experiment. However, the specific radioactivity of Fraction C was randomly variable. Possibly this variation was related to difficulties experienced in obtaining the material of Fraction C in an anhydrous state. Since the specific radioactivities of all the other constituents of the cell material and the carbon dioxide were constant, it may be assumed that the specific radioactivity of the material in Fraction C was actually constant and that the apparent variation of the specific radioactivity of Fraction C was due to variations in the degree of hydration of the polysaccharide specimens prepared from each sample.

The results obtained with radioactive Ochromonas cells which had been grown by either Method 1 or Method 2 clearly showed that the material in Fraction C was being metabolised to carbon dioxide during endogenous respiration. The material in Fraction C was tentatively identified as a beta-linked glucoside (Chapter 4). Using cells which had been made radioactive by Method 1, it appeared that there was another product of endogenous respiration in addition to carbon dioxide. However these cells were not uniformly radioactive and it was possible that this "product" was an artifact. Since the specific radioactivities (millimicrocuries per mg.) of all the fractions and the carbon

dioxide, obtained from the cells which had been grown by Method 2, remained nearly constant during 10 hours of endogenous respiration, then any but a very minor amount of non-radioactive cell material could have been acting as an endogenous reserve material in these cells.

Manometry.

During endogenous respiration of dark-grown cells in the absence of exogenous substrates the R.Q. was found to be 0.86 ± 0.08 (standard deviation, 11 determinations). This value was obtained by measuring, manometrically, the oxygen taken up by the cells in an atmosphere of 1.5 per cent carbon dioxide in air using the carbon dioxide-buffer technique of Pardee (1949). The volume of carbon dioxide liberated during the same timewas determined by subtracting the volume of oxygen taken up from the volume change occurring over the same quantity of cells which were respiring in an atmosphere of air in a separate Warburg flask. The R.Q. was then equal to the ratio (volume of carbon dioxide evolved)/(volume of oxygen taken up).

The R.Q. was determined by this means since it was found that the rate of endogenous respiration of dark-grown Ochromonas cells was dependent on the presence of carbon dioxide in the atmosphere. This finding was contrary to that of Reazin (1954) who found that the respiration rate of dark-grown Ochromonas cells was the same whether it was determined by the Warburg direct method, the Warburg in-

direct method or the method of Pardee (1949) using a carbon dioxide buffer.

In the present investigation, Q_{O_2} (microlitres of oxygen taken up per mg. dry weight of cells per hour) in the presence of 1.5 per cent carbon dioxide was 14.07 ± 5.85 (standard deviation, 11 determinations), while in the absence of carbon dioxide (potassium hydroxide solution in the centre-well of the Warburg flask) Q_{O_2} was 31.25 ± 10.82 (standard deviation, 8 determinations). Therefore R.Q. determined by the Warburg direct method might not be valid. It was not even certain that the R.Q. determined in the presence of 1.5 per cent carbon dioxide was valid, since this concentration was 50 times that normally found in air. The small concentration of carbon dioxide in air (0.03 per cent) could not be reproduced inside a Warburg flask using the carbon dioxide buffer systems of Pardee (1949) or Krebs (1951), so it was not possible to determine the Q_{O_2} of dark-grown Ochromonas cells in air.

If the R.Q. value of 0.86, determined in the presence of 1.5 per cent carbon dioxide, was acceptable, then this would indicate that some material other than carbohydrate was acting as the endogenous reserve material in dark-grown Ochromonas. Reazin (1954) found that the R.Q. of dark-grown Ochromonas was 0.8 and without further experimental evidence he made the same suggestion. However, the

evidence available from the present investigation clearly indicated that the material in Fraction C was the endogenous reserve of Ochromonas and it was believed that this material was a polysaccharide.

Therefore a more thorough examination was made of the material in Fraction C in an attempt to establish the nature of the reserve material.

Chapter 6

PURIFICATION AND ANALYSIS OF A POLYSACCHARIDE RESERVE MATERIAL ISOLATED FROM *OCHROMONAS MALHAMENSIS*.

It was shown in Chapter 5 that the reserve material which supported endogenous respiration of *Ochromonas* was a constituent of an aqueous extract (Fraction C) of the cell material. When *Ochromonas* cells were made radioactive by growth on ($^{14}C_6$) glucose the reserve material accounted for a large proportion of the radioactivity in Fraction C. Most of the radioactive material in Fraction C was identified with a neutral polysaccharide which was obtained by selective precipitation with hexadecyl trimethylammonium borate (Chapter 4). This neutral polysaccharide was tentatively identified as a beta-linked glucoside.

Archibald et al. (1958) had already partially characterised a polysaccharide which they had isolated from *Ochromonas*. However there was no certainity that their polysaccharide was the same as the radioactive polysaccharide obtained during this investigation. Consequently an attempt was made to characterize the radioactive polysaccharide and in so doing a novel method of purification was employed.

Materials and Methods.

It was apparent from the brief examination of Fraction C described in Chapter 4, that precipitation with hexadecyl trimethylammonium bromide in the presence of borate effected some purification of the polysaccharide which made up the largest proportion of this fraction. Chromatographic analysis of acid hydrolysates of the partially purified polysaccharide showed that either the polysaccharide was a polymer of more than one sugar or it was still accompanied by some impurities.

Antonopoulos, Borelius, Gardell, Hamnstrom and Scott (1961) had effected a separation of a mixture of acidic polysaccharides. The polysaccharides were precipitated on a cellulose column as the hexadecyl-pyridinium complexes and the complexes were separated by extracting the column with a salt gradient. Prior to this report, Scott (1960) had suggested that keratosulphate might be purified by column elution of the borate-paraffin chain quaternary nitrogen complex. The procedure used to purify the radioactive Ochromonas polysaccharide was derived from a combination of the methods described by Antonopoulos et al. (1961) with the suggestion made by Scott (1960).

Column elution of a polysaccharide-borate-quaternary nitrogen complex.

A glass column 2.5 x 42 cm. was maintained at 38° by circulating water from a heated reservoir through a

jacket surrounding the column. The column had a standard B24 socket at each end. These standard sockets were fitted with rubber stoppers of the same dimensions as the socket. A short length of stainless-steel tubing with an internal diameter of 1.5 mm. pierced the lower stopper and provided the column with an outlet. Resting on top of this stopper in ascending order were, a 1.5 mm-thick polyethylene ring, a disc of Whatman number one filter paper, a 2.5 mm-thick perforated teflon disc and another disc of filter paper. All of these articles made a close fit with the inside of the column. The volume of liquid hold up between the outlet and the cellulose packed in the column was 0.2 ml. This small hold-up volume kept remixing of the column effluent to a minimum.

Sixty gm. of Whatman standard grade cellulose powder (H. Reeve Angel and Co. Ltd., London) were suspended in 600ml. of 0.1 M sodium tetraborate solution which had been adjusted to pH 9.45 with sodium hydroxide. This suspension was stirred magnetically under full vacuum of a water pump for one hour. This procedure was necessary to remove dissolved gases and gas trapped in the interstices of the cellulose powder, otherwise large bubbles of gas formed in the column when the temperature of the cellulose was raised to 38°. All solutions that were added to the column were treated in this way. The suspension of cellulose was poured down a glass rod into the column. The cellulose

was packed down in the column by applying a pressure of 2 to 3 lbs. per square inch at the upper end of the column. This cellulose provided an inert surface on which the polysaccharide-borate-hexadecyl pyridinium complex was precipitated. A filter-paper disc with a nylon thread fixed to the centre was lowered on to the top of the cellulose. The disc prevented the eluent from scouring the surface of the cellulose and the thread facilitated withdrawal of the disc at a later stage. The flow of liquid from the outlet at the bottom of the column was controlled by means of a screw clip applied to a length of 1.5 mm. - bore polyethylene tubing which connected the column to the scanning device.

The scanning device consisted of a hydrogen lamp light source, a M290 grating monochromator (Hilger-Watts Ltd., London) and a suitable photocell. The column effluent flowed through a 0.5cm. silica cell mounted between the monochromator and the photocell. The photocell output was logarithmically amplified before it was fed to the input of a rectilinear recording milliampmeter (Texas Instruments Inc., Houston, Tex., U.S.A.) which plotted the extinction of the column effluent. The scanning device was set to record absorption at a wavelength of 260 millimicrons since 1-hexadecyl pyridinium bromide had a characteristic absorption maximum at this wavelength. The column effluent that had passed through the scanning device was collected in

tubes that were held by a time-controlled fraction collector. The 1.5 mm.-bore polyethylene tubing, connecting the scanning device to the column and the scanning device to the fraction collector, was irradiated with infrared lamps to keep the temperature of the liquid within the tubing above 35°.

A solution of 200 ml. of 0.026 M 1-hexadecyl pyridinium bromide in 0.1 M sodium tetraborate, which had been adjusted to pH 9.45, was added to the column from an electrically heated separating funnel maintained at 38°. It was necessary to keep the temperature at 38° since 1-hexadecyl pyridinium bromide has a critical solution temperature of 33°. The separating funnel was equipped with a capillary bore stem which pierced the stopper at the top of the column and reached to within 2 mm. of the filter paper disc situated on top of the cellulose. The flow from the column was maintained at 4 to 5 ml. per minute.

When the 200 ml. of solution had run in level with the cellulose the flow of liquid from the column was stopped and the filter paper disc was withdrawn. One ml. of 0.1 M sodium tetraborate solution pH 9.45 was added to the column and allowed to run in level with the cellulose. A 50 mg. sample of the radioactive fraction C material, which had been selectively precipitated with hexadecyl trimethyl ammonium-borate between pH 8.5 and 9.8 and recovered as described in Chapter 4, was dissolved in 1.5 ml. of 0.1 M

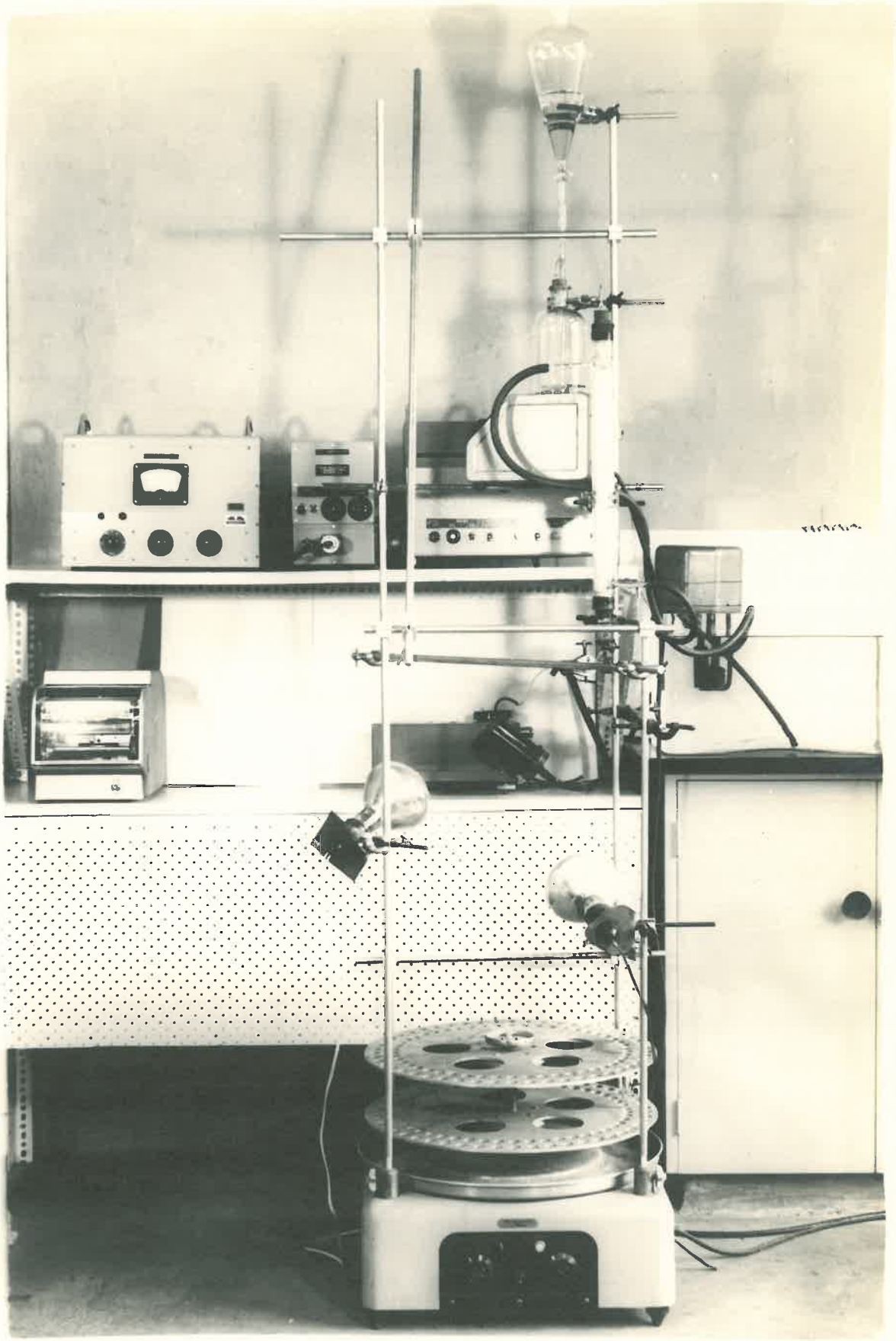
tetraborate solution, added to the column and allowed to drain into the cellulose. This solution was followed by the addition of another 1.0 ml. of the 0.1 M tetraborate solution. The filter paper disc was replaced at the top of the column and the column was irrigated with another 200 ml. of 0.026 M 1-hexadecyl pyridinium bromide in 0.1 M sodium tetraborate pH 9.45 at a flow rate of 2 ml. per minute. When this solution had passed into the column excess 1-hexadecyl pyridinium bromide was washed from the column with 0.1 M sodium tetraborate solution pH 9.45 at a flow rate of 3 ml. per minute. The washing of the column was continued until the absorption of the effluent reached a steady low value.

The polysaccharide-borate-quaternary nitrogen complex, which had been precipitated on the surface of the cellulose in the column, was eluted with a borate-phosphate mixture of decreasing pH. A solution of 750 ml. of 0.09 M sodium dihydrogen phosphate was allowed to drain from a separating funnel with a capillary bore stem into the bottom of a stoppered bottle containing 750 ml. of 0.1 M sodium tetraborate which had been adjusted to pH 9.45. The bottle contents were stirred magnetically. The mixture of continuously decreasing pH was expelled from the bottle by the pressure of the liquid in the funnel. The mixture issuing from the bottle flowed through 1.5 mm.-bore polyethylene tubing to an outlet 2mm. above the filter paper disc at the

Figure 5

Apparatus for the elution of polysaccharide-1-hexadecylpyridinium-borate complexes from a cellulose column.

In order from the left the power pack, D.C. amplifier and logarithmic amplifier are shown on the upper shelf of the stand. The chart recorder and monochromator fitted with hydrogen lamp light source, flow-cell and photo-cell are on the lower shelf of the stand. The jacketed column containing the cellulose powder can be seen to the right of the logarithmic amplifier. The column is mounted on a framework standing over a time actuated fraction collector which stood on the floor. A magnetic stirrer partly obscures the logarithmic amplifier. A bottle containing 750 ml. of tetraborate buffer (0.1 M., pH 9.45) stands on the magnetic stirrer and a separating funnel containing 750 ml. of 0.09 M. sodium dihydrogen phosphate is mounted over the bottle. The pH gradient was produced by allowing the contents of the funnel to drain into the bottle. The contents of the bottle were magnetically stirred and the buffer with a continuously changing pH was expelled from the bottle by the pressure of the liquid in the funnel. A constant temperature water bath maintained at 38° can be seen on the bench to the right of the picture. The water was continuously circulated between the bath and the jacket around the column by means of a pump. Two infrared lamps are mounted on the framework. These lamps irradiate the tubing connecting the column to the flow-cell and the flow-cell to the fraction collector thus maintaining the temperature of the liquid in the tubing at 38°.



head of the column. The flow of the column effluent was controlled at 0.5 ml. per minute. Fractions of the column effluent were collected every 10 minutes and the pH of the fractions was determined after they had cooled to room temperature (20^{o}).

Fractions of the column effluent corresponding to the peaks traced by the recorder were pooled. Before this could be done it was necessary to dissolve the precipitated complex by acidifying the fractions with one or two drops of 2N-acetic acid. The complex precipitated because the pH of the solution increased slightly when the fractions cooled from 38^{o} to room temperature. The polysaccharide was recovered from the hexadecyl pyridinium-borate-polysaccharide complex by treatment of the pooled fractions with a cation-exchange resin and removal of the boric acid by distillation with methanol as described in Chapter 4.

The complete apparatus for the column elution of the polysaccharide complex is shown in Figure 5.

The specific rotation of a solution of polysaccharide in water was determined using a Hilger Microptic polarimeter (Hilger and Watts Ltd., London).

An infrared spectrum of the polysaccharide was determined as described in Chapter 4.

Acid hydrolysis of polysaccharide 1. Polysaccharide 1, which had been separated from Fraction C in the cellulose column, was hydrolysed in 2N-sulphuric acid (1 mg. per ml.)

for 4 hours at 100° in a sealed tube. The cooled hydrolysate was neutralized with barium carbonate. The precipitated barium sulphate and the excess barium carbonate were packed by centrifuging and the supernatant, neutralized, hydrolysate was removed. The precipitate was washed three times with water. The washings were combined with the original supernatant and the solution was reduced to dryness in a vacuum desiccator.

The dry residue was dissolved in a small volume of water and samples of this solution equivalent to approximately 50 micrograms of solid material were examined by paper chromatography with the solvent mixture ethyl acetate-pyridine-water (120:50:40 by volume) (Smith 1960) using Whatman number 1 paper. The dry chromatograms were sprayed with 0.1 M oxanilic acid and then heated at 110° for 10 minutes to detect reducing sugars. Separate chromatograms were dipped in a solution of 0.3 per cent (w/v) lead tetra-acetate in chloroform, allowed to dry and then dipped in a solution of 0.03 per cent rosaniline base in 10 per cent acetic acid in acetone to detect non-reducing carbohydrates (Sampson et al. 1961).

A partial hydrolysate of polysaccharide 1. was obtained by treating it with 0.5 N hydrochloric acid (10 mg. per ml.) at 100° in a sealed tube for 20 minutes. The hydrolysate was dried in a vacuum desiccator over sodium hydroxide flakes to remove hydrochloric acid. The dry material was

dissolved in water and samples of the solution were examined by paper chromatography with the solvent system previously described.

Acid hydrolysis of polysaccharide 2. Polysaccharide 2 was hydrolysed with sulphuric acid exactly as described for polysaccharide 1. The neutralised hydrolysate was examined by paper chromatography with the same solvent system used for polysaccharide 1. Additional samples of the hydrolysate were subjected to electrophoresis on Whatman 3MM paper with sodium arsenite buffer pH 9.6, at 400 volts for 16 hours. In addition the hydrolysate was assayed for total carbohydrate by the method of Hallivell (1960), for total hexose by the method of Dische, Shettles and Osnos (1949) and for reducing sugars by the method of Nateles (1960).

Polysaccharide 2 was partially hydrolysed and the hydrolysate was examined exactly as described for polysaccharide 1.

Enzymic hydrolysis of polysaccharide 1. If the radioactive polysaccharide in Fraction C was similar to the polysaccharide isolated by Archibald et al. (1958), then the radioactive polysaccharide should be degraded by two enzymes which have been shown to catalyse the hydrolysis of beta-1, 3-linked glucans (Reese and Mandels 1959).

A solution of 1 mg. of polysaccharide 1 dissolved in

0.275 ml. of 0.0275 M citric acid-sodium citrate buffer pH 4.9 (Hemington and Dawson 1959) was added to a solution containing 5.25 units of an exohydrolytic beta-D-1, 3-glucanase (kindly donated by Dr. E.T. Reese, Quartermaster Research and Engineering Centre, Natick, Mass., U.S.A.) in 0.025 ml. of water. The exohydrolytic beta-D-1, 3-glucanase was obtained from the culture filtrate of a Basidiomycete species by precipitation with acetone and partially purified by fractional precipitation with ethanol. This enzyme preparation had a specific activity of 350 units per mg. One unit of enzyme produced 1 mg. of reducing sugar, measured as glucose, from 3 mg. of laminarin in 1.0 ml. of citrate buffer pH 4.9 at 40° in one hour (Reese and Mandels 1959). A drop of toluene was placed on the surface of the reaction mixture which was incubated at 40° for 8 hours. The reaction was stopped by boiling the reaction mixture.

Samples of the boiled reaction mixture were analysed by paper chromatography with the solvent mixture ethyl acetate-pyridine-water as described previously.

Enzymic hydrolysis of polysaccharide 2. Polysaccharide 2 was treated with the exohydrolytic beta-D-1, 3-glucanase exactly as described for polysaccharide 1 and the products of the reaction were examined by paper chromatography. An oligosaccharide, which was a product of the activity of

this enzyme on polysaccharide 2, was obtained in greater yield by repeating the above reaction with ten times the quantities of reactants. The boiled reaction mixture was streaked along an origin line, 55 cm. in length, on a sheet of Whatman 3MM paper. The chromatogram was developed with the solvent mixture ethyl acetate-pyridine-water previously described. A strip, 1.5 cm. wide, was cut from one side of the dry chromatogram down the line of development. This strip was treated with the lead tetraacetate-rosaniline reagent previously described to locate the oligosaccharide band. A strip, corresponding to the position of the detected oligosaccharide, was cut from the remainder of the chromatogram. To elute the oligosaccharide, this strip was coiled and placed in a nylon-mesh bag where the paper was moistened to saturation with water. The upper edges of the bag were held between the wall of a 25 ml. centrifuge tube and a polyethylene stopper, so that the bag hung down inside the tube. The tube was centrifuged at 900 g for 10 minutes in a bench centrifuge. The eluate collected at the bottom of the centrifuge tube. This procedure was repeated six times and the combined eluates were reduced to dryness in a rotary evaporator.

Since the exo-beta-1, 3-glucanase had a restricted specificity (Johnson, Kirkwood, Misaki, Nelson, Scaletti and Smith 1963), then this oligosaccharide still might have possessed beta-1, 3-glycosidic links. Therefore the

oligosaccharide was treated with two other enzymes which catalysed the hydrolysis of beta-glycosidic bonds in order to establish whether the oligosaccharide still possessed bonds of this type.

The oligosaccharide was dissolved in 0.75 ml. of water and 0.25 ml. of this solution was mixed with 0.035 ml. of 0.4 M sodium acetate-acetic acid buffer pH 5.5. and 0.5 mg. of a beta-glucosidase preparation obtained from almond emulsin (California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A.). A drop of toluene was placed on the surface of the reaction mixture which was left at 37° for 15 hours. One mg. samples of cellobiose, maltose, and polysaccharide 2 were treated with the beta-glucosidase at the same time under the same conditions.

After 15 hours 0.05 ml. samples of all reaction mixtures were examined by chromatography on Whatman number 1 paper with the ethyl acetate-pyridine-water solvent mixture described previously. Reducing sugars and non-reducing carbohydrates were detected with the oxanilic acid and lead tetraacetate-rosaniline reagents.

A further 0.25 ml. portion of the oligosaccharide solution was mixed with 0.01 ml. of 0.1 M citric acid-sodium citrate buffer pH 4.9 and 0.2 mg. of an endohydrolytic beta-D-1, 3-glucanase (also a gift of Dr. E.T. Reese). A drop of toluene was placed on the surface of the liquid and the reaction mixture was left at 38° for 12 hours. Samples of

0.05 ml. of the boiled reaction mixture were examined by paper chromatography as described before.

Partial enzymic hydrolysis of polysaccharide 2 and isolation of a disaccharide.

It was found that the endohydrolytic beta-D-1, 3 glucanase degraded polysaccharide 2 to a mixture of di- and tri-saccharide sub-units. The nature of the predominant glycosidic bond in polysaccharide 2 could be determined if the disaccharide was isolated and its structure was established. However, the enzyme preparation that was available contained a small amount of an oligosaccharidase which slowly degraded the di- and tri- saccharides to glucose. Therefore a preliminary experiment was performed to determine a time of reaction for which an optimum amount of disaccharide would be obtained.

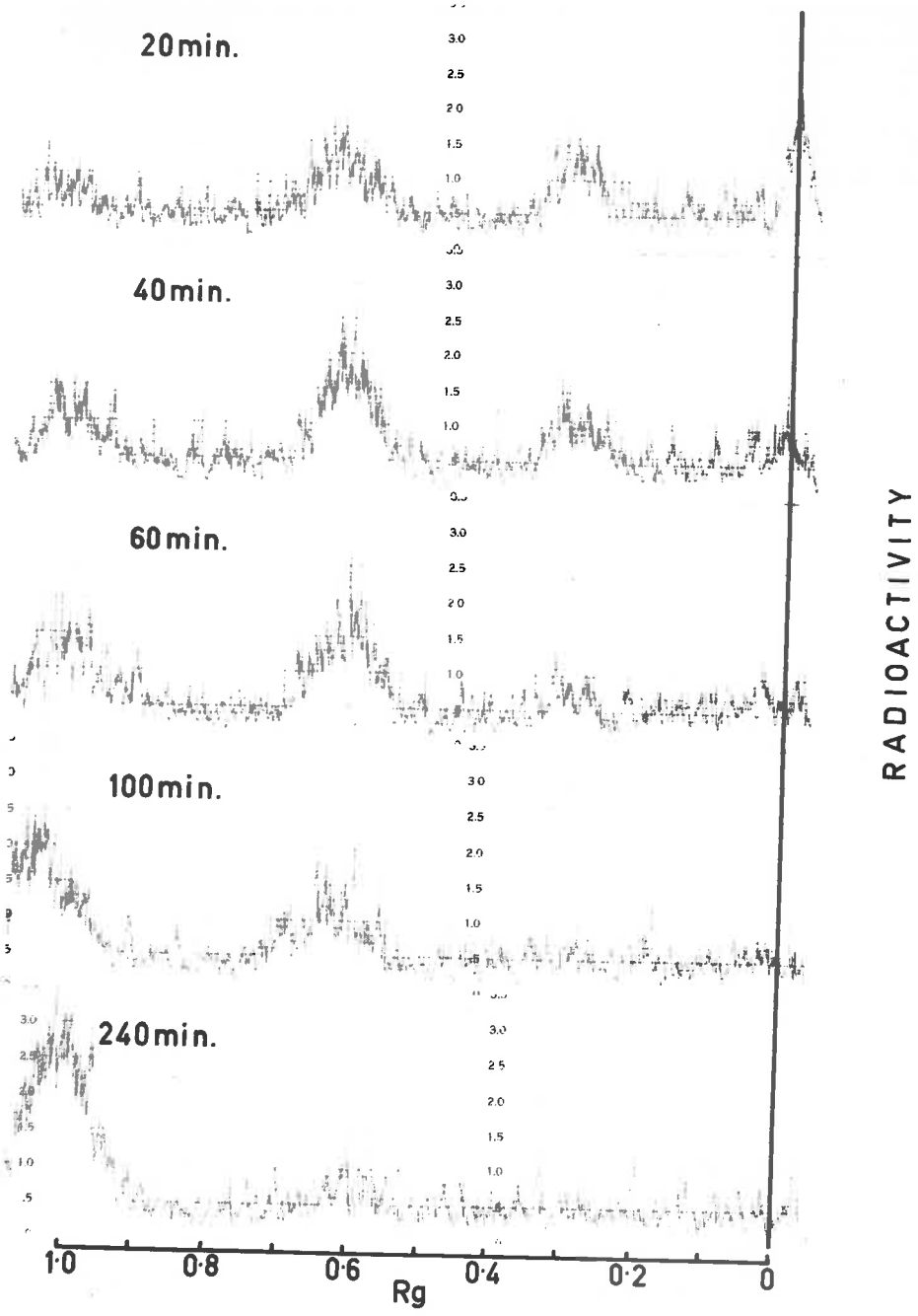
A solution containing 0.15 mg. of the endo-glucanase in 0.025 ml. of water was added to 1.5 mg. of polysaccharide 2 dissolved in 0.425 ml. of 0.05 M citric acid-sodium citrate buffer pH 4.9. The reaction was allowed to proceed at 40°. Samples of 0.05 ml. were withdrawn at 20 minute intervals and the reaction was stopped by heating the samples at 100° for 1 minute. The heated samples were examined by chromatography on Whatman number 1 paper with the solvent mixture propan-1-ol-water-ethyl acetate (7:2:1 by volume) (Albon and Gross 1952). The development was for 40 hours so that the solvent front went off the end of the

Figure 6

Treatment of polysaccharide 2 with an endo-type beta-D-1,3-glucanase.

A solution of 1.45 mg. of polysaccharide 2 in 0.45 ml. of 0.05 M citric acid-sodium citrate buffer pH 4.9 was maintained at 40°. A solution of 0.15 mg. of the endo-type glucanase in 0.025 ml. of buffer was added to the polysaccharide solution. Samples of 0.05 ml. were removed at intervals and boiled. The boiled samples were analysed by paper chromatography with the solvent propan-1-ol-water-ethyl acetate (7:2:1 by volume). Radioactive components separated on the chromatogram were detected by an Actigraph 2 paper-strip scanner (Nuclear Chicago Corporation). The chart recordings are shown in the figure. Reducing sugars were detected on the chromatograms with the oxanilic acid reagent. Radioactive components with Rg values of 1.00, 0.62 and 0.25 were identified as glucose, disaccharide and trisaccharide respectively. Rg is the ratio of the distance the component moves to the distance glucose moves.

FIGURE 6.


20min.
40min.
60min.
100min.
240min.
3.0
2.5
2.0
1.5
1.0
1·0
0·8
0·6
0·4
0·2
0
Rg
RADIOACTIVITY

paper. After the development was completed the dry chromatograms were cut into strips down the line of development. Radioactive areas were detected on these strips by passing them through an Actigraph 2 radioactive paper-strip scanner. The amounts of oligosaccharides formed were estimated from the areas under the peaks of radioactivity recorded. Under these conditions a reaction time of 45 minutes was found to produce an optimum amount of disaccharide (Figure 6).

In order to obtain sufficient disaccharide for analysis the quantities of reactants in the reaction mixture were increased so that 20 mg. of polysaccharide 2 might be treated with the endo-glucanase under exactly the same conditions as described before. The reaction was allowed to proceed 45 minutes and the reaction mixture was boiled. Ions were removed from the boiled reaction mixture by allowing it to pass first through a column of the cation-exchange resin Dowex 50 in the acid form (100-200 mesh) and then through a column of the anion-exchange resin Amberlite CG400 in the formate form (100-200 mesh). The effluent solution was freeze dried.

The oligosaccharides in the dry material were separated by chromatography on a 1 x 10 cm. column made from a mixture of celite 535 and activated charcoal powder (1:1 by weight) (Whistler and Durso 1950). Celite 535 (Johns-Manville) was treated with 8N-hydrochloric acid at 80° for

10 minutes and then left to stand overnight at room temperature. Next day the celite was washed free of chlorides with deionised water. Water was removed from the celite by washing it with ethanol and the ethanol was removed by washing with diethyl ether. The powder was air dried to remove ether and then it was dried at 100° for 6 hours. The activated charcoal powder (British Drug Houses Ltd.) was acid washed. The suspension of celite and charcoal in water was supported by a porosity 3 sintered glass disc at the bottom of the column. The upper surface of the celite-charcoal mixture was protected by a filter paper disc.

The freeze-dried oligosaccharide mixture was dissolved in 3 ml. of water and this solution was added to the top of the column. Initially the column was eluted with 150 ml. of 0.01N-formic acid. Elution was continued using a gradient of increasing concentration of ethanol in 0.01N-formic acid (Taylor and Whelan 1962). The simple gradient was produced using three chambers of a gradient mixer (Peterson and Sober 1959). The first and second chambers of the gradient mixer contained 130 ml. each of 0.01N-formic acid while the third chamber contained 130 ml. of 30 per cent (v/v) ethanol in 0.01N-formic acid. The column effluent was collected in tubes that were held by a time-controlled fraction collector. Initially the flow of liquid from the column was 24 ml. per hour, but this rate gradually increased

to a maximum of 50 ml. per hour. The total hexose in 0.25 ml. samples of each fraction was determined by the method of Dische et al. (1949).

Fractions containing separated oligosaccharide peaks were pooled and the solutions were reduced to dryness in a rotary evaporator. The dry residues were dissolved in water and portions of these solutions were examined by paper chromatography with the propanol-water-ethyl acetate solvent mixture previously described. Further portions of these solutions were examined by electrophoresis on Whatman 3MM paper using an electrolyte of 0.05 M sodium tetraborate at 1200 volts for 7 hours (Frahn and Mills 1956). Borax on the dried paper suppressed the colour reaction between the sugars and the oxanilic acid reagent. However colour development with the aniline-phosphoric acid-butan-1-ol reagent of Frahn and Mills (1959) was not affected. The identity of the oligosaccharides from the column was established by comparison with the components of a partial acid hydrolysate of polysaccharide 2 and with authentic disaccharides.

Analysis of a disaccharide prepared from polysaccharide 2.

A weighed amount of the dry disaccharide dissolved in water was assayed for total hexose (Dische et al. 1949) and for reducing sugars (Mateles 1960).

In order to establish the structure of the disaccharide it was oxidised with lead tetraacetate by a method similar to that described by Perlin (1955). Perlin had shown that it was possible to distinguish disaccharides with different types of linkages by examining the products of lead tetraacetate oxidation.

A solution of 5 mg. of lead tetraacetate in 0.49 ml. of glacial acetic acid was added to a solution of 1 mg. of the disaccharide in 0.01 ml. of water. The reaction was allowed to proceed at 28° for 10 minutes when the reaction was stopped by the addition of 0.2 ml. of glacial acetic acid containing 2 mg. of oxalic acid. The precipitate of lead oxalate was packed down by centrifuging and 0.05 ml. samples of the supernatant liquid were assayed for pentose in the presence of glucose by the method of Dische, Ehrlich, Munoz and Von Sallman (1953). The rest of the supernatant liquid was passed through a 5 x 40 mm. column of the cation-exchange resin Dowex 50 in the acid form (100-200 mesh). The column effluent was reduced to dryness in a vacuum desiccator. The dry material was treated with 0.5 ml. of 1N-hydrochloric acid at 100° for 30 minutes. This solution was reduced to dryness in a vacuum desiccator over sodium hydroxide. The dry material was examined by paper chromatography with the solvent mixtures ethyl acetate-pyridine-water previously described and water saturated phenol. Radioautographs were made from the chromatograms.

Figure 7

Elution of polysaccharide-l-hexadecyl pyridinium-borate complexes from a cellulose column.

A 50 mg. sample of the polysaccharide in Fraction C, which had been partially purified by selective precipitation with hexadecyl trimethylammonium borate between pH 8.5 and 9.8, was precipitated on a cellulose column as a l-hexadecyl pyridinium borate complex. Excess hexadecyl pyridinium bromide was washed from the column and the polysaccharide complexes were eluted from the cellulose with a continuously decreasing pH gradient at 38°.

The continuous line shows the extinction of the column effluent at 260 millimicrons. The initial broad flat peak is due to hexadecyl pyridinium added to the column for the preliminary washing and precipitation of the polysaccharide complex. The small depression (arrowed) in this peak was caused by the addition of the polysaccharide solution. The two peaks recorded further on were the l-hexadecyl pyridinium borate complexes of the two polysaccharides which were separated by elution of the column with a decreasing pH gradient.

The broken line represents the pH of the column effluent recorded at 20°.

FIGURE 7.

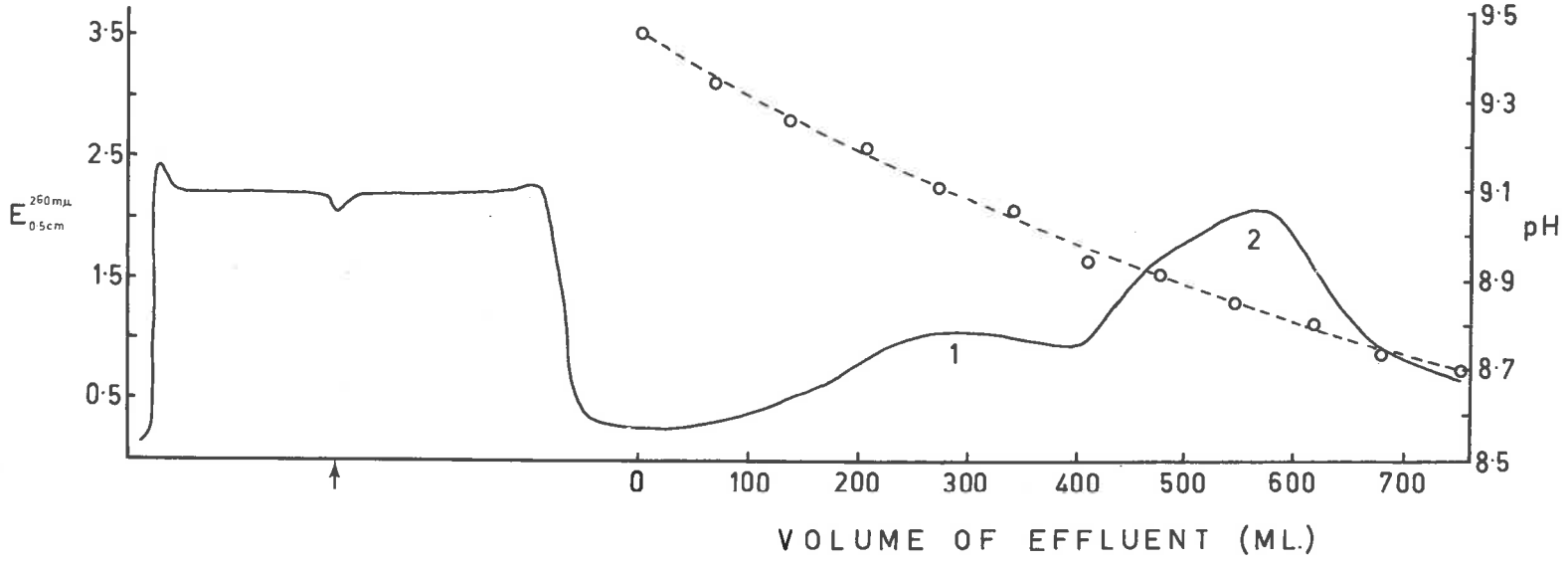
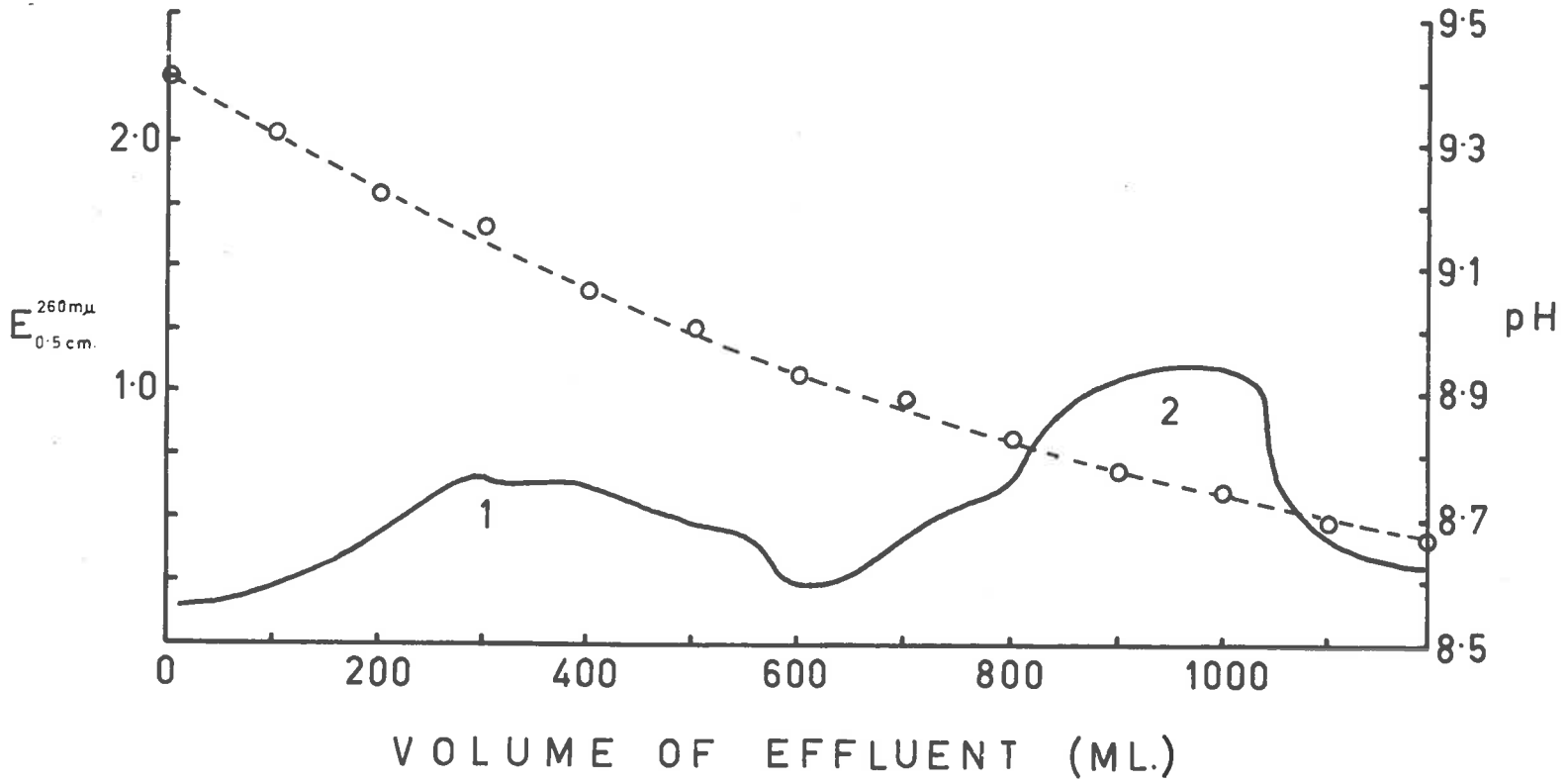

3·5
2·5
1·5
0·5
$E^{260m\mu}_{0.5cm}$
9·5
9·3
9·1
8·9
8·7
8·5
pH
1
2
0
100
200
300
400
500
600
700
VOLUME OF EFFLUENT (ML.)

Figure 8

Elution of the polysaccharide-l-hexadecyl pyridinium-borate complexes from a cellulose column.

The conditions were the same as those described in the legend of Figure 7 except that only 20 mg. of polysaccharide were added to the cellulose column and the pH gradient was produced with 1200 ml. of buffer instead of 750 ml. of buffer. The continuous line represents the recording of the extinction at 260 millimicrons of the column effluent. The broken line shows the pH of fractions of the column effluent. The pH was determined at 20°. The recording of the initial washing of the column, precipitation of the complex and removal of excess l-hexadecyl pyridinium was omitted from this figure.

FIGURE 8.


$E^{260m\mu}_{0\cdot5\,cm.}$
2·0
1·0
9·5
9·3
9·1
8·9
8·7
8·5
pH
1
2
0
200
400
600
800
1000
VOLUME OF EFFLUENT (ML.)

Results.

Column elution of the polysaccharide-borate-hexadecyl pyridinium complex.

Figure 7 shows a typical recording of the extinction of the column effluent at 260 millimicrons during the elution of a polysaccharide-borate-hexadecyl pyridinium complex from a cellulose column. When the partially purified polysaccharide of Fraction C was precipitated on the column and the column was eluted with a gradient of decreasing pH two peaks of 260 millimicron-absorbing material were detected in the column effluent. Since the two peaks were overlapping, the curves of each peak were interpolated to the base-line extinction and a series of fractions was selected under each peak so that contamination from the other peak was minimal. Under these conditions, 2.4 and 28 mg. of polysaccharide were recovered from the complexes which were in the fractions corresponding to peaks 1 and 2 respectively; 50 mg. of the partially purified polysaccharide were added to the column originally.

By reducing the quantity of partially purified polysaccharide to 20 mg. and extending the pH gradient over a volume of 1200 ml. instead of 750 ml., it was possible to separate completely the two peaks of 260 millimicron-absorbing material (Figure 8). The contamination of one peak by the other was eliminated. However, the necessity of starting with a smaller amount of the partially purified

polysaccharide and the increase in losses attendant with handling the larger volume of column effluent appeared to offset any of the advantages gained by these measures. During the column elution of several quantities of the partially purified polysaccharide-borate-hexadecyl pyridinium complex under the same conditions, the peak 1 material was consistently eluted at a room temperature pH of 9.4 and the peak 2 material at a pH of 9.1.

Polysaccharide 2 had an $[\alpha]_D^{25}$ of + 6.7° (c 0.215 in water). The polysaccharide isolated from Ochromonas by Archibald et al. (1958) had an $[\alpha]_D$ of + 10°. Polysaccharide 2 had an infrared spectrum identical with that of the cruder material (Chapter 4 and Figure 4). Unfortunately there was insufficient polysaccharide 1 to determine the specific rotation or the infrared spectrum.

Acid hydrolysis of polysaccharide 1. When an acid hydrolysate of polysaccharide 1 was examined by paper chromatography with the solvent ethyl acetate-pyridine-water, only a single component was detected on the chromatogram. This component was a reducing hexose with an Rg value of 1.00. Authentic mannose, mannitol and galactose had Rg values of 1.18, 0.91 and 0.86, respectively, on the same chromatogram.

The detection powers of the oxanilic acid reagent for reducing hexoses were checked using glucose and mannose.

Various amounts of solutions of these two sugars were spread over 7 square cm. areas on Whatman number 1 paper. The paper was dried and then treated with the oxanilic acid reagent as described in the methods section. Under ultra-violet light, one microgram of either sugar was readily detected by its yellow fluorescence. In the same manner it was found using mannitol that 2 micrograms was the smallest quantity which could be detected with the lead tetraacetate-rosaniline reagent. The quantity of acid hydrolysate spotted on the chromatogram was equivalent to 0.05 mg. of the original polysaccharide. Since only one component was detected on the chromatogram, any other reducing hexoses or non-reducing carbohydrates that might have been present would have been less than 2 or 4 per cent by weight, respectively, of the unhydrolysed polysaccharide. Therefore, within the limits of detection, polysaccharide 1 was composed solely of glucose.

When a chromatogram of a partial hydrolysate of polysaccharide 1 was sprayed with the oxanilic acid reagent to detect reducing sugars, a series of reducing oligo-saccharides were revealed. A straight-line plot was obtained when $\log \left(\frac{1-Rf}{Rf}\right)$ (the Rm value) was plotted against the predicted degree of polymerisation. Therefore these oligosaccharides were probably all members of a homologous series (French and Wild 1953).

A component with an Rg of 0.46 had been detected in a partial hydrolysate of the Fraction C material prepared from Ochromonas cells (Chapter 4). It was suggested that this component was not a member of the homologous series of oligosaccharides that were also detected in this partial hydrolysate. This component could not be detected in the partial hydrolysate of polysaccharide 1.

Acid hydrolysis of polysaccharide 2. A chromatogram of an acid hydrolysate of polysaccharide 2 was examined and found to have only one component and this component was a reducing hexose with an Rg value of 1.00. Since the amount of hydrolysate spotted on the chromatogram was equivalent to 0.055 mg. of unhydrolysed polysaccharide then, within the same limits of detection established for polysaccharide 1, polysaccharide 2 appeared to be composed solely of glucose residues.

A partial hydrolysate of polysaccharide 2 yielded a series of oligosaccharides. When these oligosaccharides were separated by paper chromatography they were found to have Rg values which were identical to the Rg values of the oligosaccharides obtained from polysaccharde 1. The Rg 0.46 component, which was present in the partial hydrolysate of the Fraction C material, could not be detected in the partial hydrolysate of polysaccharide 2. Since this Rg 0.46 component could not be detected in the partial

hydrolysates of both polysaccharides 1 and 2, it was apparent that the Rg 0.46 component was an impurity which was separated from the two glucose polymers by column elution of the polysaccharide-borate-hexadecyl pyridinium complexes.

Paper electrophoresis of the acid hydrolysate of polysaccharide 2 revealed a single component with an Mr value of 0.22. Arsenite buffer pH 9.6 was used as the electrolyte and mannose, mannitol, galactose, glucose and glycerol had Mr values of 0.39, 1.15, 0.35, 0.21 and 0.32 respectively. Thus glucose was the only component that could be detected in an acid hydrolysate of polysaccharide 2.

Analysis of a neutralised acid hydrolysate of polysaccharide 2 yielded the following values with standard deviations from samples of equal volume containing the equivalent of 55.6 micrograms of the original polysaccharide:

Total carbohydrate	52.2 ± 1.7 micrograms	(4	determinations)
Total hexose	59.0 ± 0.8 "	"	"
Reducing value (glucose standard)	58.4 ± 1.9 "	"	"

On the basis of the total hexose present, 96 per cent of the polysaccharide was accounted for as glucose since 55.6 micrograms of polysaccharide theoretically would yield after hydrolysis 61.7 micrograms of glucose.

The amount of hexose was well equated by the amount of

reducing sugar, measured as glucose. If other hexoses, in addition to glucose, were present in polysaccharide 2, then it would be expected that these two values would have been different, since different hexoses give different colour yields in the hexose determination and different hexoses differ in their reducing powers. It was difficult to explain why the total carbohydrate value was less than the other two values.

Enzymic hydrolysis of polysaccharide 1. Polysaccharide 1 was treated with an exohydrolytic beta-D-1,3-glucanase. When a chromatogram of the products of this reaction was examined, three components could be detected. Two of these components, with Rg values of 1.00 and 0.11, produced the yellow fluorescence characteristic of reducing hexoses when the chromatogram was treated with oxanilic acid and examined under an ultraviolet light. The third component could only be detected with the lead tetraacetate-rosaniline reagent. This third component, which had an Rg of 0.01 was shown to be the citrate that was used to buffer the reaction mixture. When the Rg 0.01 material was examined by paper chromatography with the solvent mixture butan-1-ol-acetic acid-water (110:30:75 by volume), it had an Rf value of 0.41. Citric acid and glucose had Rf values of 0.42 and 0.33, respectively, on the same chromatogram. The Rg 0.01 material was acid to bromcresol green.

As judged by the intensity of colour generated with the lead tetraacetate-rosaniline reagent, it was estimated that there was approximately 2 parts of glucose to one part of the Rg 0.11 material in the reaction mixture.

Enzymic hydrolysis of polysaccharide 2. When the products of the reaction between polysaccharide 2 and the exohydrolytic beta-D-1,3-glucanase were examined by paper chromatography, they were found to be the same as the products from polysaccharide 1. However, the relative quantities of the products from polysaccharide 2 were different from the relative amounts obtained from polysaccharide 1. For polysaccharide 2, the intensity of the colour generated by glucose with the lead tetraacetate-roaniline reagent was several times the intensity of the colour produced by the Rg 0.11 material.

Since polysaccharide 2 was available in larger quantities than polysaccharide 1, it was possible to isolate the Rg 0.11 material produced by the action of the exo-glucanase on polysaccharide 2. The Rg 0.11 material was treated with two enzymes, beta-glucosidase and an endohydrolytic beta-D-1,3-glucanase. When the products of the reaction with the beta-glucosidase were examined by paper chromatography, only a trace of glucose and a non-reducing component with a Rg of 0.56 could be detected on the chromatogram in addition to the Rg 0.11 material.

The beta-glucosidase completely hydrolysed polysaccharide 2 and cellobiose to produce only glucose, while a small amount of glucose was liberated from maltose. The endo-glucanase was equally without effect on the Rg 0.11 material; yet under the same conditions this enzyme hydrolysed polysaccharide 2 to glucose and a small quantity of a disaccharide.

Partial enzymic hydrolysis of polysaccharide 2. When polysaccharide 2 was treated with an endohydrolytic beta-D-1,3-glucanase a series of oligosaccharides and glucose were produced. These products were separated by paper chromatogrpahy with the solvent mixture propanol-water-ethyl acetate (Figure 6). However, the endoglucanase preparation contained a small amount of an oligosaccharidase, so that the di- and tri-saccharides, which were the products of the endo-glucanase activity (Reese and Mandels 1959), were being slowly degraded to glucose. Since the purpose of the experiment was to produce sufficient disaccharide for a structural analysis it was necessary to choose a time of reaction between the endoglucanase and polysaccharide 2 when the maximum amount of disaccharide was present.

The oligosaccharides produced by the action of the endoglucanase on polysaccharide 2 were separated by column chromatography. The components of the oligosaccharide

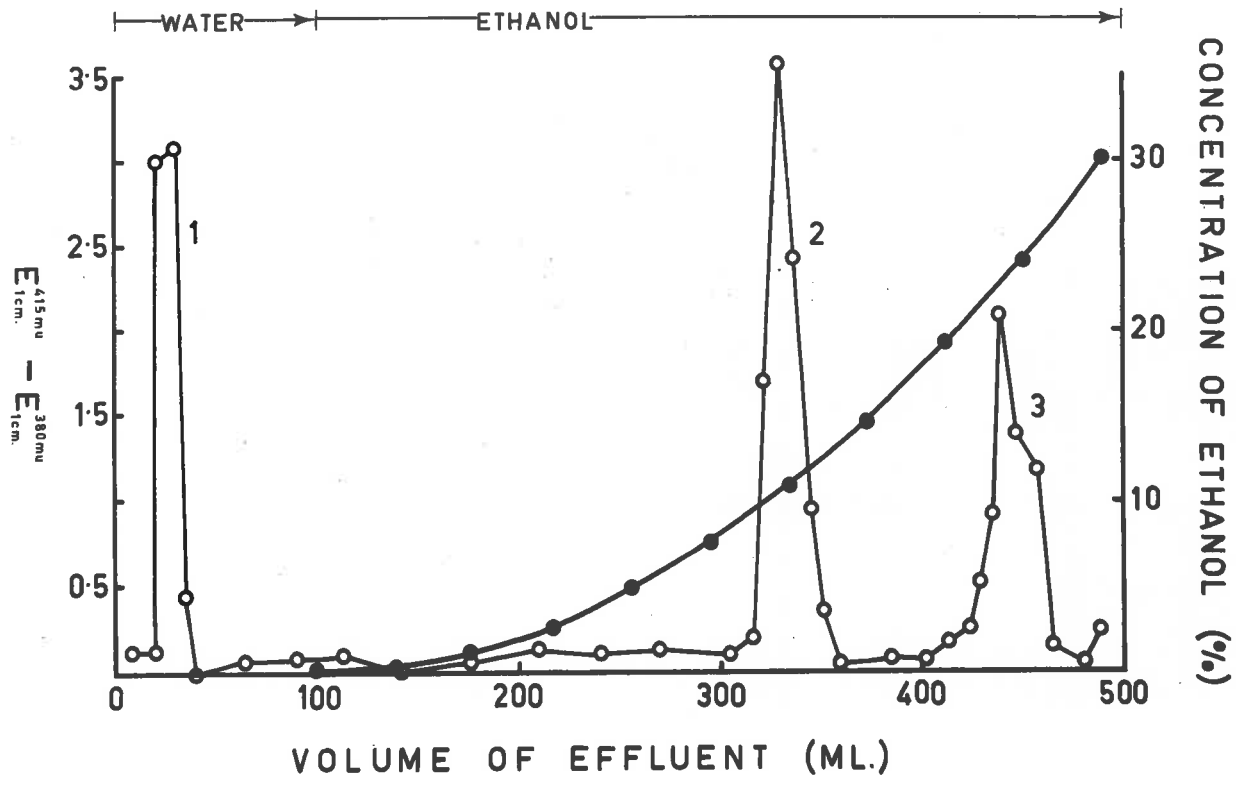
Figure 2

Column chromatography of an oligosaccharide mixture prepared from polysaccharide 2.

The oligosaccharide mixture was obtained by treating polysaccharide 2 with an endo-type beta-1,3-glucanase. The constituents of the mixture were separated by chromatography on a 1.1x10 cm. column of activated charcoal powder and celite 535 (1:1 by weight). Glucose (1) was eluted from the column with 10% N-formic acid. Subsequently the disaccharide (2) and trisaccharide (3) were eluted with a continuous gradient of increasing concentration of ethanol in 10% N-formic acid.

- ○ Total hexose determined by the method of Dische et al. (1948).
- ● Concentration (%) of ethanol in the eluant determined by calculation using the formula of Peterson and Sober (1959).

FIGURE 9.


WATER
ETHANOL
3·5
2·5
1·5
0·5
1
2
3
0
100
200
300
400
500
30
20
10
$E^{415mu}_{1cm.} - E^{380mu}_{1cm.}$
CONCENTRATION OF ETHANOL (%)
VOLUME OF EFFLUENT (ML.)

mixture that were eluted from the celite-charcoal column are shown in Figure 9. From the 20 mg. of polysaccharide 2 that were treated with the endo-glucanase, a total of 13.25 mg. were recovered in the three peaks eluted from the column; 1.9 mg. in peak 1, 7.2 mg. in peak 2 and 4.15 mg. in peak 3.

When the hexose-containing materials in peaks 1, 2 and 3 were examined by paper chromatography with the solvent propanol-water-ethyl acetate, one component was detected for each peak and these had Rg values of 1.00, 0.58 and 0.30 respectively. Glucose and the two oligosaccharides with the greatest mobility in a partial acid hydrolysate of polysaccharide 2 had Rg values of 1.00, 0.56 and 0.28 on the same chromatogram. When the materials in peaks 1, 2 and 3 were examined by paper electrophoresis with borate as electrolyte, one reducing-component was detected for each peak and these had Mg values (mobility relative to the mobility of glucose) of 1.00, 0.70 and 0.64 respectively. When the electrophoresis papers were passed through the Actigraph 2 radioactive paper-strip scanner only a single band of radioactivity was detected for each component and this band had an Mg value identical to the Mg value of the reducing component in the material from each peak. The Mg values of the three components with the greatest mobility in a partial acid hydrolysate of polysaccharide 2 were 1.00, 0.71 and 0.64 on the same electrophoresis paper.

Cellobiose and maltose had M_G values of 0.37 and 0.42, respectively, also on the same electrophoresis paper.

Therefore the first three peaks of hexose-containing material separated on the column were respectively : glucose and the di- and tri-saccharides produced by acid hydrolysis of polysaccharide 2.

Analysis of a disaccharide. The disaccharide, which had been eluted from the celite-charcoal column, was analysed for total hexose and reducing value. An amount of 1.00 mg. of this disaccharide gave the following values with standard deviations:

Total hexose	1.074 ± 0.008	(4 determinations)
Reducing value (glucose standard)	0.772 ± 0.012	(")

In theory 1 gm. of disaccharide would be equivalent to 1.052 gm. of glucose when the disaccharide was assayed for total hexose, since 1 mole of disaccharide equal to 342.3 gm. would yield after hydrolysis 2 moles of glucose equal to 360 gm. Thus the experimental value for the total hexose in the disaccharide was slightly higher than the theoretical value, but this difference was probably within the limits of experimental error.

It might be expected that a reducing disaccharide composed of the one sugar would have half the reducing

power of the two monosaccharide residues. The disaccharide obtained from polysaccharide 2 had more than half the reducing power of the equivalent weight of glucose. This observation probably was explained by the fact that the lowest member of a polymeric series of sugars, with the properties characteristic of that series, was the disaccharide and not the monosaccharide (Whelan, Bailey and Roberts 1953). The molar ratio of the reducing power of maltose to glucose was 1:0.927, while the ratio for maltose to maltotriose and higher homologues was 1:1. The reducing power of sugars was not stoichiometric with respect to the aldehydic function, but it was influenced by the spatial arrangement of hydroxyl groups around the aldehydic group.

Perlin (1955) reported that when reducing hexose disaccharides were oxidized with lead tetraacetate, the amounts of formic acid and formaldehyde produced and the type of oxidised disaccharide were characteristic of the link between the two hexose residues. Thus 1:2, 1:3, 1:4 and 1:6-linked hexose disaccharides yielded three carbon dialdehyde, pentose, tetrose and unstable three carbon monoaldehyde derivatives, respectively, when these disaccharides were oxidized with lead tetraacetate. When the disaccharide eluted from the celite-charcoal column was oxidized with lead tetraacetate and the reaction mixture was assayed for pentose with the orcinol reagent, 1.14 moles

of pentose were found per mole of disaccharide oxidized. This value was higher than the theoretical yield of 1.052 moles of pentose that might have been expected. However the products of lead tetraacetate oxidation of hexose disaccharides are thought to be formyl esters (Perlin 1955) and it was not known if the formyl ester of a pentose would give the same colour yield as a free pentose in the orcinol reaction. The product of lead tetraacetate oxidation of the disaccharide was hydrolysed and the hydrolysate was examined by paper chromatography with the solvent ethyl acetate-pyridine-water. Three radioactive areas were detected on the chromatogram and these had Rg values of 1.36, 1.00 and 0.69. The proportions of the total radioactivity in these three components were 0.73: 1.00: 0.35, respectively. Authentic arabinose had an Rg of 1.36 on the same chromatogram. The identities of the Rg 1.36 and 1.00 components were established by co-chromatography and radioautography with authentic arabinose and glucose employing water saturated phenol as solvent. The shape of the spots detected on the chromatogram with the oxanilic acid reagent was identical with the shape of the areas of radioactivity detected by the radioautograph. Furthermore, the Rg 1.36 component showed the red-brown fluorescence characteristic of aldopentoses, and the Rg 1.00 component showed a yellow fluorescence, characteristic of aldohexoses, when the chromatogram was

treated with oxanilic acid and viewed under ultraviolet light. The third radioactive component with an Rg of 0.69 could not be detected with oxamilic acid. This component may have been a residue of the formyl ester of the hexo-pentose disaccharide, which was produced by the lead tetraacetate oxidation and was not hydrolysed to pentose and hexose (Perlin 1955).

Perlin (1955) showed that lead tetraacetate cleaved the reducing hexose of a 1,3-linked disaccharide at the 1,2-hemiacetal glycol to produce a pentose derivative. When the reducing hexose was glucose the product of oxidation was a derivative of arabinose. Therefore these results show that the disaccharide member of the homologous series of oligosaccharides obtained from polysaccharide 2, was a beta-1,3-linked glucose disaccharide.

Discussion.

Acidic polysaccharides have been precipitated quantitatively from aqueous solution by the addition of paraffin chain quaternary ammonium salts (Terayama 1955, Scott 1955). Neutral polysaccharides were not precipitated by the quaternary salts in neutral solution. When neutral polysaccharides were converted to polyanions, by forming a borate complex, precipitation could be brought about (Barker, Stacey and Zweifel 1957, Palmstierna, Scott and Gardell 1957). Selective precipitation of neutral polysaccharides in the

presence of borate was achieved by controlling the pH of the reaction (Barker et al. 1957). However, the complete separation of a mixture of neutral polysaccharides by this technique was only possible where the constituent polysaccharides could be precipitated at pH values differing by more than 1.0 to 1.5 units.

Acidic polysaccharide mixtures have been separated by precipitating the hexadecyl pyridinium complexes on a cellulose column and then extracting the complexes from the column with a salt gradient (Antonopoulos et al. 1961). Keratosulphate has a low charge density and it did not form an insoluble complex with quaternary ammonium salts. Scott (1960) suggested that keratosulphate might be precipitated as a borate complex on a cellulose column and then the column could be extracted with a salt gradient to effect purification of this compound.

In the present investigation a neutral polysaccharide mixture was precipitated on a cellulose column with hexadecyl pyridinium bromide in the presence of borate and the column was extracted with a gradient of gradually decreasing pH. The use of a quaternary nitrogen compound with a characteristic and strong absorption maximum in the ultraviolet region greatly facilitated the detection of separated components in the column effluent. If 1-hexadecyl pyridinium chloride (critical solution temperature 22°) had been available and it was used instead of the bromide, then many of

the difficulties associated with keeping the contents of the elution apparatus above 35° would have been avoided.

The technique reported here may be of some use in effecting separation of closely related water-soluble neutral polysaccharides. The two polysaccharides that were separated during the present investigation both appeared to be essentially beta-1,3-linked glucosides. During an examination of the properties of these two polysaccharides the only difference noted was in the extent of hydrolysis catalysed by an exohydrolytic beta-D-1,3-glucanase. The two polysaccharides were biologically different; polysaccharides 1 and 2 which had been prepared from cells that had been made radioactive by Method 1, had specific radioactivities of 10.2 and 13.2 millimicrocuries per mg. respectively.

Archibald et al. (1958) isolated a polysaccharide material from Ochromonas malhamensis and purified it by fractional precipitation with hexadecyl trimethylammonium bromide in the presence of borate and fractional precipitation with acetone. However, it was not evident from their report whether they were aware of more than one component in their final polysaccharide preparation. If their preparation still contained the two closely related polysaccharides reported in this work, then their figure for the degree of polymerisation, which was determined by periodate oxidation, may have to be modified.

The polysaccharide isolated by Archibald et al. (1958)

contained approximately 10 per cent of a sugar which was tentatively identified as mannose. The two polysaccharides obtained during the present investigation appeared to have less than 2 per cent of any reducing sugar other than glucose. Goldstein Smith and Unrau (1959) found that seaweed laminarin was heterogeneous; some of the beta-1, 3-linked glucoside chains were terminated by mannitol residues. Annan, Hirst and Manners (1962) estimated that the mannitol content was 2 per cent and they concluded that 50 per cent of the polymer chains were terminated by mannitol and the rest of the chains ended with a reducing glucose residue. Such a small content of mannitol would not have been detected by the methods employed to examine the polysaccharides obtained during the present investigation.

Archibald et al. (1958) reported that the polysaccharide isolated form Ochromonas was slowly degraded by an exohydrolytic beta-D-1,3-glucanase; in one experiment 9 per cent of the polysaccharide was converted to glucose. Although no measurements were made, it appeared from the chromatograms of the reaction products that more than 9 per cent of the two polysaccharides isolated during this work were converted to glucose by the exo-glucanase.

An interesting observation was the inability of beta-glucosidase and an endohydrolytic beta-D-1,3-glucanase to degrade the residue left from the action of the exo-glucanase on polysaccharide 2. The beta-glucosidase and the endo-glucanase

readily degraded the complete polysaccharide 2 to glucose and disaccharide respectively, without producing any resistant residue. Nelson, Scaletti, Smith and Kirkwood (1962) reported that the exohydrolytic beta-D-1,3-glucanase specifically cleaved the beta-glycosidic bond of the penultimate 3-substituted pyranosyl residue starting at the non-reducing end of the laminarin polymer; thus releasing one glucose unit at a time. The enzyme could pass single residue branches linked beta-1,6- to the beta-1,3-linked glucose chain (Johnson, Kirkwood, Misaki, Nelson, Scaletti and Smith 1963), but otherwise appeared to be restricted in its specificity. Emulsin beta-glucosidase was found to have rather a broad specificity; it could attack almost all linkages to a beta-D-glucopyranosyl residue and split off glucose residues from the non-reducing end of the polymer chain (Gottschalk 1950). However, shielding of beta-links had been noted in one or two instances so that the inability of beta-glucosidase to degrade a polysaccharide did not mean the polysaccharide was devoid of beta-linked residues. (Bailey and Pridham 1962). Without further information about the composition of the resistant residue it was not possible to resolve the apparent inconsistency of the action of beta-glucosidase and the endo-glucanase on this residue and the complete polysaccharide 2.

In summary, glucose was shown to be the sole product of complete acid hydrolysis of polysaccharide 2. Partial

enzymic or acid hydrolysis of polysaccharide 2 produced the same homologous series of oligosaccharides. The glucose residues of the disaccharide of this series were shown to be joined by a beta-1,3 glycosidic bond. It was concluded that polysaccharide 2 which was a substrate for the endogenous respiration of Ochromonas malhamensis, was essentially a beta-1,3 glucoside and this polymer was similar to the polysaccharide isolated from the same organism by Archibald et al. (1958).

ADDENDUM to CHAPTER 6

During the preparation of the typescript of this thesis a report came to notice concerning the structure of the reserve polysaccharide of Ochromonas malhamensis (Archibald, Cunningham, Manners, Stark and Ryley 1963). The authors of this report obtained a crude polysaccharide from a water extract of the organism by precipitation with ethanol and treatment with hexadecyl trimethylammonium bromide. The crude preparation was fractionally precipitated with acetone and a polysaccharide was obtained which was found to be composed of beta-1,3-linked D-glucose residues. From the amount of formaldehyde produced during periodate oxidation of this polysaccharide the degree of polymerisation was estimated to be approximately 34. A small quantity of formic acid was also produced during periodate oxidation indicating that there were either three non-reducing end groups or two internal 1,6-glucosidic linkages per molecule. Additional evidence suggested that the formic acid production was due to the presence of non-reducing end groups implying that there were two branch points per molecule.

Branching of the Ochromonas reserve polysaccharide could explain why it was not completely hydrolysed to glucose by an exo-hydrolytic beta-D-1,3-glucanase (Chapter 6). This enzyme was shown to specifically catalyse the hydrolysis of

the glucosidic bond of a 3-substituted pyranosyl residue located one residue removed from the non-reducing end of a chain (Nelson, Scaletti, Smith and Kirkwood 1963a ; this report also appeared during the typing of this thesis and was a full account of a study previously published in abstract form Nelson, Scaletti, Smith and Kirkwood 1963b). Therefore hydrolysis of a polysaccharide chain would cease when a branch point was reached. When all the polysaccharide chains had been hydrolysed as far as the branch point a limit polysaccharide would be produced which was resistant to any further activity of the enzyme. These additional facts explain the production of a resistant residue from the Ochromonas reserve polysaccharide. However there was still no explanation why this resistant residue was also resistant to the action of a beta-glucosidase and an endo-hydrolytic beta-D-1,3-glucanase while these two enzymes readily degraded the complete polysaccharide to monosaccharide and disaccharide products.

ARCHIBALD, A.R., CUNNINGHAM, W.L., MANNERS, D.J., STARK, J.R. and RYLEY, J.F. (1963). Biochem. J. 88, 444.

NELSON, T.E., SCALETTI, J.V., SMITH, F. and KIRKWOOD, S. (1963a). Canad. J. Chem. 41, 1617.

NELSON, T.E., SCALETTI, J.V., SMITH, F. and KIRKWOOD, S. (1963b). Fed. Proc. 22, 297.

Chapter 7

DISCUSSION.

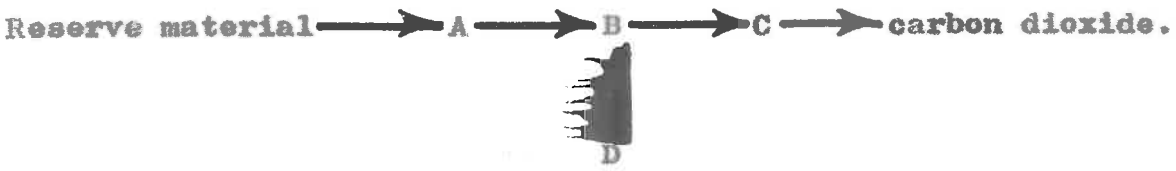
Isotopic techniques for assessing endogenous respiration during the respiration of exogenous substrates were mentioned in Chapter 1. It was suggested that these same techniques could be used to recognize endogenous reserve materials among the constituents of radioactive cells during endogenous respiration. Cells in which all of the constituents had the same specific radioactivity were thought to be a prerequisite for this type of experiment. Ideally the organism should be grown from a small inoculum in a medium supplying a uniformly (^{14}C) labelled substrate as the sole source of carbon in order to obtain uniformly radioactive cells. The constituents of cells which had been grown under these conditions would all have the same specific radioactivity (radioactivity per atom of carbon).

However, it is not always possible to grow cells under such conditions; for example some organisms require more than one carbon substrate for growth. Ochromonas has an absolute requirement for either a sugar or glycerol and a member of the tricarboxylic acid cycle (Hutner, Provasoli and Filfus 1953). Furthermore, growth is far from optimal unless amino-acids such as histidine, methionine, glutamic

acid and arginine are also included in the medium. Also it has not been established whether carbon dioxide may be a source of carbon for the dark-grown organism. Thus it was not practicable to grow Ochromonas with a uniformly labelled carbon source. As a compromise the organism was grown from a small inoculum in a medium containing ($^{14}C_6$) glucose where the glucose was a major source of carbon.

Where it is not possible to grow the cells in a medium in which all the carbon substrates are generally labelled and of the same specific radioactivity, then the constituents of these cells may have quite different specific radioactivities. It may happen that the endogenous reserve material of such cells is not, or is only slightly labelled so that it will not be detected by the type of experiment envisaged above.

Assuming for the moment that all of the endogenous reserve materials of non-uniformly radioactive cells are extensively labelled, there are several possibilities which must be examined before any firm conclusions can be drawn from the results obtained with these cells. Since the cells are aerobic it is only necessary to consider the situation where the carbon of the endogenous reserve material is completely oxidized to carbon dioxide. This may be represented by the equation:



A, B and C represent intermediates in the pathway of breakdown of the reserve material to carbon dioxide. D represents a compound in equilibrium with the intermediate B. The reaction B $\rightleftharpoons$ D is a side reaction not in the main pathway of breakdown of the reserve material. Since the reserve material is being completely oxidized to carbon dioxide, there is no net transfer of carbon between B and D, although there may be a considerable exchange of carbon between these two compounds. Only the concentrations of the initial substrate and the ultimate product of this pathway will change.

If all the constituents of the cells have the same specific radioactivity, that is the cells are uniformly radioactive, then only the total radioactivity of the reserve material and the carbon dioxide will alter during endogenous respiration in the absence of exogenous substrates. The endogenous reserve material is easily recognized under these conditions.

If compound D has a lower specific radioactivity than the intermediates of the direct pathway, that is the cells are not uniformly radio-active, then, as a result of the exchange reaction between B and D, the total radio-

activity of D will increase until D has the same specific radioactivity as B. From measurements of the total radioactivity of the cell constituents it will appear that the reserve material was catabolised to two end products, carbon dioxide and D. This was probably the situation that was observed during endogenous respiration of Ochromonas cells which had been isotopically labelled by oxidative assimilation of ($^{14}C_6$) glucose (Method 1, Chapter 3). When these cells were respiring endogenously, the radioactivity disappearing from the polysaccharide (Fraction C) appeared in Fraction A and the carbon dioxide liberated by the cells (Chapter 5). A transfer of radioactivity into Fraction A was not observed during the endogenous respiration of cells which had been made radioactive by growth on ($^{14}C_6$) glucose (Chapter 3, Method 2). Apparently the material in Fraction A of the Method 1 cells was not in isotopic equilibrium with the intermediates in the pathway of oxidation of the polysaccharide.

Alternatively, the specific radioactivity of compound D may be greater than the specific radioactivity of the true reserve material. Because of the equilibrium between B and D, compound D will lose radioactivity until it has the same specific radioactivity as B. In this case it will appear that there are two endogenous reserve materials being oxidized to carbon dioxide.

An organism may possess two endogenous reserve materials

which are utilised simultaneously e.g. Sarcina lutea (Dawes and Holms 1958). Using non-uniformly radioactive cells it will not be possible to decide from the measurement of the total radioactivities of the cell constituents during endogenous respiration whether there are two genuine reserve materials or whether there is a situation similar to that mentioned in the previous paragraph. These possibilities may be distinguished by determining the specific radioactivities (radioactivity per unit weight is sufficient for this purpose) as well as the total radioactivities of the cell constituents and of the carbon dioxide released. The specific radioactivity of a genuine reserve material will remain constant during endogenous respiration, while the specific radioactivity of a compound like D will change until it acquires the same specific radioactivity as the compound with which it is in equilibrium. At the same time the specific radioactivity of the carbon dioxide will alter; if the specific radioactivity of D increases then that of the carbon dioxide will decrease and vice versa.

The foregoing is a simple illustration of the situations which may occur using non-uniformly radioactive cells. In fact the situation may be quite complicated, e.g. there may be more than a single compound such as D and these compounds may be in equilibrium with the direct pathway of breakdown

at points between reactions yielding carbon dioxide. Therefore it is advisable to use cells which are uniformly radioactive or only slightly removed from this condition.

Although it was not possible to grow Ochromonas under conditions that would have ensured that the cells were uniformly radioactive, it was apparent (Chapter 5) that the constituents of the radioactive cells which could be obtained did have almost the same specific radioactivities (radioactivity per atom of carbon). Therefore the situations just discussed did not have to be considered when the cell constituents of Ochromonas were being examined for the substrate of endogenous respiration.

Using the isotopic techniques discussed previously it was established that a water-soluble polysaccharide was the substrate of endogenous respiration in Ochromonas (Chapter 5). Subsequently the polysaccharide was shown to consist of two separate beta-1,3-linked glucosides (Chapter 6). These two polysaccharides appeared to be biologically differentiated since they had different specific radioactivities when they were isolated from cells which had been made radioactive by oxidative assimilation of ($^{14}C_6$) glucose. It was not known whether the two were differentiated during the breakdown of the polysaccharide reserves in endogenous respiration. These two polysaccharides may have a precedent in the two glycogen pools which were shown to be the substrates of aerobic endogenous respiration

in yeast (Eaton 1960, 1961). During endogenous respiration of yeast one of these glycogen pools was rapidly metabolized while the other glycogen was only slowly metabolized. The rapidly metabolized glycogen was found to act also as a substrate for endogenous fermentation but the other glycogen was not a substrate under these conditions.

Reazin (1956) investigated the pathways of aerobic breakdown of glucose in Ochromonas. When the organism metabolized (1-^{14}C) glucose or (2-^{14}C) glucose in the presence of arsenite the isotope was found to be present in the methyl or carbonyl carbon atoms, respectively, of the pyruvate which accumulated under these conditions. Furthermore, when the cells oxidized (1-^{14}C) glucose and (6-^{14}C) glucose the ratio of the per cent of isotope in the carbon dioxide liberated by the cells from the two types of glucose was always approximately 1.0. The latter findings were confirmed by Kauss and Kandler (1962). Therefore it appeared that Ochromonas oxidized glucose exclusively by way of the Embden-Meyerhof pathway. Presumably the same pathway was used by Ochromonas for the breakdown of the reserve polysaccharide during endogenous respiration. There have been no reports concerning the mechanisms of terminal oxidation of the products of the Embden-Meyerhof pathway in Ochromonas.

When a polysaccharide was completely metabolized by an organism so that carbon dioxide was the sole final product

then this organism would be expected to have a respiratory quotient of 1.00. During the initial ten hours of endogenous respiration it was found with uniformly radioactive Ochromonas cells that the quantity of (^{14}C) carbon dioxide liberated was equal to the amount of (^{14}C) that had been lost from the intracellular polysaccharide (Chapter 5). Therefore it might have been expected that the R.Q. determined during these ten hours would be equal to 1.00.

Contrary to expectation, Reazin (1954) using the Warburg direct method found that during endogenous respiration Ochromonas had a R.Q. of 0.8. During the present investigation Ochromonas was found to have a R.Q. of 0.86 measured in an atmosphere of 1.5 per cent carbon dioxide in air. However the rate of endogenous respiration was found to be dependent on the presence of carbon dioxide in the atmosphere. In the absence of carbon dioxide Qo_2 was 31 while in the presence of 1.5 per cent carbon dioxide Qo_2 was 14. Therefore R.Q. determined by one or the other method might have been invalid, or both values were invalid, because the rate of respiration in the Warburg flask with potassium hydroxide or carbon dioxide-buffer in the centre well was not the same as the rate of respiration in the flask with air. Whichever value was correct both were less than the theoretical value of 1.0.

Carbon dioxide or the bicarbonate ion may be directly responsible for controlling the respiration rate, but it is

more likely that the effect is mediated by some compound formed from carbon dioxide. Ochromonas cells cannot take up carbon dioxide in a flask with potassium hydroxide in the centre well; any change in volume is due to the exchange of oxygen. In a Warburg flask with carbon dioxide-buffer in the centre well the cells can incorporate carbon dioxide, but the net change in volume is again solely due to exchange of oxygen since any carbon dioxide taken up or generated by the cells is replaced or absorbed by the carbon dioxide buffer. The net change of carbon dioxide that occurs in a flask without these centre well additions may not be equal to the volume of carbon dioxide generated by the cells if the cells are taking up carbon dioxide at the same time. Under these conditions where the measurements are made in a closed system any fixation of carbon dioxide by the cells will cause a reduction in the value of the R.Q.

As it was found that during endogenous respiration of Ochromonas R.Q. was less than 1.0 while it was expected that this value might be equal to 1.0, then the difference could be due to a fixation of carbon dioxide during endogenous respiration.

Chapter 8.

INCORPORATION OF CARBON DIOXIDE BY OCHROMONAS MALHAMENSIS WHICH HAD BEEN GROWN IN THE DARK.

It was shown in the previous chapters that a polysaccharide was the substrate for the endogenous respiration of Ochromonas which had been grown in the dark. Carbon dioxide was the sole product of the catabolism of this polysaccharide during endogenous respiration. It might be expected from these results that Ochromonas would have a R.Q. of 1.00, however the R.Q. was in fact less than 1.00. It was suggested that these apparently contradictory facts might be reconciled if the organism incorporated carbon dioxide during endogenous respiration.

Heterotrophic carbon dioxide fixation in the presence of exogenous organic carbon is well established for micro-organisms (reviewed by Wood and Stjernholm 1962). It is not so well recognized that heterotrophic carbon dioxide fixation may occur in the absence of exogenous carbon sources. An example of the latter type of fixation has been observed with Escherichia coli (Palmstierna 1956); this organism was found to incorporate (^{14}C) carbon dioxide into an alkali-stable polyglucose reserve material in the absence of any external carbon or

nitrogen sources.

The results presented in this chapter showed that a washed suspension of Ochromonas cells was able to incorporate carbon dioxide. The question remained, what role did carbon dioxide fixation play in endogenous respiration of Ochromonas? In an attempt to answer this question the pathways by which the organism might incorporate carbon dioxide were examined.

Materials and Methods.

Basically the same procedures as those devised by Calvin and his colleagues (Benson, Bassham, Calvin, Goodale, Haas and Stepka 1950) were employed to examine the pathways of carbon dioxide incorporation in Ochromonas. It was possible to discern the first stable products of carbon dioxide fixation by exposing the cells to (^{14}C)-bicarbonate for short intervals of time and then analysing extracts of these cells by two-dimensional chromatography and radioautography.

Incorporation of sodium (^{14}C) bicarbonate by intact cells.

A culture of Ochromonas was grown for three days in the dark and the cells were harvested and washed by centrifuging as described in Chapter 2. The packed cells were suspended in water. The cell suspension was diluted with water until a sample which was diluted one in ten with

water had an extinction of 1.0 at a wavelength of 580 millimicrons. Then 10.3 ml. of the cell suspension were transferred to a 40 ml. filtering crucible with a grade 3 sintered glass filter. The cell suspension was agitated and aerated by a stream of air blown up through the filter. The suspension was gassed in this manner for 30 minutes at room temperature (26°) to allow the cells to consume any exogenous organic carbon sources which were not removed by the washing procedure. Then 0.2 ml. of a solution containing 200 microcuries and 9.54 micromoles of sodium (^{14}C) bicarbonate was added rapidly to the cell suspension with a syringe. At short intervals of time 1.0 ml. samples of the cell suspension were removed quickly with a syringe and immediately squirted into centrifuge tubes containing 4 ml. of 95 per cent ethanol at 75°. When the ethanolic suspensions had cooled to room temperature the cell material was separated into fractions as described in Chapter 3 and these fractions were assayed for radioactivity by the methods also described in that chapter.

Paper chromatography of Fraction A and estimation of the radioactivity of the separated components.

Fraction A was dissolved in 0.025 ml. of water and the solution was applied as a spot approximately 7 m.m. in diameter to the corner of a sheet of Whatman number 1 paper by means of a capillary pipette. The chromatogram was

developed in two dimensions (see Chapter 2 for details). Water-saturated phenol was used as the solvent for development in the first direction (Consden, Gordon and Martin 1944). Phenol (British Drug Houses, Ltd., Analar grade) was freshly distilled and immediately saturated with deionised water. The solution was stored in an air-tight, brown glass bottle in the dark. When the solvent was prepared and stored in this manner it remained colourless for several months and did not produce darkened and uneven fronts which might have interfered with the development in the second direction. When the development with phenol was complete the paper was dried in a fume hood at room temperature for 24 hours. The paper was developed in the second direction at 90° to the direction of the first development with the solvent butan-1-ol-acetic acid-water (222: 57: 150 by volume). In both instances development was halted when the solvent front reached the end of the paper.

Since it was necessary to elute the individual radioactive areas from the chromatogram for the purpose of identification, the radioactivities of the compounds in these areas were assayed in the eluates by the liquid scintillation technique. By assaying the radioactivity of the components eluted from the chromatogram by the liquid scintillation technique, rather than assaying the

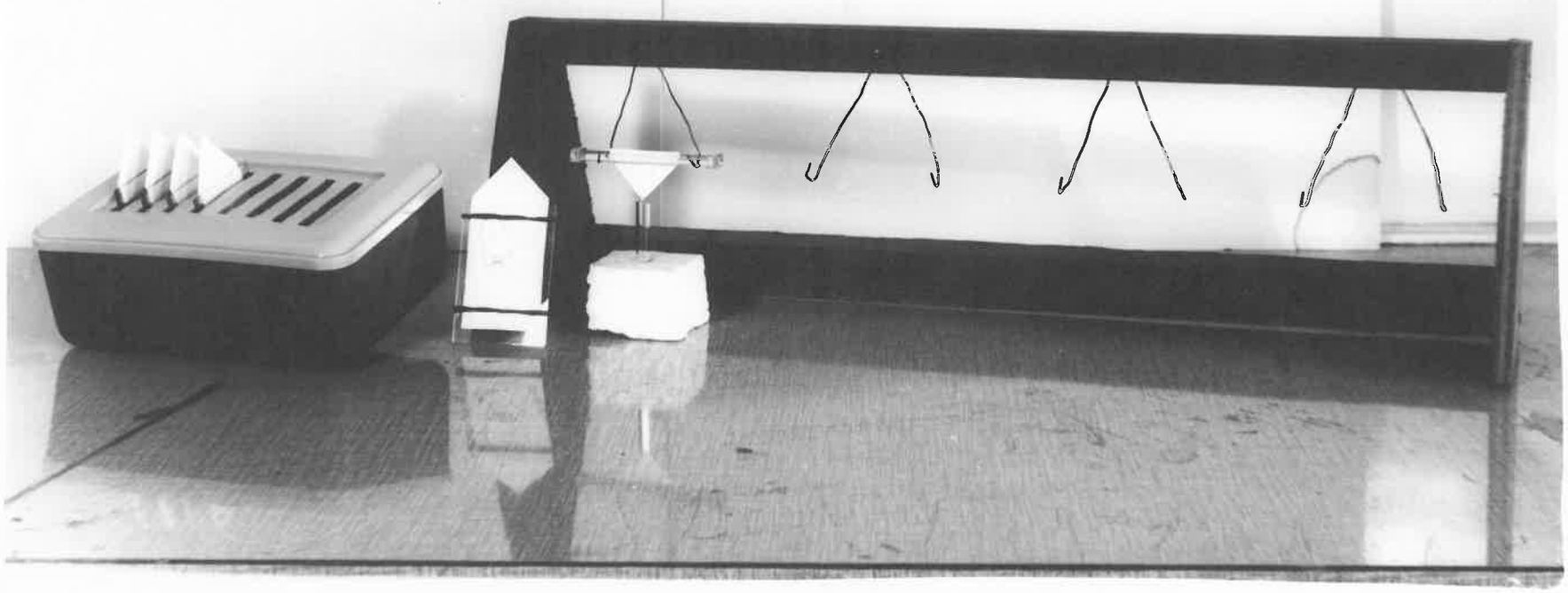
radioactivity with an end-window Geiger-Muller detector while the compounds were still on the chromatogram, advantage was taken of the greater efficiency (more than twenty-fold) of radioactive assay possible with the former method. The efficiency of the elution procedure was checked as described further on and the small losses incurred during this procedure were not sufficient to preclude the use of this technique.

Radioactive areas were located on the developed chromatogram by radioautography (see Chapter 2 for details). These areas were cut out of the chromatogram and the material was eluted from the papers using a technique adapted from that devised by Davis, Dubbs and Adams (1962). Sheets of Whatman number 1 paper were thoroughly washed in deionised water and dried. This paper was cut into rectangular strips measuring 4 x 10 cm. and the strips were cut to a point beginning 3 cm. from one end. These strips, together with the radioactive area cut out of the chromatogram, were mounted between two glass slides (5 x 8 cm.) so that the pointed end of the strip projected beyond the edge of the slides and the cut-out from the chromatogram was between the slides. The slides were held together by means of two rubber bands. The slides were held vertically, with the pointed end of the strip uppermost, by slots cut in the lid of a plastic box which measured 5 x 7 x 2.5 inches. Water

Figure 10A

Apparatus for the elution of radioactive components from paper chromatograms.

On the left of the picture is a plastic box with a slotted cover which holds the elution units. Immediately to the right of the box is an elution unit consisting of two slides with the cut-out from the chromatogram and the pointed piece of Whatman number 1 paper between them. Four of these elution units are shown in the plastic box ready for elution. On the right of the picture is a rack with wire cradles. One of the cradles is shown holding the two glass rods with the triangular tip cut from a pointed piece of paper held between them. The tip of paper hangs down into the mouth of a graduated tube standing below the cradle.



was poured into the box to a depth of approximately 0.5 cm. and the box was placed in the draught of a fume hood for 15 hours (Figure 10A). As water evaporated from the pointed end of the paper more water from inside the box passed up, by capillary action, between the slides and the radioactive material was eventually eluted into the tip of the paper.

After 15 hours the slides were removed from the box and the pointed end of the paper was clamped between two glass rods fastened at each end with a short section of plastic tube. The rest of the paper strip was cut off and discarded. The glass rods were slung in a wire cradle so that the point of the paper hung down into the neck of a small tube graduated at a volume of 0.5 ml. The material concentrated at the tip of the paper was eluted into the tube in 10 minutes by applying 0.3 ml. of water dropwise between the glass rods. The volume of solution in the tube was adjusted to 0.5 ml. with water. Aliquots of this solution were assayed for radioactivity by the liquid scintillation technique. The remaining solution was evaporated to dryness in a vacuum desiccator over sodium hydroxide.

The efficiency of this method of elution was checked using (carboxy $^{14}C_2$) succinic acid, ($^{14}C_6$) glucose, (^{14}C) algal protein hydrolysate and (^{14}C) Fraction A obtained from Ochromonas cells which were made radioactive by fixation

of sodium (^{14}C) bicarbonate for one hour. Aliquots of solutions of these materials containing known amounts of radioactivity were applied to circular paper discs 3 cm. in diameter. These discs were cut from dry Whatman number 1 paper which had been developed previously with the phenol-water and butanol-acetic acid-water solvent mixtures. The radioactive materials were eluted from the paper discs by the method described above and aliquots of the eluates were assayed for radioactivity. The recoveries of radioactive material from the paper discs were 102, 99, 92 and 95 per cent, respectively.

Identification of radioactive compounds separated from Fraction A.

The materials in the radioactive areas located on the two-dimensional chromatograms were identified by paper chromatography and electrophoresis.

The solutions containing the radioactive components from the two-dimensional chromatograms were dried and the dry residues were dissolved in 0.025 ml. of water. These solutions were spotted by means of a capillary pipette on Whatman 1 or Whatman 3MM paper for chromatography or electrophoresis respectively.

If the identity of the unknown compound was suspected, then the authentic compound was spotted with the unknown. In addition a series of authentic compounds, with which

the unknown might be identified, were applied to the origin line adjacent to the unknown. The chromatograms were developed in one dimension. After development a strip 1.5 inches in width was cut from the dry chromatogram down the line of development of the unknown compound. The radioactive area was located on this strip using the Actigraph 2 radioactive paper-strip scanner. The authentic compounds, which were chromatographed adjacent to the strip, were detected with an appropriate dip or spray reagent. If the radioactivity of the unknown compound detected on the strip had the same mobility as the authentic compound detected on the adjacent portions of the chromatogram, then a radioautograph was made from the strip. The identification of the unknown compound was accepted only after it had been shown that there was exact coincidence between the shape and location of the area exposed on the film and the area detected on the strip with a suitable reagent, following separate developments on at least two chromatograms with different solvent systems.

In addition to the two solvent mixtures used in the original two-dimensional analysis, the following solvent mixtures were used to identify most of the radioactive components detected on the two-dimensional chromatograms. All proportions quoted are by volume. The solvent mixtures used for identifying amino acids were:

ethanol-butan-1-ol-water-propionic acid (10:10:5:2),

ethanol-butan-1-ol-water-diethylamine (10:10:5:2),

methanol-butan-1-ol-water (2:2:1) (Hardy, Holland and Mayler 1955),

methanol-water-pyridine (20:5:1) (Redfield 1953),

For carboxylic acids the solvent mixtures employed were:

butan-2-ol-pyridine-acetic acid-water (90:4:1:15) (Holdsworth 1961),

butan-1-ol-2 methyl propan-1-ol-water-90 per cent formic acid (1:2:3:1) upper phase (Ladd and Nossal 1954),

ethanol-ammonia (S.G. 0.88)- water (15:1:4) (Cheftel, Munier and Macheboeuf 1952).

Phosphate esters were identified using the solvent mixtures:

2 ethoxy ethanol-pyridine-acetic acid-water (8:4:1:1) containing 0.15 per cent (w/v) 8 hydroxy quinoline (Runeckles and Krotkov 1959).

Aspartic and glutamic acids were also identified by paper electrophoresis at 225 volts for 15 hours with the electrolyte pyridine-acetic acid-water (3:5:192) using the apparatus described in Chapter 2.

Amino acids were detected by dipping the papers in 0.25 per cent (w/v) ninhydrin (indane 1,2,3,-trione hydrate) in acetone. The colours were allowed to develop

Table 10

The distribution of radioactivity in fractions prepared from dark-grown Ochromonas exposed to sodium (^{14}C) bicarbonate.

Sodium (^{14}C) bicarbonate (200 microcuries with a specific radioactivity of 21 microcuries per micromole) was added to an aerated suspension of Ochromonas which had been grown for 3 days in the dark. At short time intervals after the addition of the (^{14}C) bicarbonate, 1.0 ml. samples of the cell suspension were transferred into hot ethanol. The ethanolic suspensions were separated into fractions as described in Chapter 3. All values are in millimicrocuries.

Fraction	A soluble intermediates.	B lipid	C polysaccharide	D protein	Total radioactivity in all fractions.
Time					
4 seconds	1.03	0.03	0.035	0.01	1.105
9 "	3.16	0.055	0.10	0.035	3.350
20 "	9.31	0.16	0.21	0.11	9.79
40 "	23.1	0.29	0.55	0.26	24.20
60 "	36.4	0.55	0.82	0.41	38.2
3 minutes	102.3	1.25	2.50	5.42	111.5
5 "	-	1.01	4.63	9.54	-
10 "	-	2.46	7.45	15.9	-
15 "	-	2.70	9.72	20.8	-
45 "	-	-	-	47.1	-

at room temperature (Toennies and Kolb 1951). Carboxylic acids were detected by spraying the chromatograms with a solution of 0.04 per cent (w/v) bromcresol green in ethanol which had been made alkaline with a few drops of ammonia (Ladd and Nossal 1954). Phosphate esters were detected by spraying the chromatograms with a mixture of 60 per cent (w/v) perchloric acid, N-hydrochloric acid, 4 per cent ammonium molybdate, water (1:2:5:12 by volume). The chromatograms were dried in a draught oven at 85° for one minute and then they were exposed to ultraviolet light for 15 minutes (Bandurski and Axelrod 1951).

Results.

It was apparent from the data assembled in Table 10 that Ochromonas was able to incorporate radioactive carbon dioxide during endogenous respiration in the absence of any exogenous carbon sources. After the cells had been exposed to (^{14}C) bicarbonate for 3 minutes Fraction A contained 92 per cent of the total radioactivity fixed. Fraction A had been shown to contain mostly compounds of intermediary metabolism (Chapter 4). Significant amounts of radioactivity began to appear in the other fractions after one minute. Fraction B, which had been shown to consist almost entirely of lipid, became radioactive to only a limited extent. Fractions C (polysaccharide) and D (protein) incorporated more radioactivity than Fraction B.

Figure 10B

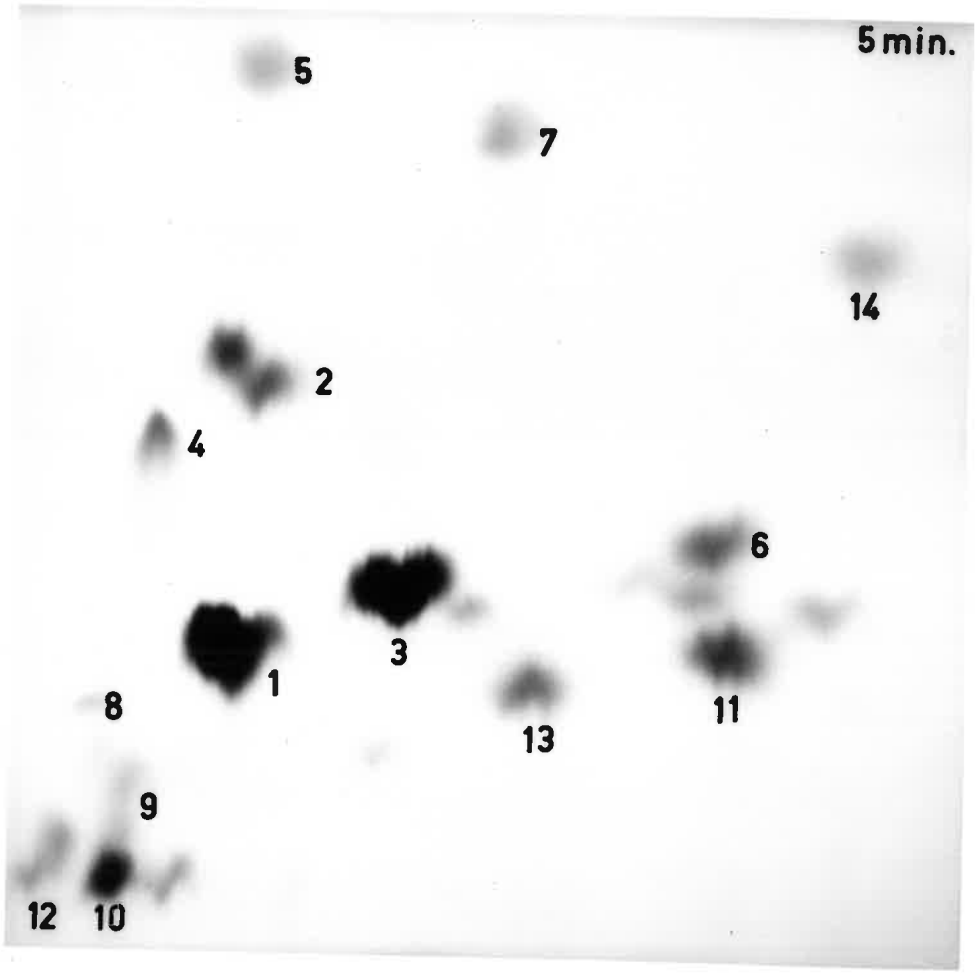
A typical radioautograph of the material in fraction A prepared from Ochromonas cells exposed to sodium (^{14}C) bicarbonate.

The radioautograph was made from a two dimensional chromatogram developed first with the solvent phenol-water and then in a direction at 90° to the first development with butanol-acetic acid-water. Radioactive components were identified as follows:

1. Aspartate
2. Malate
3. Glutamate
4. Citrate
5. Fumarate
6. Alanine
7. Succinate
8. Phosphopyruvate
9. Phosphoglycerate
10. believed to be Uridine Diphosphoglucose
11. Glutamine
12. Sugar Diphosphates
13. Serine
14. Lactate

Other minor radioactive constituents have not been identified.

FIGURE 10B.



BUTAN-1-OL—ACETIC ACID—WATER ⟶

PHENOL—WATER ⟶

Table 11

The distribution of radioactivity incorporated from (^{14}C) bicarbonate into the components of Fraction A.

The conditions of incorporation were as described in Table 10. Fraction A (soluble intermediates) was prepared from the ethanol extracts as described in Chapter 3. The values are all millimicrocuries.

Time (seconds)	Aspartate	Malate + Citrate	Glutamate	Fumarate	Alanine	Phosphate esters	UDPG?	Others
4	0.95	0.08	-	-	-	-	-	-
9	2.52	0.36	0.16	0.12	-	-	-	-
20	4.46	1.71	0.97	0.13	1.07	0.19	0.09	0.56
40	7.92	2.13	5.61	0.27	3.74	0.73	0.33	2.42
60	11.95	2.69	9.72	0.21	6.49	1.10	0.91	3.52
180	28.80	5.59	23.31	0.22	23.10	3.31	6.20	14.83

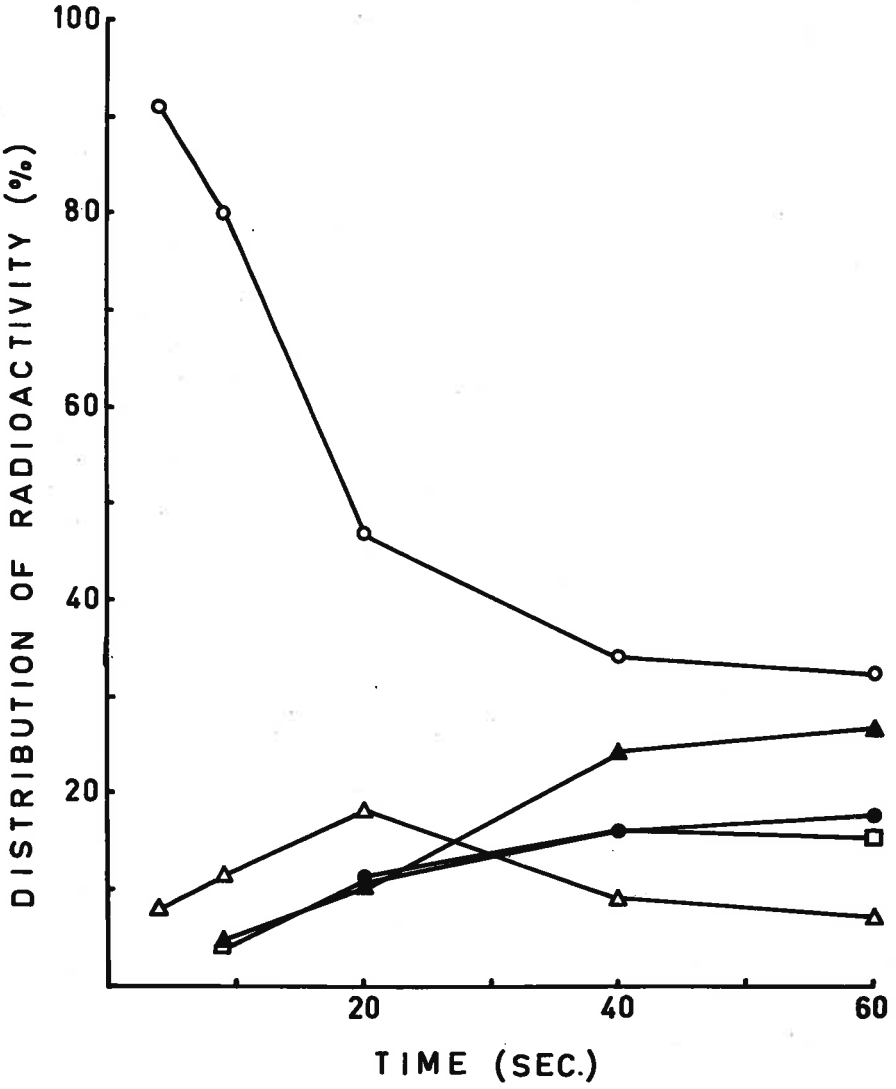
Figure 11

Incorporation of radioactivity into the constituents of fraction A obtained from Ochromonas cells after exposure of the cells to sodium(^{14}C) bicarbonate for short periods of time.

Dark-grown Ochromonas cells were suspended in water and gassed with air. Sodium(^{14}C) bicarbonate was added to the suspension and samples were quickly removed and squirted into 4 volumes of hot ethanol. Fraction A was prepared from the ethanolic suspensions and then analysed by two dimensional chromatography and radioautography. Radioactive areas on the chromatogram were eluted and assayed for radioactivity by the liquid scintillation technique. Subsequently the radioactive components were identified by paper chromatography and electrophoresis. The radioactivity assayed in each component was expressed as a percentage of the total radioactivity on the chromatogram and this was plotted against time.

○ Aspartate
△ Malate+ citrate
▲ Glutamate
● Alanine
□ Other components.

FIGURE 11.


100
80
60
40
20
DISTRIBUTION OF RADIOACTIVITY (%)
20
40
60
TIME (SEC.)

Radioactivity was being incorporated into the material in Fraction D at twice the rate it was being incorporated into Fraction C.

The components of Fraction A were separated by two-dimensional paper chromatography and the radioactive compounds were located by radioautography. A typical radioautograph of the components of Fraction A, which was prepared from a sample of the Ochromonas cell suspension taken 5 minutes after the addition of the (^{14}C) bicarbonate, is shown in Figure 10B and the identities of the radioactive areas are indicated. The radioactive areas were eluted from the chromatograms and assayed for radioactivity. The distribution of radioactivity among the components separated on the chromatograms is shown in Table II. The proportion of the total radioactivity on the chromatogram that was present in each component was plotted against time and the result is shown in Figure 11.

In the earliest samples that were taken after the addition of the (^{14}C) bicarbonate aspartic acid clearly was the major radioactive compound in Fraction A. In addition to aspartate, malate and citrate were the only other radioactive compounds that could be detected on the chromatogram of Fraction A prepared from the first sample ($\frac{1}{4}$ seconds). In subsequent samples radioactivity progressively accumulated in glutamate and alanine.

Fumarate, lactate, glutamine, glycine, serine and asparagine were identified among a number of compounds which became radioactive to only a limited extent and appeared on the chromatograms of samples that were taken after 10 seconds exposure to the radioactive bicarbonate. Among the unidentified radioactive materials and not far removed from the origin there were a number of components which contained phosphate. Prominent among these phosphate containing components, in samples taken after 60 seconds exposure to the radioactive bicarbonate, there was a compound which was tentatively identified as uridine diphosphate glucose. It was interesting to note that the increase in the radioactivity of this compound coincided with the increase of radioactivity in the polysaccharide of Fraction C (Table 10). Uridine diphosphate-sugar compounds have been shown to act as glycosyl donors in the biosynthesis of a number of polymers such as glycogen, cellulose, chitin etc. (Hassid 1962). Of particular interest with regard to the present investigation Feingold, Neufeld and Hassid (1958) reported that a soluble enzyme preparation from the mung bean (Phaseolus aureus) catalysed the formation of a beta-1,3-linked polysaccharide from the glucose units donated by uridine diphosphate glucose.

Examination of Figure 11 showed that at the earliest times of fixation the curve plotted for aspartate was the only one with a negative slope. Therefore it was concluded

that aspartate was the primary stable product of the fixation of (^{14}C)-bicarbonate by Ochromonas which had been grown in the dark.

Discussion.

Since there are no known enzyme systems that catalyse the incorporation of carbon dioxide directly into aspartate it was assumed that oxaloacetic acid was the primary fixation product and that aspartate was produced from oxaloacetate by a transamination reaction. Because of its instability oxaloacetate would not be expected to be present on the chromatograms. However if oxaloacetate was in ready equilibrium with aspartate then the appearance of radioactivity in aspartate would reflect a fixation in oxaloacetate.

At the time that the above experiments were performed Kauss and Kandler (1962) published a report concerning heterotrophic carbon dioxide fixation by Ochromonas malhamensis. These authors followed heterotrophic carbon dioxide fixation with cells which had been grown for 5 days in the light. Their experiments were conducted in a darkened "lollipop". In contrast to the results of the experiments reported in the present investigation these authors found that malic acid was the major radioactive compound present in an ethanol extract of Ochromonas cells

which had been exposed to potassium (^{14}C) bicarbonate for 15 seconds; aspartate only had half as much radioactivity as malate at this time. Furthermore, glutamic acid did not gain more than 2.1 per cent of the total radioactivity present in the ethanol extracts at any time during the experiment. These differences probably were a reflection of the different past histories of the cells used in the two investigations; Kauss and Kandler used cells which had been grown for 5 days in the light while the cells used in the present work had been grown for 3 days in the dark.

Possible mechanisms for the incorporation of carbon dioxide into oxaloacetate.

A number of enzyme systems have been shown to catalyse carboxylation reactions where oxaloacetate is a product. Phosphopyruvate carboxykinase (nucleoside triphosphate: oxaloacetate carboxy-lyase, transcarboxylating E.C. 4.1.1.32) an enzyme discovered in avian liver by Utter and Kurahashi (1954) was found to catalyse the reversible reaction -

Phosphopyruvate + CO_2 + Nucleoside diphosphate $\rightleftharpoons$ Oxaloacetate + Nucleoside triphosphate

Bandurski and Greiner (1953) described an enzyme which they obtained from spinach leaves. This enzyme, orthophosphate:

oxaloacetate carboxy-lyase, phophorylating (E.C.4;1;1;31), catalysed the irreversible formation of oxaloacetate according to the equation:-

$$\text{Phosphopyruvate} + CO_2 \rightleftharpoons \text{Oxaloacetate} + iP$$

Another reaction involving the carboxylation of phosphopyruvate was catalysed by phosphopyruvate carboxy transphosphorylase (Siu, Wood and Stjernholm 1961). This reaction was found to be reversible and was shown to proceed according to the equation:-

$$\underset{iP}{\text{Phosphopyruvate}} + CO_2 + \rightleftharpoons \text{Oxaloacetate} + \text{pyrophosphate}$$

An enzyme catalysing the carboxylation of pyruvate was discovered in liver mitochondria by Utter and Keech (1960). This enzyme (pyruvate: carbon dioxide ligase, ADP, E.C. 6.4.1.1) catalysed the reaction:

$$\text{Pyruvate} + CO_2 + ATP \rightleftharpoons \text{Oxaloacetate} + ADP + iP.$$

and it was found to require the presence of catalytic amounts of acetyl coenzyme A for activity. The activity of this enzyme was inhibited by avidin.

In addition there are two carboxylation reactions which produce dicarboxylic acids that could be direct precursors of oxaloacetate. Ochoa, Mehler and Kornberg (1948) discovered an enzyme (L-malate:NADP oxidoreductase, decarboxylating, E.C. 1.1.1.40) which catalysed the reductive carboxylation of pyruvate to malate. If this reaction was coupled with the reaction catalysed by malate dehydrogenase (L-malate:NAD oxidoreductase, E.C.1.1.1.37) the overall reaction would represent the carboxylation of pyruvate to form oxaloacetate:

$$\text{Pyruvate} + CO_2 + PNH_2 \rightleftharpoons \text{Malate} + PN$$

$$\text{Malate} + PN \rightleftharpoons \text{Oxaloacetate} + PNH_2$$

$$\text{Sum: Pyruvate} + CO_2 \rightleftharpoons \text{Oxaloacetate}$$

where PN represents either NAD or NADP and PNH_2 represents either $NADH_2$ or $NADPH_2$.

The equilibrium of the malate dehydrogenase catalysed reaction was shown to be far in the direction of malate formation (Stern, Ochoa and Lynen 1952, Burton and Wilson 1953) and this would be an obstacle to this pathway of formation of oxaloacetate. The overall reaction would not proceed in the direction of oxaloacetate formation unless

there was some sink for the end products. The removal of oxaloacetate by a transamination reaction to form aspartate could be the means by which the coupled reactions might be pulled in the direction of carboxylation. Malate dehydrogenase from a number of sources was found to be specific for NAD (Burton and Wilson 1953). "Malic" enzyme originating from animals and plants was usually specific for NADP (Wood and Stjornholm 1962). Although "malic" enzyme from Lactobacillus arabinosus was specific for NAD it was not possible to demonstrate carbon dioxide fixation with this enzyme; the reaction would proceed only in the direction of decarboxylation (Korkes, del Campillo and Ochoa 1950, Kaufman, Korkes and del Campillo 1951). A "malic" enzyme prepared from Ascaris lumbricoides was active with either NAD or NADP (Saz and Hubbard 1957). If "malic" enzyme activity was demonstrated in Ochromonas the occurrence of the fully coupled reactions envisaged above would depend upon the cofactor specificity of the "malic" enzyme since it appeared that malate dehydrogenase from all sources was NAD-specific.

A reaction catalysed by the enzyme propionyl CoA carboxylase (propionyl CoA:carbon dioxide ligase, ADP, E.C. 6.4.1.3), in which propionyl CoA was carboxylated to form methyl malonyl CoA, was discovered by Flavin, Ortiz and Ochoa (1955). The results of an investigation of the effect of vitamin B_{12} on the oxidation of (^{14}C) propionate

by vitamin B_{12}-deficient Ochromonas malhamensis suggested to White and Arnstein (1961) that vitamin B_{12} might be acting as a cofactor for the enzyme methyl malonyl CoA isomerase (2 methyl malonyl CoA: CoA-carbonyl mutase, E.C. 5.4.99.2) in this organism. It might be implied from this that Ochromonas had the enzyme propionyl CoA carboxylase which would be necessary for the conversion of propionate to methyl malonyl CoA. If Ochromonas was able to incorporate carbon dioxide through the activity of this enzyme, then coupling with a reaction discovered by Swick and Wood (1960), which was catalysed by theenzyme malonyl CoA: pyruvate carboxyl transferase (E.C.2.1.3.1.) would lead to the production of oxaloacetate.

Propionyl CoA + ATP + CO_2 ⇌ Methyl malonyl CoA + ADP + iP

Methyl malonyl CoA + Pyruvate ⇌ Propionyl CoA + Oxaloacetate

Sum: Pyruvate + ATP + CO_2 ⇌ Oxaloacetate + ADP + iP

It would appear that the overall reaction was the same as that demonstrated by Utter and Keech (1960) with the enzyme pyruvate carboxylase. The coupled reaction described above would depend on the presence of catalytic amounts of

propionyl CoA. The reaction catalysed by pyruvate carboxylase was shown to depend on the presence of acetyl CoA or propionyl CoA. However pyruvate carboxylase would not catalyse the transcarboxylation reaction -

Malonyl CoA + Pyruvate $\rightleftharpoons$ Acetyl CoA + Oxaloacetate

Therefore the type of coupled reaction described above could not account for the reaction catalysed by the pyruvate carboxylase of Utter and Keech (1960). Both of the enzymes involved in the coupled reaction and pyruvate carboxylase required biotin for activity since they were all inhibited by avidin (Halenz and Lane 1960, Swick and Wood 1960, Utter and Keech 1960). Kaziro, Leone and Ochoa (1960) showed that purified propionyl-CoA carboxylase contained biotin. Here again the transamination of oxaloacetate to aspartate would pull the coupled reaction in the direction of carboxylation.

From the results described in this chapter it was not possible to decide which of the enzymes discussed above might be responsible for the incorporation of (^{14}C) bicarbonate into oxaloacetate and hence into aspartic acid during fixation of carbon dioxide by Ochromonas.

Incorporation of (^{14}C) bicarbonate by thiamine or biotin deficient Ochromonas cells.

From a study of the distribution of radioactivity incorporated from (^{14}C) bicarbonate into the components of ethanol extracts of biotin or thiamine deficient cells, Kauss and Kandler (1962) suggested that there were two independent mechanisms for the fixation of carbon dioxide in this organism. The incorporation of radioactivity into malic acid was greatly reduced in the thiamine deficient cells. This effect was thought to be due to a decrease in the amount of reduced pyridine nucleotide available as a result of the damage incurred by the respiratory system during thiamine deficiency. A decrease in the amount of reduced pyridine nucleotide meant that less carbon dioxide could be incorporated into malic acid by the "malic" enzyme.

Biotin deficient cells were characterized by a total inability to incorporate radioactive bicarbonate into aspartate. Therefore it was suggested that the incorporation of bicarbonate into aspartate was catalysed by either pyruvate carboxylase or phosphopyruvate carboxykinase. The inclusion of phophopyruvate carboxy kinase was based on the premise that this enzyme contained bound biotin (Lichstein 1957). However it was unlikely that this enzyme needed biotin for activity since it was not inhibited by avidin.

Furthermore, protein-bound biotin and the enzyme were not eluted in the same fraction from an avidin azocellulose column (Somenza, Prestige, Menard-Joker and Bettex-Galland 1959). The presence of the other two enzymes catalysing the incorporation of carbon dioxide into oxaloacetate (phophopyruvate carboxylase and phosphopyruvate carboxytransphosphorylase) was precluded since these enzymes were not inhibited by avidin (Zenk 1962 and Siu and Wood 1962, respectively). Therefore the two mechanisms of carbon dioxide fixation suggested by the results of Kauss and Kandler (1962) were limited to the reactions catalysed by "malic" enzyme and pyruvate carboxylase.

Incorporation of radioactivity into the secondary products of (^{14}C) bicarbonate fixation.

It was found that malic and citric acids were early radioactive products of (^{14}C) bicarbonate fixation by dark-grown Ochromonas cells (Table 11). Radioactivity from (^{14}C) bicarbonate may be incorporated into malate by two pathways. The first possible pathway was a direct incorporation through the reaction catalysed by "malic" enzyme. Another indirect pathway was by incorporation into oxaloacetate which could be reduced to malate in the reaction catalysed by malate dehydrogenase.

Presumably radioactive citric acid was formed by

condensation of radioactive oxaloacetate with acetyl CoA. If Ochromonas had a tricarboxylic acid cycle, this would explain the passage of radioactivity from oxaloacetic acid to alpha-oxoglutaric acid and into glutamic acid by transamination of the alpha-oxoglutaric acid.

The incorporation of (^{14}C) bicarbonate into alanine would not be explained by a simple reversal of the reactions catalysed by "malic" enzyme or pyruvate carboxylase and transamination of the pyruvate produced. However they were formed, the dicarboxylic acids would be labelled in the beta carboxyl group and this group was converted to carbon dioxide in the reverse reactions catalysed by these two enzymes. Pyruvate might become radioactive if labelling of the dicarboxylic acids was not confined to the beta carboxyl groups. The label could become distributed between the two carboxyl groups if the dicarboxylic acids were in equilibrium with a symmetrical compound such as fumarate. Radioactive fumarate was found in the earliest samples taken after exposure of intact cells to (^{14}C) bicarbonate (Table 11). Although the amount of radioactivity in fumarate at any time was quite small and did not increase much with time, this need not invalidate its participation in the scheme. If the pool size of fumarate was small and static and there was a rapid turnover of ^{14}C then the maximum possible specific radioactivity might be reached in a very short time;

thereafter there would be no increase in the total radioactivity of this compound. Radioactive pyruvate was not seen on the radioautographs since this compound would be volatile in the acid solvent and lost from the chromatogram.

Chapter 9

INCORPORATION OF SODIUM (^{14}C) BICARBONATE BY BROKEN CELL PREPARATIONS OF OCHROMONAS MALHAMENSIS.

It was apparent from the results presented in Chapter 8 that Ochromonas, which had been grown in the dark, incorporated (^{14}C) bicarbonate primarily into dicarboxylic acids in the absence of exogenous carbon substrates. However, there were several known enzymes which might have catalysed a primary carboxylation reaction yielding dicarboxylic acids. The results obtained by Kauss and Kandler (1962) from (^{14}C) bicarbonate incorporation experiments with biotin and thiamine deficient Ochromonas cells indicated that there were at least two pathways of carbon dioxide incorporation in this organism. Furthermore it seemed that these two pathways started from the carboxylation reactions catalysed by "malic" enzyme and pyruvate carboxylase.

In the present investigation a more direct approach was employed in an attempt to define the primary carboxylation reactions of heterotrophic carbon dioxide fixation by Ochromonas. Broken cell preparations of the organism were examined for their ability to catalyse various C1 + C3 condensations. In the following sections two carboxylation reactions are described. One of these reactions was shown to be catalysed by "malic" enzyme, but the nature of the other reaction was not determined.

Methods and Materials.

Preparation of broken cells. The cells from a 300 ml. culture of Ochromonas, which had been grown for 3 days in the dark, were harvested and washed by centrifuging as described in Chapter 2. The washed cells were suspended in approximately 6 ml. of a freshly prepared solution of 1 mM cysteine hydrochloride in 10 mM tris-HCl buffer pH 8.0 at 4°. This suspension was transferred to an ice-cold plastic cup from a type OM-2000 microhomogeniser attachment fitted to a Servall Omnimixer (Ivan Sorvall Inc., Norwalk, Conn., U.S.A.). The cup was fitted to the homogeniser and then lowered into an ice bath. The cold cell suspension was homogenised at maximum speed (blade speed 50,000 rpm.) for 1 minute. The cup was immediately detached from the apparatus, to prevent the transfer of heat from the motor and allowed to stand in an ice bath for five minutes. Then the homogenate was centrifuged at 900g for 10 minutes at 4° to remove large fragments and unbroken cells from the preparation. The supernatant suspension was carefully removed with a teat pipette and the packed residue was discarded.

Whenever possible this 900g supernatant was added to the various reaction mixtures immediately after preparation, otherwise it was kept on ice. For the spectrophotometric assay of "malic" enzyme the 900g supernatant was centrifuged

at 57,300g for 30 minutes at 4° in a Spinco model L centrifuge and the supernatant liquid was used as the source of enzyme.

To determine the relative distribution of "malic" enzyme and malate degydrogenase between the soluble and particulate fractions of the cell a 900g supernatant of a broken cell preparation was divided into two portions. One portion was kept in an ice bath while the other portion was centrifuged at 57,300g for 30 minutes at 4°. The supernatant liquid was removed and kept in the ice bath while the packed residue was suspended in an amount of the 1 mM cysteine, 10 mM tris-HCl buffer equal to the volume of the supernatant. The enzyme activities were assayed spectrophotometrically in these three preparations. At the same time these preparations were tested for their ability to fix (^{14}C) bicarbonate in the presence of pyruvate and $NADPH_2$.

Protein was estimated by a modification of the method of Westley and Lambeth (1960) which was described in Chapter 4.

Fixation of sodium (^{14}C) bicarbonate by broken cell preparations. All reactions were allowed to proceed at 28° in glass centrifuge tubes. The standard assay mixture used in these experiments contained 100 micromoles of tris-HCl buffer pH 8.0, 15 micromoles of magnesium chloride,

25 micromoles of reduced glutathione, 20 micromoles and 10 microcuries of sodium (^{14}C) bicarbonate and 0.5 ml. of a 900g supernatant of a broken cell preparation (approximately 0.9 mg. of protein). The various substrates which were added to the standard assay mixture are listed in the tables of results. When it was expected that oxaloacetate might be a product of the reaction 5 micromoles of $NADH_2$ and 400 units of malate dehydrogenase were included in the reaction mixture to reduce unstable oxaloacetate to malate.

The reactions were always started by the addition of the broken cell preparation to the rest of the reactants. The reactions were stopped by making the reaction mixtures 2.5N with respect to formic acid. Unreacted (^{14}C) bicarbonate was flushed from the reaction mixtures by adding a few small pieces of solid carbon dioxide. Then the reaction mixtures were treated with four volumes of ethanol. Subsequently the ethanolic solutions were flushed by bubbling carbon dioxide gas through the mixtures for 60 minutes. Finally the mixtures were centrifuged at 900g for 10 minutes. The supernatant liquid was collected and the volume was measured. Aliquots of the supernatant liquid were assayed for radioactivity by the liquid scintillation technique.

In some instances insufficient radioactivity was fixed to permit a satisfactory analysis of the products when the

standard assay mixture was used. These experiments were repeated using reaction mixtures containing 50 microcuries of sodium (^{14}C) bicarbonate but otherwise identical to the standard mixture.

Analysis of the radioactive products in ethanolic extracts of broken cell preparations which had been incubated with (^{14}C) bicarbonate. The ethanolic solutions, which were prepared from the reaction mixtures as described above, were distilled to dryness under reduced pressure at less than 35°. The brown oily residue was emulsified with 0.5 ml. of water and the emulsion was shaken with 2.5 ml. of a chloroform-methanol mixture (3:1 by volume). The two phases of the resultant biphasic mixture were separated by a brief centrifuging and all but a thin layer of the upper phase was carefully removed. Without disturbing the lower phase the phase interface was washed with a mixture having the same solvent composition as the upper phase which had been removed (Folch et al. 1957). The washings and the upper phase were combined while the lower phase, which contained the cell lipid, was discarded. This procedure was analogous to that which was described in Chapter 3 for the preparation of Fraction A. Therefore the upper phase consisted largely of ethanol-soluble intermediates of metabolism.

The upper phase was concentrated to dryness in a vacuum

desiccator over sodium hydroxide. The dry residue was dissolved in 1.0 ml. of water and the solutes were separated into acidic, basic and neutral fractions by treatment with ion-exchange resins as described in Chapter 4. The subfractions were assayed for radioactivity by the liquid scintillation technique.

Radioactive subfractions were evaporated to dryness in a vacuum desiccator over sodium hydroxide. The dry residues were dissolved in 0.05 ml. of water and these solutions were spotted on Whatman number 1 papers for analysis by chromatography in one dimension. Chromatograms of the acidic fraction were developed with the solvent mixture butan-1-ol-2 methyl propan-1-ol-water-90 per cent formic acid (1:2:3:1 by volume, upper phase) (Ladd and Nossal 1954). The basic fraction contained a large amount of tris which was used to buffer the reaction mixtures. Because of the excess tris in this fraction the spot made at the origin of the chromatogram was grossly overloaded and the components separated on the chromatogram were all badly streaked. The tris was separated from the radioactive materials in the basic fraction by paper electrophoresis on Whatman 3 MM paper using the electrolyte pyridine-acetic acid-water (3:5:192 by volume) at 580 volts for 5 hours in the apparatus described in Chapter 2. This procedure separated a complete amino acid mixture into 4 bands

consisting of aspartic acid, glutamic acid neutral amino acids and basic amino acids. Tris migrated with the band of basic amino acids and radioactive materials were always associated with the other bands. Radioactive areas were located on the electrophoresis strip by passing it through an Actigraph 2 radioactive paper-strip scanner. The radioactive areas were cut from the paper and the paper was eluted as described in Chapter 8. The eluted material was spotted on Whatman number 1 paper. The chromatogram was given a double development with the solvent mixture butan-2-ol-ammonia (S.G.0.88)-water (300:33:67 by volume).

Radioactive areas were detected on the dry one-dimensional chromatograms using the Actigraph 2 radioactive paper-strip scanner. The radioactive areas were eluted and the eluates were assayed for radioactivity by the liquid scintillation technique. Radioactive compounds were identified by paper chromatography as described in Chapter 8.

Isolation and degradation of radioactive malate. Radioactive malate was isolated from a reaction mixture by paper chromatography. Its identity was confirmed by paper chromatography with the solvent mixtures used for the identification of carboxylic acids and described in Chapter 8. The compound was finally eluted from the chromatogram and the radioactivity in the eluate was assayed by the liquid

scintillation technique. The eluate was reduced to dryness in a vacuum desiccator over sodium hydroxide.

The radioactive malate was quantitatively converted to lactic acid and carbon dioxide using a washed cell suspension of Lactobacillus arabinosus (Blanchard, Korkes, del Campillo and Ochoa 1950). The dry material was dissolved in 1.0 ml. of a solution containing 20 micromoles of sodium-L-malate. This solution was transferred to the outer compartment of a 30 ml. bottle equipped with an inner well having acapacity of 1.5 ml. The outer compartment was also supplied with 2.0 ml. of water and 0.2 ml. of a buffer-cofactor solution (Nossal 1952) while 1.0 ml. of 5N-sodium hydroxide solution was added to the inner well. The bottle was sealed with a skirted rubber vaccine cap.

Lactobacillus arabinosus 17-5 was grown in the medium described by Nossal (1951). The cells were harvested from the culture and washed by centrifuging. The washed cells were suspended in 0.1 M potassium chloride and 0.4 ml. of the cell suspension was added to the outer compartment of the sealed reaction vessel by means of a syringe. The bottle was incubated at 35^{0} for 30 minutes and then allowed to stand at room temperature for a further 60 minutes to ensure that the carbon dioxide evolved was completely absorbed in the alkali. The alkali was recovered quantitatively from the inner well and an amount of carrier sodium carbonate

was added to the solution so that the total amount of carbonate was approximately 35 micromoles. Carbonate was precipitated from the alkali as barium carbonate by the methods described in Chapter 5. The barium carbonate was plated and assayed for radioactivity as described in Chapter 2.

Lactate was recovered from the reaction mixture in the outer compartment as follows. The reaction mixture was transferred from the bottle to a centrifuge tube. Protein was precipitated by treating the mixture with 4 volumes of ethanol. The solution was centrifuged to pack the precipitate. The supernatant liquid together with several ethanol washes of the precipitate were concentrated almost to dryness under a stream of nitrogen at 40°. The concentrated solution was passed through two 1 x 5 cm. columns packed with the ion exchange resins Dowex 50 (8 per cent cross-linked, 100-200 mesh, in the acid form) and Dowex 1 (4 per cent cross-linked, 50-100 mesh, in the acetate form) in succession. The lactate exchanged on to the latter resin was eluted with 25 ml. of 6N-formic acid and the eluate was evaporated to dryness in a rotary evaporator. The residue was dissolved in 2.0 ml. of a solution containing 180 micromoles of lithium lactate.

The lactate in this solution was oxidized to acetic acid and carbon dioxide using acid permanganate according to the method described by Katz, Abraham and Chaikoff (1955).

The reaction was performed in the bottle equipped with a centre well which was described previously. The carbon dioxide evolved during the reaction was absorbed in sodium hydroxide, precipitated as barium carbonate and assayed for radioactivity as outlined above. Acetic acid was recovered from the reaction mixture in the outer compartment of the bottle by steam distillation and the distillate was titrated to a phenol red end-point with sodium hydroxide solution. The neutralised distillate was concentrated to a small volume in a rotary evaporator and assayed for radioactivity by the liquid scintillation technique.

Spectrophotometric enzyme assays. All of the assays were performed in a Shimadzu type RS-27 automatic recording spectrophotometer. In all assays the rates of reactions were determined from the difference in extinction between 15 and 45 seconds after the initiation of the reaction.

"Malic" enzyme (E.C.1.1.1.40). The activity of this enzyme was determined by measuring the increase in extinction at 340 millimicrons of the following reaction mixture. The complete system, in 1 cm. light-path silica cuvettes, consisted of 100 micromoles of tris-HCl buffer pH 8.0, 25 micromoles of magnesium chloride, 10 micromoles of cysteine hydrochloride, 2 micromoles of potassium malate, 0.1 micromole of NADP, 0.1 ml. of enzyme preparation (57,300g supernatant of an Ochromonas broken cell preparation)

and water to make a final volume of 3.0 ml. This assay system was virtually identical to that employed by Rutter and Lardy (1958). These authors conducted their assay at pH 7.5 and used a glycyl glycine buffer.

The reactions were started by the addition of malate to the other reactants in the test cell while an equivalent volume of water was added to the other reactants in the reference cell. One unit of enzyme activity was defined as that amount which caused a change in extinction of 0.01 per minute under the above conditions (Rutter and Lardy 1958).

Lactate dehydrogenase (E.C.1.1.1.27). This enzyme was assayed according to the method described by Neilands (1955). The lactate-dependent reduction of NAD catalysed by this enzyme was assayed by measuring the increase in extinction at 340 millimicrons.

Pyruvate kinase (E.C. 2.7.1.40). The method of Bucher and Pfleiderer (1955) was used to assay the activity of pyruvate kinase. In this method the pyruvate kinase catalysed reaction was coupled with the reaction catalysed by lactate dehydrogenase, the overall reaction being measured by the decrease in extinction of $NADH_2$ at 340 millimicrons.

Malate dehydrogenase (E.C.1.1.1.37). Malate dehydrogenase activity in broken cell preparations was determined by measuring the decrease in extinction at 340 millimicrons of the following reaction mixture. The complete system in 1 cm. light path silica cuvettes contained 100 micromoles of tris-HCl buffer pH 8.0, 0.4 micromoles of $NADH_2$, 25 micromoles of magnesium chloride, 0.1 ml. of enzyme solution (900g supernatant), 2 micromoles of oxaloacetate and water to make the final volume 3.0 ml. The reference cell contained all the reactants except oxaloacetate which was replaced by an equivalent volume of water. The reaction was started by the addition of oxaloacetate to the other reactants in the test cell.

Chemicals. Oxaloacetic acid, $NADH_2$, $NADPH_2$, GDP, ATP, reduced glutathione, sodium pyruvate, glucose 6 phosphate dehydrogenase (E.C.1.1.1.49), lactate dehydrogenase (E.C.1.1.1.27) free of pyruvate kinase, p-chloromercuribenzoate and the barium salt of glucose 6 phosphate were all purchased from the Sigma Chemical Co., St. Louis, Mo., U.S.A. Glucose 6 phosphate barium salt was converted to the sodium salt by dissolving it in 0.1N hydrochloric acid and precipitating the barium as barium sulphate by the addition of sodium sulphate in slight excess. The resultant solution was adjusted to a pH of 7.8 with sodium hydroxide solution.

NAD and NADP were obtained from C.F. Boehringer und Soehne, Mannheim, Germany. Avidin was obtained from

Nutritional Biochemicals Corp. Cleveland, Ohio, U.S.A. Malate dehydrogenase was purchased from Worthington Biochemical Corp., Freehold, N.J., U.S.A. Coenzyme A and ATP came from Pabst Laboratories, Milwaukee, Wis., U.S.A. D-biotin was obtained from the California Foundation for Biochemical Research, Los Angeles, Calif., U.S.A. Propionic anhydride was purchased from Fluka A.G. Chemische Fabrik, Buchs S.G., Switzerland.

The tricyclohexylamine salt of phosphopyruvic acid was prepared in these laboratories by Mr. B. Bubela. This preparation was found to be free of pyruvic acid when assayed with lactate dehydrogenase. Acetyl coenzyme A and propionyl coenzyme A were prepared from the appropriate acid anhydrides by the method of Stadtman (1957).

Results and Discussion.

Aspartic acid was the first stable radioactive product of (^{14}C) bicarbonate incorporation by intact Ochromonas cells during endogenous respiration in the absence of exogenous carbon sources (Chapter 8). There were four known reactions which might have yielded oxaloacetate as a direct product.

These reactions were catalysed by the enzymes -

Phosphopyruvate carboxylase

$$\text{Phosphopyruvate} + CO_2 \longrightarrow \text{Oxaloacetate} + iP,$$

Phosphopyruvate carboxykinase

$$\text{Phosphopyruvate} + CO_2 \rightleftharpoons \text{Oxaloacetate} + GTP, + GDP$$

Phosphopyruvate carboxytransphosphorylase

$$\text{Phosphopyruvate} + CO_2 \rightleftharpoons \text{Oxaloacetate} + \text{pyrophosphate} + iP$$

Pyruvate carboxylase

$$\text{Pyruvate} + CO_2 + ATP \rightleftharpoons \text{Oxaloacetate} + ADP + iP.$$

The participation of these enzymes in the carbon dioxide fixation reactions of Ochromonas was discussed in Chapter 8.

In addition there were two coupled reactions which could produce oxaloacetate as an indirect product of carbon dioxide fixation. The first of these coupled reactions was due to the concerted action of "malic" enzyme and malate dehydrogenase -

$$\text{pyruvate} + CO_2 + NADPH_2 \rightleftharpoons \text{malate} + NADP$$
$$\text{malate} + NAD \rightleftharpoons \text{oxaloacetate} + NADH_2$$

$$\text{Sum:} \quad \text{pyruvate} + ATP + CO_2 \rightleftharpoons \text{oxaloacetate} + ADP + iP$$

Using a standard reaction mixture a number of experiments were performed with Ochromonas broken cell preparations

Table 12

Incorporation of radioactivity from sodium (^{14}C) bicarbonate by a broken cell preparation of Ochromonas.

The standard reaction mixture contained the following components with the quantities expressed in micromoles:

tris-HCl buffer pH 8.0	100
$MgCl_2$	15
GSH	25
Sodium (^{14}C) bicarbonate	20 (10 microcuries)

After the various additions (listed across) were added to the the standard mixture the reactions were started by the addition of 0.5 ml. of the broken cell preparation. With the exception of Experiment 1, the reaction mixtures all contained 5 micromoles of $NADH_2$ and 400 units of L-malate: NAD oxidoreductase (E.C. 1.1.1.37). The $NADPH_2$ regenerating system used in Experiment 1 consisted of 5 micromoles of glucose-6-phosphate and 0.5 mg. of D-glucose-6-phosphate: NADP oxidoreductase (E.C.1.1.1.49). The reactions were allowed to proceed for 20 minutes at 28° before they were stopped by acidifying the mixture with formic acid. Unreacted (^{14}C) bicarbonate was flushed from the reaction mixtures which were then treated with 4 volumes of ethanol. Radioactivity was assayed in the ethanolic extracts by the liquid scintillation technique.

Table 12

Incorporation of radioactivity from sodium (^{14}C) bicarbonate by a broken cell preparation of Sphaeroplana.

Expt.	Additions (micromoles)	Radioactivity (millimicrocuries)
1. a.	Nil.	0.375
b.	Pyruvate 5.	3.09
c.	Pyruvate 5, $NADH_2$ 5.	5.15
d.	Pyruvate 5, NADP 2, $NADPH_2$ regenerating system.	18.86
e.	NADP 2, $NADPH_2$ regenerating system.	0.65
2. a.	Nil.	0.40
b.	Phosphopyruvate 5.	0.62
c.	Phosphopyruvate 5, iP 20	0.66
d.	Phosphopyruvate 5, ADP 5.	1.07
e.	Phosphopyruvate 5, ADP 5.	0.63
3. a.	Nil.	0.38
b.	Pyruvate 5.	3.20
c.	ATP 5.	3.79
d.	Pyruvate 5, ATP 5.	4.32
e.	Pyruvate 5, ATP 5 acetyl CoA 1	3.42
4. a.	Nil.	1.43
b.	ATP 5.	3.69
c.	Propionyl CoA 2.	2.30
d.	Propionyl CoA 2, ATP 5.	4.50
e.	Propionyl CoA 2, ATP 5, Pyruvate 5.	4.77

in an attempt to detect any of the enzymes catalysing the six reactions outlined above. The results of these experiments are shown in Table 12.

Incorporation of (^{14}C) bicarbonate by broken cell preparations in the presence of pyruvate. In experiment 1 of Table 12 it was found that the addition of pyruvate to the standard reaction mixture significantly increased the amount of radioactivity fixed by a broken cell preparation of Ochromonas (reaction b). The addition of $NADH_2$ together with pyruvate did not elicit any increase in the amount of radioactivity fixed over that already observed with pyruvate alone (reaction c). When $NADH_2$ was replaced by a system generating $NADPH_2$ (glucose 6 phosphate dehydrogenase, glucose 6 phosphate and NADP) the amount of radioactivity fixed was six times the amount fixed in the presence of pyruvate alone (reaction d). No significant fixation of radioactivity was observed when pyruvate was omitted from the latter reaction mixture (reaction e). These results suggested that an NADP-dependent "malic" enzyme was present in Ochromonas broken cell preparations.

Reaction mixture d of experiment 1, Table 12 was partitioned between a chloroform-methanol-water mixture and the upper phase was treated with ion-exchange resins. All of the radioactivity in the reaction mixture passed through

Table 13

Distribution of radioactivity incorporated into compounds after exposure of broken cell preparations of Gehrenonas to sodium (^{14}C) bicarbonate.

The conditions of the reactions were the same as described in Table 12 with the exception that experiment 2 was conducted with 50 microcuries instead of 10 microcuries of sodium (^{14}C) bicarbonate. Fraction A was prepared from the ethanol extracts of the reaction mixtures. Fraction A in turn was subfractionated with ion exchange resins into acidic, basic, and neutral fractions. The acidic and basic fractions were examined by paper chromatography and the radioactivity in the separated components was determined. Values for radioactivity are in millimicrocuries.

Experiment 1

Additions (micromoles)	Malate	Succinate	Fumarate	Others
Pyruvate 5, NADP 2, $NADPH_2$ regenerating system	14.20	1.11	1.83	0.84

Table 13

Experiment 2

Additions (micromoles)	Rf								
	0.01	0.10	0.23	0.34	0.41	0.48	0.70	0.81	Amino acids
a. Nil	0.69	0.67	-	1.39	-	-	1.08	-	2.54
b. Pyruvate 5, ATP 5.	3.49	0.76	-	6.51	-	-	2.62	-	3.74
c. Propionyl CoA 2, ATP 5.	5.22	1.25	-	5.20	1.53	0.59	6.47	1.11	3.90
d. Propionyl CoA 2, Pyruvate 5, ATP 5.	5.80	2.18	0.45	4.34	1.50	0.51	4.21	1.08	6.37

a column of cation-exchange resin but 99 per cent of the total radioactivity was retained by a column of anion-exchange resin. All of the radioactivity retained by the anion-exchange resin was eluted by formic acid. The formic acid eluate was examined by paper chromatography using the solvent mixture butan-1-ol-2 methyl propan-1-ol-water-formic acid. The distribution of radioactivity among the components separated on the chromatogram is shown in experiment 1 of Table 13. The sum of the radioactivities of the components eluted from the chromatogram accounted for 95 per cent of the total radioactivity originally applied to the paper. The radioactive components were identified by chromatography and radioautography. Malate contained 79 per cent of the total radioactivity recovered from the chromatogram; lesser amounts were present in fumarate (10 per cent) and succinate (6 per cent).

Degradation of radioactive malate. The radioactive malate isolated from reaction mixture d, experiment 1, Table 12 was degraded to acetate and carbon dioxide. The radioactivity recovered in these products accounted for 98 per cent of the radioactivity in the malate at the beginning. The distribution of the radioactivity among these products indicated that the malate was labelled in the following manner (the figures are per cent of the total radioactivity):

COOH---CH_2---CHOH---COOH
79.4 17.3 3.3

Unfortunately the acetate was not sufficiently radioactive to permit further degradation to one-carbon units. Therefore it was not possible to determine the distribution of radioactivity between the methylene and hydroxymethylene carbon atoms of the radioactive malate.

The presence of the largest proportion of the total radioactivity in the beta carboxyl group of malate was consistent with a reductive carboxylation of pyruvate as catalysed by "malic" enzyme. The small proportion of the total radioactivity in the alpha carboxyl group could have entered this position if malate was in equilibrium with fumarate, a symmetrical dicarboxylic acid. The identification of radioactive fumarate and succinate in the reaction mixture lent support to this notion. Much more puzzling was the presence of a significant proportion of the total radioactivity in the methylene and/or hydroxymethylene carbon atoms of malate.

It was shown (Chapter 8) that intact Ochromonas cells incorporated radioactivity from (^{14}C) bicarbonate into alanine. It was suggested that pyruvate might become radioactive if the radioactivity incorporated into the beta carboxyl group of malate or oxaloacetate became distributed between both carboxyl groups as a result of the

Table 14

The influence of cysteine in the homogenising medium on the incorporation of radioactivity from sodium (^{14}C) bicarbonate by broken cell preparations.

The conditions of the reactions were the same as described in Table 12. *Ochromonas* cells were suspended in 10mM tris-HCl buffer. The suspension was divided into two equal portions. Solid cysteine hydrochloride was added to one portion so that the final concentration of cysteine was 1mM ; the other portion was untreated. Both suspensions were homogenised and the broken cell preparations were used in the reaction mixture as described in Table 12.

Expt.	Additions (micromoles)	Cysteine in cell preparation	Radioactivity fixed (millimicrocuries)
1.	Propionyl CoA 2, pyruvate 5, ATP 5.	+	4.74
2.	Propionyl CoA 2, pyruvate 5, ATP 5.	-	0.09
3.	Pyruvate 5, NADP 2, $NADPH_2$, regenerating system.	+	12.70
4.	Pyruvate 5, NADP 2, $NADPH_2$, regenerating system.	-	0.14

fumarase catalysed equilibrium with symmetrical fumarate. Then carboxyl-labelled pyruvate could be produced by a reversal of the reactions catalysed by "malic" enzyme or pyruvate carboxylase and radioactive alanine would be the product of a transamination reaction with radioactive pyruvate. However the small proportion of the total radioactivity found in the alpha carboxyl group of malate indicated that the distribution of radioactivity between the two carboxyl groups, via the fumarase catalysed reaction, was not occurring to a significant extent in the broken cell preparation. If the distribution of radioactivity among the carbon atoms of malate produced by the broken cell preparation was a true reflection of the situation in the intact cell, then radioactive alanine must be formed by some means other than reversal of the "malic" enzyme or pyruvate carboxylase catalysed reactions.

Requirement of cysteine for the fixation of (^{14}C) bicarbonate by broken cell preparations.

The ability of Ochromonas broken cell preparations to fix (^{14}C) bicarbonate was found to be dependent on the presence of cysteine in the homogenising medium. The fixation of (^{14}C) bicarbonate normally observed in the presence of pyruvate and $NADPH_2$ or pyruvate, propionyl CoA and ATP could not be demonstrated with preparations which had been made in the absence of cysteine (Table 14).

Table 15

Malate-dependent reduction of NADP by a broken cell preparation of Ochromonas.

Silica cuvettes (light path, 1 cm.) contained the following components (quantities are in micromoles) in a final volume of 3.0 ml.: tris-HCl buffer pH 8.0 100, $MgCl_2$ 25, cysteine hydrochloride 10 and the additions listed below. Each reaction mixture also contained 0.1 ml. of a 57300g. supernatant of a broken cell preparation of Ochromonas (0.61 mg. of protein). The rates of the reactions were determined by observing the change in extinction at 340 millimicrons during the time 15 to 45 seconds after the addition of the last reactant listed below. In experiments 5 to 8 cysteine was omitted from the reaction mixture.

Expt.	Additions (micromoles)	Change in E per minute at 340 millimicrons.
1.	NADP 0.1	0
2.	NAD 0.1	0
3.	NADP 0.1, malate 2.	0.136
4.	NAD 0.1, malate 2.	0
5.	NADP 0.1, malate 2, no cysteine.	0.072
6.	p-CMB $3x10^{-4}$, NADP 0.1, malate 2, no cysteine.	0.042
7.	p-CMB $3x10^{-3}$, NADP 0.1, malate 2, no cysteine.	0.032
8.	p-CMB $3x10^{-2}$, NADP 0.1, malate 2, no cysteine.	0.002
9.	p-CMB $3x10^{-2}$, NADP 0.1, malate 2	0.058

Malate dependent reduction of NADP.

Since the carboxylation reaction catalysed by "malic" enzyme was coupled with oxidation of $NADPH_2$ it would be possible to follow the reaction in a spectrophotometer by measuring the change in extinction at 340 millimicrons. The compounds participating in this reaction were also potential substrates for the reactions catalysed by malate and lactate dehydrogenases. Lactate dehydrogenase activity could not be demonstrated in an Ochromonas broken cell preparation using the assay system described by Neilands (1955) but the same preparation did possess malate dehydrogenase activity. Therefore it was possible malate dehydrogenase might interfere with the "malic" enzyme assays. However this possibility was circumvented by centrifuging the broken cell preparation at 57,300g. Malate dehydrogenase activity was associated with the particulate material while "malic" enzyme activity remained in the supernatant liquid under these conditions (Table 16). Therefore the 57,300g supernatant of Ochromonas broken cell preparations was used for the spectrophotometric measurements of "malic" enzyme activity.

The results of a series of spectrophotometric assays demonstrating the malate dependent reduction of NADP are shown in Table 15. Experiments 1 and 2 showed that NAD and NADP were not reduced in the absence of malate. In the

presence of malate no reduction of NAD could be observed (experiment 4), hence the 57,300g supernatant was devoid of malate dehydrogenase activity under these conditions. When NAD was replaced by NADP (experiment 3) the extinction of the reaction mixture increased at the rate of 0.223 per minute per mg. of protein in the presence of malate.

It was apparent from experiments 5 to 9 that thiol groups were necessary for the activity of "malic" enzyme. When cysteine was omitted from the reaction mixture the rate of reaction was 58 per cent of that observed with the complete system. When p-chloro mercuribenzoate (p-CMB) was added to the assay system minus cysteine at concentrations of 10^{-7}, 10^{-6} and 10^{-5} M the rate of the reactions were 58 per cent, 44 per cent and 3 per cent, respectively, of the rate in an unsupplemented system. When excess cysteine was added to the assay system prior to the addition of p-CMB to a concentration of 10^{-5} M (reaction 9) the rate was 75 per cent of that in the control (reaction 5). This indicated that the inhibitory effect of p-CMB was due to its property of forming a mercaptide with thiol groups.

The relatively crude 57,300g supernatant used in the assays described above would have provided a large number of p-CMB binding sites other than those essential for "malic" enzyme activity. Furthermore cysteine carried over in the 57,300g supernatant also would bind p-CMB. Therefore the "malic" enzyme activity was probably more sensitive to p-CMB

Table 16

The distribution of "malic enzyme" and malate dehydrogenase activities between supernatant and particulate fractions obtained from a broken cell preparation of Ochromonas.

The conditions for experiment 1 were the same as described in Table 15. In experiment 2 the reaction mixture contained the following components in a final volume of 3.0 ml., (quantities are expressed in micromoles) : tris-HCl buffer pH 8.0 100, $MgCl_2$ 25, and the additions listed below. In both experiments 1 and 2 the rates of reaction were determined by observing the change in extinction at 340 millimicrons. In experiment 3 the standard reaction mixture described in Table 12 was used and the conditions of the experiment were also the same as in Table 12. For all 3 experiments a broken cell preparation of Ochromonas was divided into two portions. One portion (900g supernatant) was kept on ice while the other portion was centrifuged at 57,300g for 30 minutes. The supernatant was removed and kept on ice (57,300g supernatant) while the particulate material was suspended in a volume of the cysteine-tris buffer equal to the volume of the supernatant (57,300g particulate).

Table 16

Expt.	Additions (micromoles)	Cell preparation	Change in E per minute at 340 millimicrons.	Radioactivity fixed millimicrocuries
1.	Malate 2, NADP 0.1	900g supernatant	0.118	
	Malate 2, NADP 0.1	57300g supernatant	0.086	
	Malate 2, NADP 0.1	57300g particulate	0.005	
2.	$NADH_2$ 0.4, oxaloacetate 2	900g supernatant	0.150	
	$NADH_2$ 0.4, oxaloacetate 2	57300g supernatant	0	
	$NADH_2$ 0.4, oxaloacetate 2	57300g particulate	0.138	
	$NADPH_2$ 0.4, oxaloacetate 2	900g supernatant	0.002	
3.	Nil	900g supernatant		0.70
	Pyruvate 5, NADP 2, $NADPH_2$ regenerating system	900g supernatant		15.53
	Pyruvate 5, NADP 2, $NADPH_2$ regenerating system	57300g supernatant		11.13
	Pyruvate 5, NADP 2, $NADPH_2$ regenerating system	57300g particulate		6.76

than these experiments indicated. Rutter and Lardy (1958) reported that the activity of a purified "malic" enzyme from pigeon liver was inhibited 100 per cent by 10^{-6} M p-CMB.

Distribution of "malic" enzyme and malate dehydrogenase between soluble and particulate fractions of Ochromonas cells.

It was apparent from the results shown in Table 16 that the enzyme catalysing the malate dependent-reduction of NADP was largely confined to the 57,300g supernatant obtained from a broken cell preparation of Ochromonas (experiment 1). Summation of the enzyme activities found in the 57,300g supernatant and particulate fractions showed that only 77 per cent of the "malic" enzyme activity in the original 900g supernatant was recovered in these two fractions. Approximately 73 per cent of the original enzyme activity was recovered in the 57,300g supernatant.

Concurrently these same three preparations (900g supernatant, 57,300g supernatant and 57,300g particulate) were tested for their ability to fix (^{14}C) bicarbonate in the presence of pyruvate and $NADPH_2$ using the standard assay mixture (experiment 3). Under these conditions the 57,300g supernatant was able to fix 71 per cent and the 57,300g particulate material 4 per cent of the amount of radioactivity fixed by the 900g supernatant. Thus a 25 per cent loss in overall activity was incurred during the

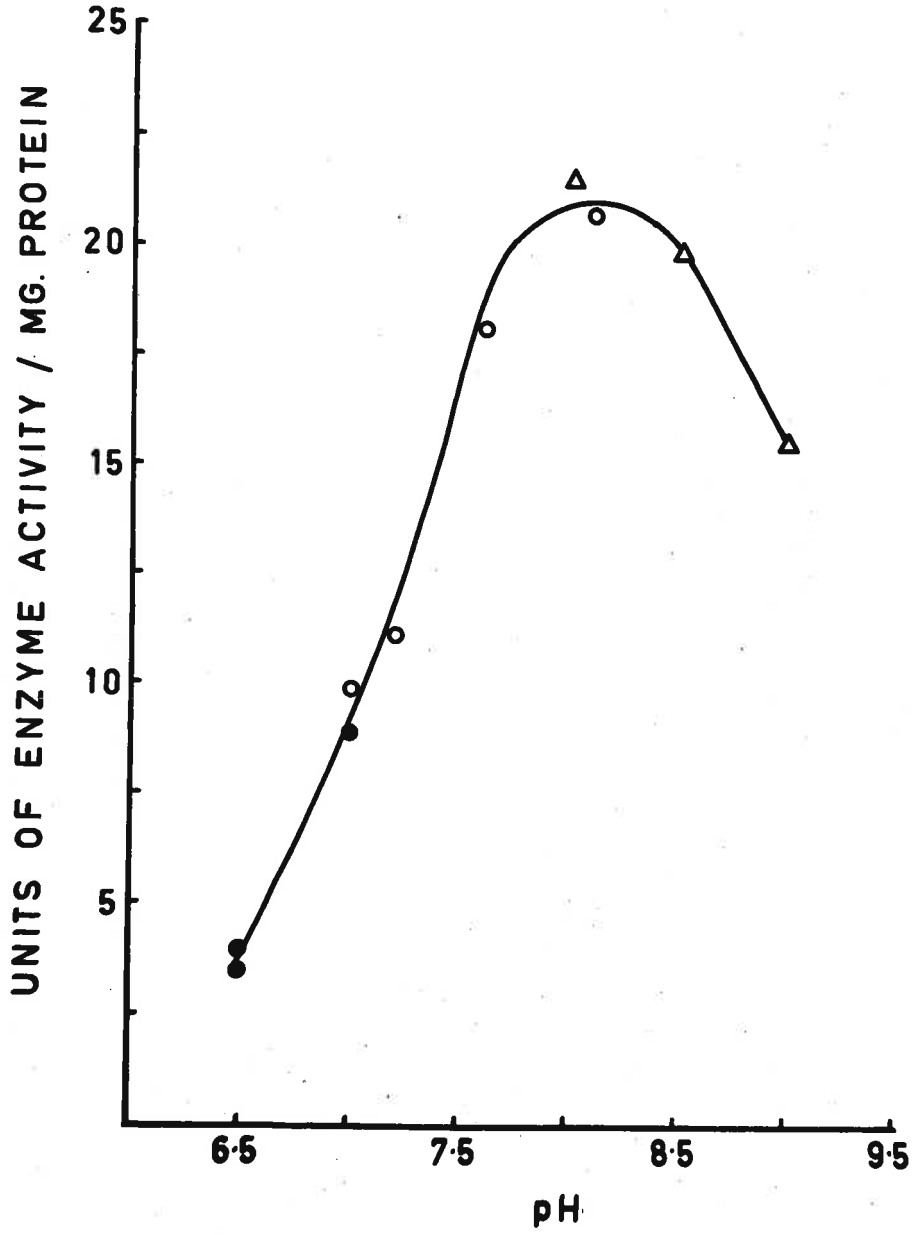
Figure 12

Effect of pH on the malate-dependent reduction of NADP.

Silica cuvettes (light path, 1 cm.) contained the following components in a total volume of 3.0 ml. : 100 micromoles of buffer, 25 micromoles of $MgCl_2$, 10 micromoles of cysteine hydrochloride, 0.1 micromole of NADP and 0.1 ml. of a 57300g. supernatant of an Ochromonas broken cell preparation. The reactions were started by the addition of 2 micromoles of potassium malate to the reactants in the test cell while an equivalent volume of water was added to the reactants in the reference cell. The change in extinction at 340 millimicrons was recorded by a Shimadzu type RS-27 automatic recording spectrophotometer. The rate of reaction was obtained from the difference in extinction at 15 and 45 seconds after the addition of the malate. One enzyme unit was that amount causing a change in extinction of 0.01 per minute under the above conditions. Protein was determined by a method modified from that of Westley and Lambeth (1960) (see chapter 4, fraction D).

● sodium phosphate-citric acid buffers.
○ sodium phosphate-potassium phosphate buffers.
△ tris-HCl buffers.

FIGURE 12.



preparation of the 57,300g fractions. The ratio of enzyme activities in the 900g and 57,300g supernatant preparations was the same for both the spectrophotometric and isotopic assay systems using the same broken cell preparation; implying that both assays were measuring the same enzyme activity. Furthermore these assay systems were measuring the reaction from opposite directions thus demonstrating the reversibility of the reaction.

The distribution of malate dehydrogenase was examined with the same enzyme preparations (experiment 2). This enzyme was shown to occur in the 57,300g particulate material; no activity could be detected in the 57,300g supernatant. When $NADPH_2$ was substituted for $NADH_2$ in the assay system only a trace of activity was observed. Therefore malate dehydrogenase was specific for NAD.

The effect of pH on "malic" enzyme activity.

Ochromonas "malic" enzyme exhibited a moderately sharp maximum in activity at pH 8.0 in the presence of 6.6×10^{-4} M malate. The pH at which maximum activity was observed with a purified "malic" enzyme from pigeon liver was found to be dependent on the concentration of malate in the reaction mixture (Rutter and Lardy 1958). Maximum activity was observed at pH 7.7 in the presence of 10^{-3} M malate and this maximum had shifted to pH 7.2 when the malate concentration was reduced to 10^{-4} M. (Figure 12.)

Specific activity of "malic" enzyme. It was interesting that the specific activity of "malic" enzyme in the 57,300g supernatant was in the vicinity of 22 units of enzyme activity per mg. of protein. A 40,000g supernatant obtained from a homogenate of pigeon liver (an especially good source of NADP-linked "malic" enzyme) was reported to have a specific activity of 9.6 units of enzyme activity per mg. of protein. (Rutter and Lardy 1958). Therefore Ochromonas would appear to be an even better source of the enzyme than pigeon liver.

If the 57,300g supernatant, obtained from broken cell preparations of Ochromonas, was frozen and thawed once a heavy precipitate formed. When this precipitate was removed by centrifuging at 57,300g for 30 minutes the protein concentration of the supernatant was only 30 per cent of the protein concentration in the unfrozen supernatant. In some instances the specific activity of the "malic" enzyme in the frozen and thawed supernatant was as high as 59 units of enzyme activity per mg. of protein. However this level of specific activity was not consistently attained.

Coupling of "malic" enzyme and malate dehydrogenase.

It was suggested in the discussion of Chapter 8 that the coupling of the "malic" enzyme and malate dehydrogenase catalysed reactions was a possible pathway for the incorporation of (^{14}C) bicarbonate into oxaloacetate and hence

aspartate:

$$\text{pyruvate} + CO_2 + PNH_2 \rightleftharpoons \text{malate} + PN$$

$$\text{malate} + PN \rightleftharpoons \text{oxaloacetate} + PNH_2$$

$$\text{Sum: pyruvate} + CO_2 \rightleftharpoons \text{oxaloacetate}$$

where PN and PNH_2 represent the oxidized and reduced forms of either NAD or NADP.

The results presented above showed that Ochromonas possessed an enzyme which catalysed the NADP-dependent reductive carboxylation of pyruvate to form malate. Ochromonas broken cell preparations were also shown to have malate dehydrogenase activity, but this enzyme required NAD as a cofactor and was virtually inactive with NADP. Because of their different nucleotide-cofactor specificities it was not possible for the reactions catalysed by these two enzymes to be fully coupled through substrate and cofactors.

The "malic" enzyme was shown to be present in the soluble portion of Ochromonas broken cell preparations. Although the malate dehydrogenase activity was in the particulate portion of the broken cell preparations, it was not known whether the enzyme was membrane bound. There has been no agreement among investigators of mitochondrial function concerning the permeability or impermeability of

the mitochondrial membrane to solutes of metabolic importance (Bellamy and Bartley 1960). Therefore coupling of the "malic" enzyme and malate dehydrogenase catalysed reactions through malate could not be eliminated on these grounds.

It was shown that the oxidation of malate catalysed by malate dehydrogenase was an endergonic reaction and the equilibrium greatly favoured the reduction of oxaloacetate (Burton and Wilson 1953). "Malic" enzyme from avian liver or Kalanchoe crenata was shown to have a low affinity for pyruvate and carbon dioxide (Utter 1959, Walker 1962). Also the equilibrium position of the "malic" enzyme catalysed reaction in air was far in the direction of decarboxylation (Johnson 1960). Thus incorporation of (^{14}C) bicarbonate into oxaloacetate would be rather unlikely unless there were some means of pulling the reactions in the direction of carboxylation.

If "malic" enzyme was the chief agent of (^{14}C) bicarbonate fixation in the intact cell, then malate should be the primary fixation product. However, it was definitely established with intact cells that aspartate and hence oxaloacetate became radioactive prior to malate (Chapter 8). These findings were not consistent with the incorporation of (^{14}C) bicarbonate into malate and oxidation of malate to oxaloacetate as catalysed by "malic" enzyme and malate dehydrogenase respectively.

Ochromonas broken cell preparations were found to contain high concentrations of "malic" enzyme and yet this enzyme appeared to have only a minor part in the bicarbonate fixing activities of the cell during endogenous respiration. A possible alternate function for this enzyme may be to catalyse the reverse of the carboxylation reaction, i.e. the oxidative decarboxylation of malate. In this capacity "malic" enzyme may be catalysing the formation of $NADPH_2$ for reductive biosynthesis.

When Ochromonas was cultured under the conditions described in this report, lipid accounted for 20 per cent of the dry weight of cell material. Cells which had been cultured for periods of ten days were reported to contain 33 per cent of lipid (Aaronson and Baker 1961). In view of the preference which the fatty acid-synthesising enzymes show for $NADPH_2$ (Wakil, Titchener and Gibson 1959), Ochromonas would need a generous supply of this reduced nucleotide to be able to synthesise such quantities of lipid.

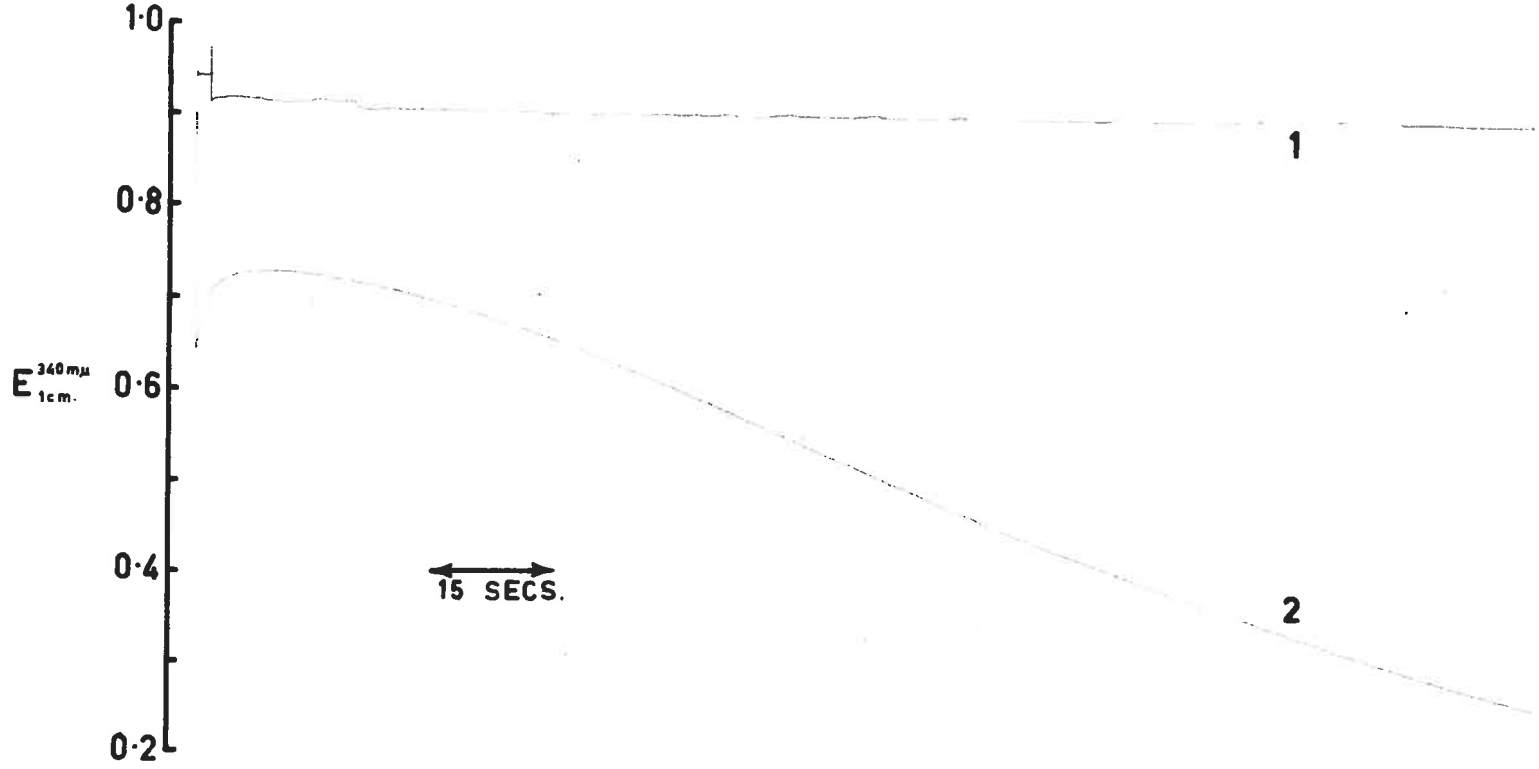
There have been two separate reports that Ochromonas did not have a functioning pentose phosphate pathway (Reazin 1956, Kauss and Kandler 1962). Therefore, the reaction catalysed by "malic" enzyme may be a major source of $NADPH_2$ in the absence of pentose phosphate pathway dehydrogenases. If this was the major purpose of the "malic" enzyme catalysed reaction in Ochromonas, then this enzyme probably would not

Figure 13

Pyruvate kinase activity in a broken cell preparation of Ochromonas.

Silica cuvettes (light path, 1 cm.) contained the following reactants in a final volume of 3.0 ml. : 150 micromoles of tris-HCl buffer pH 7.2, 0.45 micromole $NADH_2$, 0.7 micromole ADP, 225 micromoles KCl, 24 micromoles of $MgSO_4$, 2250 units of L-lactate: NAD oxidoreductase (pyruvate kinase free) and 0.05 ml. of broken cell preparation. The reference cell contained the previous reactants with the exception of $NADH_2$ and ADP. Trace 1 was obtained with the previous reactants showing the broken cell preparation did not have $NADH_2$ oxidase activity which might have interfered with the measurement of the pyruvate kinase. Trace 2 was obtained when 2.35 micromoles of phosphopyruvate were added to the test cell.

FIGURE 13.



participate in the endogenous metabolism of the organism during respiration in the absence of exogenous substrates, since reductive biosynthesis would not be expected under these conditions.

Fixation of (^{14}C) bicarbonate by broken cell preparations in the presence of phosphopyruvate.

It was found that the addition of phosphopyruvate, in combination with a number of cofactors, to the standard reaction mixture did not cause a significant increase in the amount of radioactivity fixed from (^{14}C) bicarbonate by broken cell preparations of Ochromonas (experiment 2, Table 12). The slight increase in fixation observed when phosphopyruvate and ADP were added to the standard reaction mixture was probably not due to the carboxylation of phosphopyruvate. In addition to being potential substrates for the carboxylation reaction catalysed by phosphopyruvate carboxykinase, phosphopyruvate and ADP can also be substrates for the reaction catalysed by pyruvate kinase. This enzyme was detected in Ochromonas broken cell preparations (Figure 13). Therefore some of the added phosphopyruvate would have been converted to pyruvate and significant fixation of radioactivity already had been observed in the presence of this compound (experiment 1, reaction b).

Apparently Ochromonas broken cell preparations did not contain phosphopyruvate carboxylase or phosphopyruvate carboxytransphosphorylase (reactions b and d, respectively).

Also the same preparations did not appear to possess phosphopyruvate carboxykinase activity. However a complete range of nucleoside diphosphates was not available and tests for this enzyme were restricted to an examination of the effects of GDP and ADP on the fixation of (^{14}C) bicarbonate in the presence of phosphopyruvate. It was known that all phosphopyruvate carboxykinases examined in animal tissues were active with IDP as well as GDP (Utter 1960). The yeast enzyme used only adenosine derivatives (Cannata and Stoppani 1959). The enzyme in E. coli was specific for ADP rather than IDP (Amarasingham and Umbarger 1955). Plant enzymes appeared to be active with ADP (Mazelis and Vennesland 1957). In view of the aforementioned reports it seemed that Ochromonas broken cell preparations were not likely to catalyse a phosphopyruvate carboxylation reaction specific for a nucleoside diphosphate other than GDP or ADP.

Incorporation of (^{14}C) bicarbonate into polysaccharide.

In Chapter 8 it was shown that intact Ochromonas cells incorporated radioactivity from (^{14}C) bicarbonate into polysaccharide (Table 10). Since it was not possible to demonstrate a condensation of carbon dioxide with phosphopyruvate, then radioactivity was not transferred from oxaloacetate to polysaccharide by reversal of the reactions catalysed by phosphopyruvate carboxykinase and

phosphopyruvate carboxytransphosphorylase (the reaction catalysed by phosphopyruvate carboxylase is generally regarded to be irreversible).

The only other means of transferring radioactivity from (^{14}C) bicarbonate to polysaccharide via dicarboxylic acids was by reversal of the reaction catalysed by pyruvate kinase -

phosphopyruvate + ADP ⟶ pyruvate + ATP.

Since intact Ochromonas cells incorporated radioactivity from (^{14}C) bicarbonate into alanine, it was known that radioactive pyruvate could be formed from (^{14}C) bicarbonate, as pyruvate was an obligate precursor of alanine. The actual mechanism of this latter process has been discussed previously. Quite strong pyruvate kinase activity was demonstrated in Ochromonas broken cell preparations by spectrophotometric assay (Figure 13).

The equilibrium of the reaction catalysed by pyruvate kinase was found to be far over in the direction of pyruvate formation (McQuate and Utter 1959). There was a considerable energy barrier to be overcome if the reaction was to proceed in the direction of phosphopyruvate synthesis; ΔF^{o} for the reaction as written above was - 6.3 k cal (Burton 1960). Although the ratio between the maximal forward and back

reactions catalysed by pyruvate kinase was not markedly unfavourable to the synthesis of phosphopyruvate, it was found that the enzyme had a low affinity for pyruvate (McQuate and Utter 1959). These authors suggested that the concentrations of pyruvate and ATP necessary for a significant synthesis of phosphopyruvate probably would not be attained in vivo. Nonetheless, observations have been made which suggested that pyruvate carbon did reach glycogen via the pyruvate kinase catalysed reaction (Utter 1959, Roberman and D'Adamo 1960, Hiatt, Goldstein, Lareau and Horecker 1958).

Kauss and Kandler (1962) observed radioactive phosphate esters in ethanol extracts of Ochromonas cells which had been exposed to (^{14}C) bicarbonate in the dark. They suggested that these phosphate esters might be formed in the dark in a limited way by cyclic carboxylation of ribulose diphosphate. In light Ochromonas was found to incorporate (^{14}C) bicarbonate primarily through the autotrophic ribulose diphosphate carboxylation cycle (Kauss and Kandler 1963). Since these authors had used light-grown cells for their dark-fixation experiments, then the enzymes of the autotrophic carboxylation cycle certainly were present at the time of the dark incorporation of (^{14}C) bicarbonate. However it was not known whether the cells employed in the present investigation possessed the enzyme catalysing the carboxylation of ribulose diphosphate since these cells

were cultured in the dark.

It was shown that Micrococcus denitrificans, Hydrogenomonas ruhlandii and species of Thiobacillus only formed ribulose diphosphate carboxylase when these chemosynthetic organisms were grown autotrophically (Kornberg, Collins and Bigley 1960, Vishniac and Santer 1957). Lascelles (1960) found only traces of ribulose diphosphate carboxylase in the facultative photosynthetic bacterium Rhodopseudomonas spheroides when the organism was grown aerobically in the dark, but cells which were grown photosynthetically were rich in the enzyme. Oxygen completely suppressed enzyme formation even in the light. Fuller and Gibbs (1959) reported that ribulose diphosphate carboxylase was present only in algae which were grown in light with carbon dioxide. Nevertheless Ochromonas appeared to be an exception to the above findings since Kauss and Kandler (1963) grew the organism aerobically in the light and demonstrated fixation of (^{14}C) bicarbonate into 3 phosphoglycerate. In the present investigation Ochromonas was grown aerobically in the absence of light and the cells were exposed to (^{14}C) bicarbonate (Chapter 8). Radioautographs were made from chromatograms of ethanol extracts of these cells. After 3 minutes exposure to the isotope it was apparent that the area, near the origin of the two-dimensional chromatograms, where phosphate esters were located was acquiring an increasing proportion of the total radioactivity

on the chromatogram (Figure 10b).

In view of the uncertainty concerning the presence of the ribulose diphosphate carboxylation pathway and the improbability of reversing the pyruvate kinase catalysed reaction, it was not possible at the present stage to reach any conclusions concerning the pathway of incorporation of (^{14}C) bicarbonate into polysaccharide.

Only a small proportion of the total radioactivity fixed by intact Ochromonas cells was incorporated into polysaccharide (Table 10). It was not established whether this incorporation was the result of a net transfer of carbon to polysaccharide or an exchange between carbon dioxide and polysaccharide. Since the cells were respiring from endogenous resources then the polysaccharide was being rapidly utilized and net synthesis of the reserve material would be unlikely under these conditions. Therefore (^{14}C) bicarbonate probably was incorporated into the polysaccharide by exchange. The pathway of this incorporation probably reflects the pathway by which polysaccharide might be synthesised from carbon dioxide in the dark under conditions favourable for the accumulation of reserve materials.

Fixation of (^{14}C) bicarbonate by broken cell preparations in the presence of other dicarboxylic acid precursors.

It was apparent from the results of experiments 3 and

4 in Table 12 that the addition of pyruvate or propionyl CoA or ATP alone to the standard reaction mixture caused significant increases in the amount of radioactivity fixed from (^{14}C) bicarbonate by broken cell preparations of Ochromonas. When these three compounds were added to the standard reaction mixture in various combinations (experiment 3, reaction d, experiment 4, reactions d and e) the amounts of radioactivity fixed by the combinations were less than the sum of the amounts of radioactivity fixed by the individual compounds. Either the fixation in the presence of each of these three compounds was a manifestation of the same reaction or the reactions were different but involved a common reactant (not bicarbonate since this was present in excess).

Reaction d, experiment 3 and reactions d and e, experiment 4 were repeated using 50 microcuries of sodium (^{14}C) bicarbonate, since the amounts of radioactivity fixed in the original experiments were insufficient for the analysis and identification of the radioactive products. The reaction mixtures were treated with ethanol and partitioned between a chloroform, methanol and water mixture. The upper phase was treated with ion-exchange resins and separated into acidic, basic and neutral fractions as described in the methods section. In all cases both the acidic and basic fractions contained radioactive material

while the neutral fraction did not. The acidic fractions were examined by paper chromatography with the solvent mixture butan-1-ol, methyl propan-1-ol, water, formic acid. A series of radioactive compounds were located on the chromatograms with the radioactive paper-strip scanner and the Rf values and radioactivities of these components are shown in Table 13 experiment 2. None of these radioactive components could be shown to possess amino groups when they were treated with a ninhydrin reagent (Toennies and Kolb 1951), nor did they give a positive test for phosphate when they were treated with a molybdate reagent (Bandurski and Axelrod 1951). Malate (compound B) was the only one of these radioactive compounds which could be positively identified by paper chromatography with at least two solvent systems and radioautography. The mobilities of the major radioactive components and some authentic markers chromatographed with three solvent systems are listed below.

Solvent 1 was the butanol, methyl propanol, water, formic acid mixture of Ladd and Nossal (1954) and the values listed under this solvent are the same as those already shown in Table 13. Solvent 2 was the butanol, pyridine, acetic acid, water mixture of Holdsworth (1961) which was described previously. Solvent 3 consisted of propan-2-ol-ammonia (S.G. 0.88) - water (14:1:5 by volume) (Cheftel, Munier and Macheboeuf 1952).

	Solvents		
	1	2	3
	Rf	Rsuccinate	Rf
Citrate	0.34	0.35	0.11
Malate	0.41	0.47	0.22
Succinate	0.70	1.00	0.27
Methyl malonate	0.82	0.90	0.24
Fumarate	0.87	0.73	0.31
A	0.34	0.12	-
B	0.41	0.47	-
C	0.70	-	0.22
D	0.81	0.78	-

The acidic fractions contained 78, 84 and 76 per cent of the total radioactivity fixed in reactions b, c and d respectively of experiment 2, Table 13. The rest of the radioactivity was contained in the basic fractions. Tris was separated from the radioactive components in the basic fractions by paper electrophoresis and then the radioactive compounds were separated by paper chromatography with a butan-2-ol, ammonia, water solvent. One radioactive compound accounted for approximately 90 per cent of the total radioactivity in the basic fraction of all mixtures of experiment 2. Three other components separated on the chromatogram only contained trace amounts of radioactivity.

The major radioactive compound of the basic fraction gave the characteristic colour reaction of an amino acid when it was treated with ninhydrin (Toennies and Kolb 1951), however it could not be identified with a number of known amino acids. The mobility of the unknown compound and several authentic amino acids with three solvent systems are listed below.

	Solvents		
	1	2	3
	Rf	Rf	Rf
Unknown	0.21	0.32	0.34
Glycine	0.23	0.46	0.34
Serine	0.24	0.51	0.37
Alanine	0.40	-	0.52
Aspartic acid	0.17	0.29	0.34
Glutamic acid	0.23	0.36	0.45
Asparagine	0.12	0.40	0.20
Glutamine	0.18	0.39	0.30
Arginine	0.08	0.15	-
gamma-Amino Butyric acid	-	0.44	-

Solvent 1 was the methanol, butanol, water mixture previously described and solvent 2 consisted of ethanol-butanol-water-dicyclohexylamine (10:10:5:2 by volume) (Hardy, Holland and Naylor 1955). Solvent 3 was the methanol, water pyridine

mixture of Redfield (1953).

It was apparent from experiments 3 and 4, Table 12 that Ochromonas broken cell preparations were able to catalyse at least one other primary carboxylation reaction in addition to the reaction catalysed by "malic" enzyme. However it seemed that the crude broken cell preparation was capable of converting the products of the fixation reactions to a number of other unidentified compounds. Therefore it was not possible to define the primary carboxylation reaction under these conditions.

Biotin and (^{14}C) bicarbonate fixation by Ochromonas.

The distribution of radioactivity among the compounds found in ethanol extracts of normal Ochromonas cells which had been exposed to (^{14}C) bicarbonate in the dark was greatly altered when biotin-deficient cells were employed (Kauss and Kandler 1962). The biotin-deficient cells were characterized by a complete inability to incorporate radioactivity from (^{14}C) bicarbonate into aspartic acid. At the same time the amount of radioactivity incorporated into malate and citrate by the deficient cells was only approximately 40 and 20 per cent, respectively, of the amount incorporated by normal cells.

The effect of avidin, an inhibitor of biotin activity, on the incorporation of (^{14}C) bicarbonate by Ochromonas broken cell preparations was examined during the present

Table 17

The effect of avidin on the incorporation of radioactivity from (^{14}C) bicarbonate by broken cell preparations of Cohnomonas.

The standard reaction mixture and assay conditions were the same as described in Table 12. The broken cell preparation was divided into three equal portions. One portion was untreated (A), another was preincubated with avidin (1 unit/ml. of preparation) (B) and the third was preincubated with avidin which had been pre-treated with excess biotin (10 micrograms of biotin/unit of avidin/unit of preparation) (C). Preincubation was carried out for 15 minutes on ice.

Expt.	Additions (micromoles)	Radioactivity fixed (millimicrocuries)		
		A	B	C
1.	Pyruvate 5, NADP 2, $NADPH_2$ regenerating system	12.70	12.05	10.80
2.	ATP 5	4.91	1.54	4.65
3.	Propionyl-CoA 2, ATP 5	4.84	1.65	5.18
4.	Pyruvate 5, ATP 5	5.58	1.95	4.08

investigation.

Preincubation of the broken cell preparation with avidin failed to alter significantly the quantity of radioactivity fixed in the presence of pyruvate and $NADPH_2$ (experiment 1, Table 17). However preincubation with avidin caused a reduction of approximately 70 per cent in the amount of radioactivity fixed by the broken cell preparations in the presence of either pyruvate or ATP or propionyl CoA (experiments 2, 3 and 4). It was apparent that this effect of avidin was due specifically to its property of binding biotin since avidin which had been treated with an excess of biotin failed to cause any significant reduction in the amount of radioactivity fixed from (^{14}C) bicarbonate in the presence of pyruvate, propionyl CoA and ATP. (column 3, Table 17).

The inability of avidin to exert any effect on the activity of Ochromonas "malic" enzyme was in accord with a previous report (Kaziro et al. 1960). Biotin did appear to have a role in the undefined carboxylation reaction which was elicited by the addition of pyruvate, propionyl CoA or ATP to the broken cell preparation. Biotin has been implicated in the carbon dioxide fixation reactions catalysed by the enzymes pyruvate carboxylase (Utter and Keech 1960), propionyl carboxylase (Kaziro et al. 1960) and methyl malonyl CoA : pyruvate carboxytransferase (Swick and Wood 1960).

Thus the undefined carboxylation reaction might have been a manifestation of the activity of one of these three enzymes in the Ochromonas broken cell preparation.

Conclusions.

Ochromonas malhamensis fixed (^{14}C) bicarbonate during endogenous respiration in the absence of exogenously furnished nutrient. Therefore fixation of carbon dioxide could explain why the R.Q. of endogenous respiration was less than 1.0 when this value was determined in a closed system.

During endogenous respiration intact cells incorporated (^{14}C) bicarbonate primarily into soluble intermediates. The remainder of the radioactivity fixed by the cells was directed mostly into protein; lesser amounts were in the polysaccharide reserve material. Aspartate was the first stable radioactive product formed from (^{14}C) bicarbonate by intact cells.

Broken cell preparations of Ochromonas were able to fix (^{14}C) bicarbonate by means of at least two carboxylation reactions. One of these reactions was found to produce malate by an NADP- linked reductive carboxylation of pyruvate. This reaction was reversible and specific for NADP. In these respects the reaction was similar to those catalysed by "malic" enzyme of plant or animal origin and it was not

akin to the "malic" enzyme reaction occurring in Lactobacillus arabinosus.

The other carboxylation reactions catalysed by the broken cell preparations were not defined. The crude preparation used to demonstrate these carboxylation reactions converted the immediate products of carboxylation to a number of other compounds so that the primary reactions were obscured. However it was apparent that there were two of these undefined carboxylation reactions and both were biotin dependent.

The broken cell preparations contained considerable amounts of "malic" enzyme. However the results of (^{14}C) bicarbonate incorporation experiments with intact cells indicated that this enzyme was not active, at least in catalysing carboxylation, during endogenous respiration. The same broken cell preparations were unable to catalyse the carboxylation of phosphopyruvate in the presence of a number of likely cofactors. Therefore the undefined carboxylation reactions were left to account for the incorporation of (^{14}C) bicarbonate into oxaloacetate and hence into aspartate by a transamination reaction. If carboxylation of phosphopyruvate is discounted then pyruvate carboxylase catalysed the only other known biotin-dependent carboxylation reaction where oxaloacetate was a direct product. Pyruvate did appear to be a participant in one of the undefined carboxylation reactions but there was no

other evidence to indicate that this reaction might be catalysed by pyruvate carboxylase.

It was evident that some purification of the broken cell preparation would be necessary before the nature of the undefined reactions might be determined. If the enzymes concerned were associated with the particulate components of the cell, the first step in purification would be to solubilize these enzymes. Then the usual procedures of salt or solvent fractionation might be sufficient to separate the carboxylation enzymes in the soluble preparation from the enzymes catalysing conflicting reactions. However precautions would have to be taken to ensure the maintenance of thiol groups during these procedures since it was found that thiol groups were essential for the activity of the undefined carboxylation enzymes (Table 14). If one of the undefined reactions was catalysed by pyruvate carboxylase and this enzyme had similar properties to its counterpart in avian liver (Utter 1961), then the marked lability of the enzyme would be a further impediment to purification.

These investigations have established that Ochromonas malhamensis was capable of fixing carbon dioxide during endogenous respiration in the absence of other exogenous carbon sources. The possibility of carbon dioxide fixation during endogenous respiration of micro-organisms is not

well recognized. Therefore, when the carboxylation reactions are properly defined, it would be of interest to investigate what role carbon dioxide has in the endogenous metabolism of Ochromonas.

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