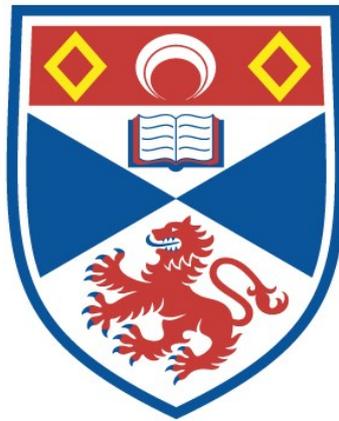


SOME INVESTIGATIONS INTO THE SARCINA
BACTERIA

Robin Henderson Christie Strang

A Thesis Submitted for the Degree of PhD
at the
University of St Andrews



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"SOME INVESTIGATIONS INTO THE SARCINA BACTERIA"

by

ROBIN HENDERSON CHRISTIE STRANG, B.Sc.

A THESIS

presented to the University of St. Andrews for the degree
of Doctor of Philosophy.

Biochemistry Department,
University of St. Andrews.

1968



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C E R T I F I C A T E

I hereby certify that ROBIN HENDERSON CHRISTIE STRANG has spent nine terms engaged on research work under my direction, and that he has fulfilled the conditions of Ordinance No.16 (St. Andrews), and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

D E C L A R A T I O N

I hereby declare that the following thesis is based on work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

The research was carried out in the Department of Biochemistry, University of St. Andrews, under the direction of Dr. D. Thirkell

ACADEMIC RECORD

I matriculated at the University of St. Andrews in October 1960, and graduated with the degree of Bachelor of Science, Second Class (1) Honours, in Biochemistry in June 1964, and was admitted as a research student to the Department of Biochemistry, University of St. Andrews.

I have been supported throughout this work by a grant from the Science Research Council.

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C O N T E N T S

	<u>Page</u>
<u>INTRODUCTION</u>	
1. Taxonomy of the Sarcina bacteria	1
2. General chemical structure and properties of carotenoids	6
3. Stereochemistry of the carotenoids	15
4. Extraction and analytical methods for carotenoids	19
5. Functions of carotenoids	42
6. Cellular location of carotenoids	55
7. Previous work on the carotenoids of the bacteria <u>S. flava</u> and <u>S. lutea</u>	67
 <u>EXPERIMENTAL SECTION.</u>	
<u>The morphology of the Sarcina bacteria</u>	
<u>The growth of cultures of <u>S. flava</u> at different temperatures:-</u>	
(a) Counting methods	73
(b) Growth with temperature on nutrient agar...	75
(c) Growth in nutrient broth	77
(d) Effect of the enrichment of the growth medium with glucose	77
Culture of the bacteria	78
<u>Bulk culture of the bacteria:-</u>	
(a) Using large aluminium dishes	79
(b) Using large developing dishes	80
(c) Bulk liquid culture	80
Extraction of the pigments	85
<u>Purification procedures:-</u>	
(a) Lipid precipitation	87
(b) Saponification	88

CONTENTS (Contd.)

	<u>Page</u>
<u>Experiments to determine the loss in absorbance of a carotene solution with time, under a variety of conditions:-</u>	
(a) The loss in absorbance of a carotene solution under various conditions.....	90
(b) The effects of sampling	91
The effect of an anti-oxidant on carotene degradation	92
<u>Pigment production of <i>S. flava</i> with variations in:-</u>	
(a) time of growth at 34°C	93
(b) temperature of culture	94
(c) pH of the medium	94
(d) enrichment of the medium with different concentrations of glucose	95
<u>Final purification of the carotenoid fractions:-</u>	
Phase separation	96
Column chromatography	98
Thin-layer chromatography	101
The use of mixed adsorbents	105
Thin-layer chromatography (2)	106
Methods of recording the results of chromatography	112
Reproducible R_f values in thin-layer chromatography	114
"Reverse-phase" chromatography	119
"Wedge-strip" method	119
Solvents	120
<u>The use of lipid standards other than carotenoids in the thin-layer chromatography of <i>S. flava</i> extracts.</u>	
(a) Co-chromatography with steroids	121
(b) Effects of lipid contaminants of the thin-layer chromatography of carotenoids.....	122

CONTENTS (Contd.)

	<u>Page</u>
Preparative thin-layer chromatography	122
Paper chromatography	127
The use of carotenoids extracted from tomatoes for experimental purposes and for the preparation of standards:-	
Column chromatography	130
Crystallisation	132
The use of Rhodamine 6G and Silica Gel G F ₂₅₄ to locate colourless lipids	132
The preparation of carotenoid standards from tomatoes	133
The controlled deactivation of alumina	134
<u>Analysis of the carotenoids</u>	
1. General methods employed:-	
(a) Quantitative estimations	135
(b) Spectroscopy	
Visual and ultra-violet	137
Mass spectrometry	138
Infra-red	139
(c) Tests for the presence of epoxy and alde- hyde groups	140
(d) Partition ratio	141
(e) Iodine isomerisation	141
(f) Acetylation	142
(g) Silane formation	143
(h) Reduction of esters with LiAlH ₄	144
(i) Oxidation with nickle peroxide	145
(j) Methylation	145

CONTENTS (Contd.)

	<u>Page</u>
2. Characterisation and analysis of the individual carotenoid fractions isolated.	
Fraction 1	146
" 2	146
" 3	147
" 4	147
Comparison of the carotenoid pigments from <u>S. flava</u> with those from other pigmented micro-organisms	148
<u>Experiments with the protoplast membrane of S. flava</u>	150
Lysis of the bacteria	150
Isolation and purification of the protoplast membranes	151
Analysis of the protoplast membranes:-	
Nitrogen estimation	153
Phosphorus estimation	154
Carotenoid content	155
Extraction of lipids from entire bacteria..	156
Preparation and testing of a water-soluble fraction of the protoplast membranes....	158
Tests on the fraction:-	
Spectrum	159
Precipitation and extraction of the carotenoids	159
Effects of light and air on solubilised protoplast membranes	163
Thin-layer chromatography of the fraction.	166

CONTENTS (Contd.)

	<u>Page</u>
Gel filtration	167
Folin-Lowry method of protein estimation	170
Electrophoresis in free-buffer film	171
Effects of light on a culture of <u>S. flava</u>	175
 <u>RESULTS SECTION</u>	
The morphology of the Sarcina bacteria	177
The growth of cultures of <u>S. flava</u> at different temperatures:	
(a) Counting methods	177
(b) Growth with temperature on nutrient agar	178
(c) Growth in nutrient broth	179
(d) Effect of the enrichment of the growth medium with glucose	179
Culture of the bacteria	179
Bulk culture of the bacteria:-	
(a) Using large aluminium dishes	180
(b) Using large developing dishes	180
(c) Bulk liquid culture	180
Extraction of the pigments	181
Purification procedures:-	
(a) Lipid precipitation	183
(b) Saponification	183
<u>Experiments to determine the loss in absorbance of a carotene solution with time, under a variety of conditions.</u>	
(a) The loss in absorbance of a carotene solution under various conditions.....	185
(b) The effects of sampling	186

CONTENTS (Contd.)

	<u>Page</u>
The effect of an anti-oxidant on carotene degradation	188
Pigment production of <u>S. flava</u> with variations in:-	
(a) time of growth at 34°C	189
(b) temperature of culture	189
(c) pH of the medium	189
(d) enrichment of the medium with different concentrations of glucose	190
<u>Final purification of the carotenoid fractions:-</u>	
Phase separation	191
Column chromatography	192
Thin-layer chromatography	193
The use of mixed adsorbents	196
Reproducible R _f values in thin-layer chromatography	197
"Reverse-phase" chromatography	198
"Wedge-strip" method	198
Solvents	199
The use of lipid standards other than carotenoids in the thin-layer chromatography of <u>S. flava</u> extracts:-	
Column chromatography	207
Crystallisation	210
The use of Rhodamine 6G and Silica Gel G F ₂₅₄ to locate colourless lipids	210
The preparation of carotenoid standards from tomatoes	212
The controlled deactivation of alumina	216

CONTENTS (Contd.)

	<u>Page</u>
Characterisation and analysis of the individual carotenoid fractions isolated:-	
Fraction 1	218
" 2	220
" 3	222
" 4	225
Comparison of the carotenoid pigments from <u>S. flava</u> with those from other pigmented micro-organisms	228
<u>Experiments with the protoplast membrane of S. flava</u>	
Lysis of the bacteria	232
Analysis of the protoplast membranes	232
Nitrogen estimation	
Phosphorus estimation	
Carotenoid content	
Preparation and testing of a water-soluble fraction of the protoplast membranes.	239
Effects of light and air on solubilised protoplast membranes	239
Thin-layer chromatography of the fraction	241
Gel filtration	241
Electrophoresis in free-buffer film	242
Effects of light on a culture of <u>S. flava</u>	242
<u>DISCUSSION</u>	244
<u>BIBLIOGRAPHY</u>	265
<u>SUMMARY</u>	278

I N T R O D U C T I O N

SECTION I

TAXONOMY OF THE SARCINA BACTERIA

According to "Bergey's Manual of Determinative Bacteriology" (edited by Breed, Murray and Smith, 1957), the genus Sarcina, with two of whose members, S. flava and S. lutea, this thesis is mainly concerned, constitute a genus within the family of Micrococcaceae. Under the heading of Micrococcaceae, the editors group six genera: Staphylococcus, Micrococcus, Gaffkya, Sarcina, and two anaerobic groups, Methanococcus, and Peptococcus.

Besides having a spherical shape these all possess the following common characteristics:

- (a) Cluster or packet forming
- (b) Catalase positive
- (c) Mostly Gram positive
- (d) Many of them are pigmented.

This family presents a considerable taxonomic disarray, (Cowan, 1963; and Baird-Parker, 1963).

If the viewpoint of the taxonomist is medical, then all those which are pathogenic to man and coagulase positive fall under the heading of Staphylococci (Shaw, Stitt and Cowan, 1951), but in a later paper, Cowan (1963) states that he considers this classification insufficiently rigorous.

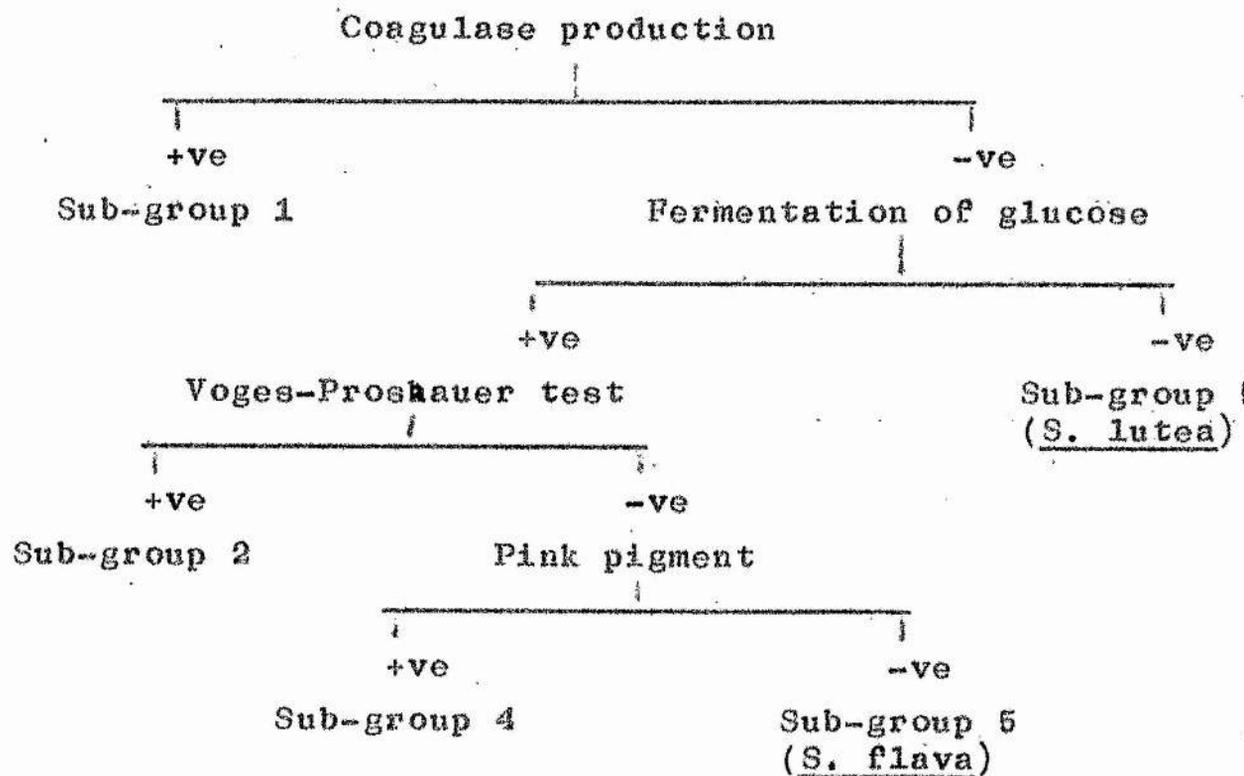
The most useful broad taxonomic criterion for the aerobic species is whether or not the bacterium will ferment anaerobically certain carbohydrates, particularly glucose.* Thus, according to the scheme of Baird-Parker (1963), and also that followed by the editors of the 7th edition of Bergey's Manual (Breed, Murray and Smith, 1957), the following should be the main divisions of the aerobic cocci:

- (a) Those which produce acid from glucose anaerobically — Staphylococci
- (b) Those which produce acid from glucose either aerobically or not at all — Micrococci

Under this scheme, the Sarcinae, which are unable to ferment glucose anaerobically, constitute a sub-group within the Micrococci, by virtue of the fact that their mode of division is so regular as to be considered a constant characteristic. They divide regularly in three planes, forming a cubical packet of eight bacteria. (Hence the name of the genus - Sarcina (Latin) - a bundle). This is in contrast to the rest of the Micrococci which are generally found in isolated pairs or irregular clumps.

* N.B. Even the aerobes are capable of slight anaerobic growth.

It may be doubted that this characteristic alone is sufficiently consistent to base a genus on it, but Baird-Parker (1963) considered that this is the case as packets are produced irrespective of the culture medium. On the other hand, however, Shaw, Stitt and Cowan (1951) state that the formation of packets is "rare even in the types named Sarcina". Their classification makes use of pigmentation which is generally considered too uncertain a criterion for taxonomy, as was admitted by Cowan (1963). According to this system, S. flava and S. lutea fall into different sub-groups.



Until some scheme of taxonomy is finally adopted, it can at best be said that the cocci present a range of characteristics which often overlap. At one end of the spectrum are found the small, metabolically active Staphylococci, and at the other end lie the relatively large, metabolically less active Sarcinae.

One point which all the recent workers agree on is that they feel that the Micrococcaceae should not include any anaerobic bacteria. This would exclude about half of those which are at the moment listed under the heading of "Sarcina" in the "Manual of Determinative Bacteriology" edited by Breed, Murray and Smith (1957), as well as the two obligate anaerobic genera, Methanococcus and Peptococcus. Those Sarcinae under consideration in this thesis, however, are aerobic.

The characteristics of the bacteria in question are as follows:-

S. flava

Spheres, 1.0-2.0 microns in diameter

Gram +ve

Gelatin stab - slowly liquefied

Litmus milk - not coagulated

Indole not produced

No reduction of nitrates to nitrites

S. flava (contd.)

Aerobic

Optimum temperature for growth - 30-35°C.

S. lutea

Spheres, 1.0-1.5 microns in diameter

Gram +ve.

Gelatin stab - slowly liquefied

Litmus milk - coagulated

Slight indole production

Nitrates usually reduced to nitrites

Aerobic

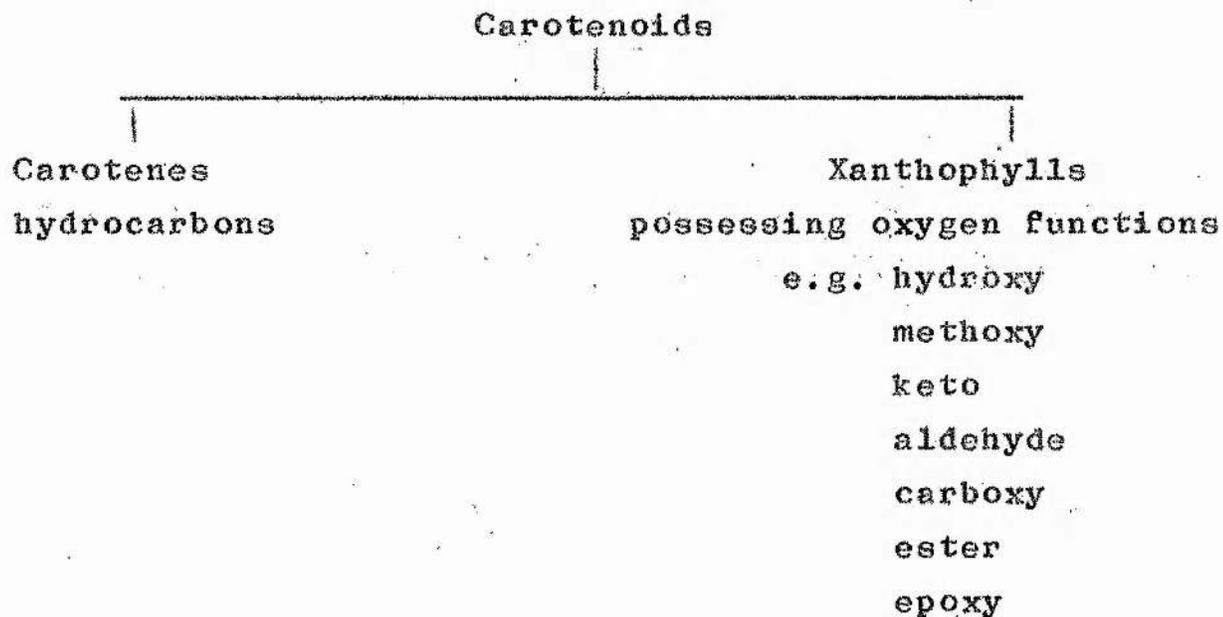
Optimum temperature for growth - 25°C.

SECTION 2.

GENERAL CHEMICAL STRUCTURE AND PROPERTIES OF CAROTENOLS.

Pigmented bacteria may either be photosynthetic or non-photosynthetic. The bacteria studied in this work were all carotenogenic and non-photosynthetic. It is as well at this point to review the general structural features and properties of this group of naturally occurring pigments.

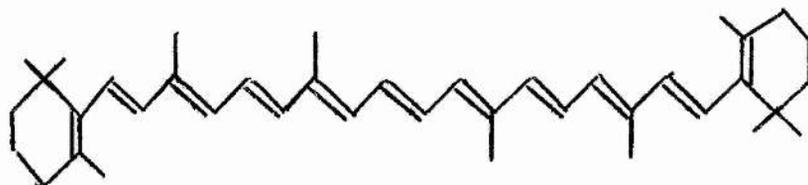
Carotenoids are grouped in two categories as follows:



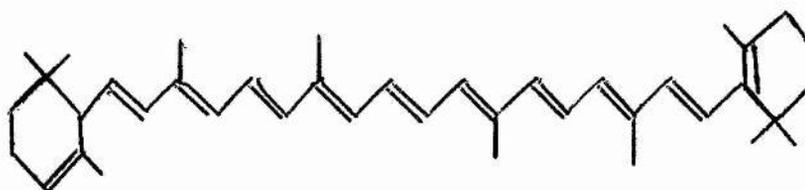
The carotenes may be aliphatic e.g. lycopene
alicyclic e.g. β -carotene
aromatic e.g. renieratene.

The more common cyclic end groups can be considered to be residues of α or β ionone. Thus β -carotene possesses two

β -ionone rings



while α -carotene has one β -ionone and one α -ionone ring.

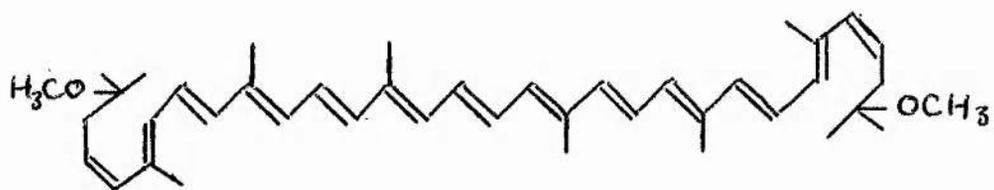


In general, carotenoids contain 40 carbon atoms in their structure, but under certain circumstances, as for instance when esterified to a fatty acid, this will be more. There are some examples of carotenoids with less than 40 carbon atoms. They are probably degradation products.

30 C	citaurin
27 C	azafrin
24 C	bixin
18 C	crocetin

Much work has been done on the carotenoid pigments present in the photosynthetic bacteria, particularly

those of the genus Rhodospirillum. It was from these bacteria that the carotenoid spirilloxanthin was first extracted.



The work has been excellently summarised by Jensen (1962). In common with all the other thoroughly analysed carotenoids from other sources, all the carotenoids so far isolated from photosynthetic bacteria contain 40 C atoms in their skeletons.

The first reported occurrence of a carotenoid with more than 40 carbon atoms as an integral part of the skeleton came from a paper delivered by Jensen and Weeks at a "Symposium on Carotenoids other than Vitamin A" in Trondheim, June 1966 (Jensen and Weeks, 1966). In the course of this thesis it will be shown that the occurrence of such carotenoids is not restricted to the bacterium from which these workers isolated their sample, viz. Flavobacterium dehydrogenans.

As can be seen from their possible structures, a wide range of polarities is encountered in carotenoids,

ranging from the non-polar carotenes, to the relatively polar xanthophylls, depending on the nature and number of oxygen-containing constituents present. They are lipids, and none are sufficiently polar to be water-soluble. Thus they are extracted from tissues with a variety of organic solvents varying in polarity from hexane to methanol.

Carotenoids vary in their sensitivity to such agents as light, oxygen and heat, acid and alkali. As to the former, all carotenoids are affected to a greater or lesser degree, and so must, when possible, be extracted and stored in the dark, under an atmosphere of nitrogen and in the cold. Of the latter, alkali is for the most part innocuous, carotenoids being considered unsaponifiable, but a few, for instance, fucoxanthin, are alkali labile. Acidic mutagenesis is much more serious, in the extraction and purification of carotenoids. Even dilute acids may cause chemical changes, such as the dehydration of allylic alcohols (Weedon, 1965). Concentrated mineral acids will react with epoxy carotenoids to cause a colour change, and in fact the formation of a blue colour with concentrated

1

hydrochloric acid is a test for the presence of such a group. Finally, even the minute amounts of hydrochloric acid formed in "aged" chloroform may cause the dehydration of allylic hydroxyl groups. If the hydroxyl group is allylic to the chromophore, then dehydration will cause a change in the spectral characteristics of the carotenoid. This too is used as a test, under controlled conditions.

Although their potential lability can be considered a drawback, there is at least one definite advantage to working with carotenoids, and that is that they are coloured. This has the practical benefit that the progress of a chromatogram can be followed with ease. This is useful not only in the purification of the individual carotenoids, but also in checking on the course of a chemical reaction involving them.

Carotenoids owe their colour to the high electron density which results from the extensive conjugation, and which enables the molecules to absorb light in the visible region.

The majority of carotenoids possess a central C_{18} unit which consists of 9 conjugated double bonds, and 4 side chain methyl groups. Not all carotenoids,

however, have nine conjugated double bonds in the central unit. Thus chloroxanthin, spheroidene, spheroidenone, and α -zeacarotene have the 7,8-dihydro system characteristic of neurosporene.

The nature of the chromophore is best indicated by the ultra-violet and visible light absorption characteristics of the carotenoid. The most usual absorption spectrum in visible light has three peaks, although variations occur which will be described later. The wavelengths of the absorption maxima increase with increasing conjugation, but the actual wavelengths depend on structural features, and on the solvent used (Weedon, 1965).

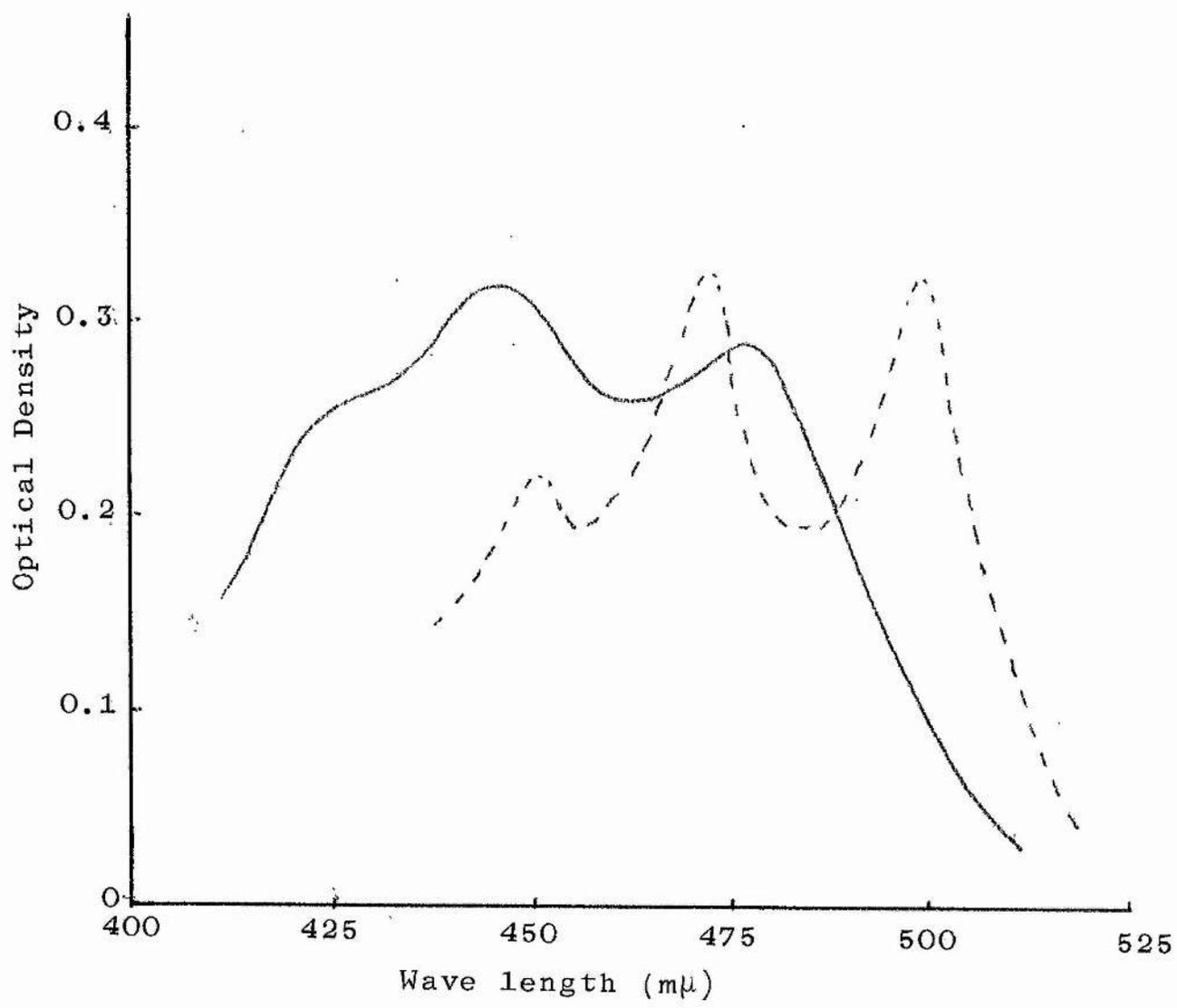
Examples:

<u>Carotenoid</u>	<u>No. of conj. double bonds</u>	<u>Principal light abs. maxima (mμ) (hexane)</u>
ξ - carotene	7	380, 401, 425.
α -zeacarotene	8	398, 421, 449.
Neurosporene	9	416, 440, 470.
Lycopene	11	443, 472, 504.

If another chromophore, e.g. C=O, is conjugated to the polyene chain, it exerts an influence on the spectrum

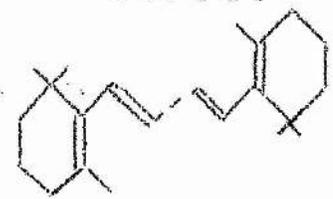
displacing the absorption maxima to longer wavelengths, and often causing a loss of fine structure (Goodwin, 1955). In the case of echinenone (4-keto- β -carotene) the spectrum in light petroleum (B.P. 60-80°C) has a single peak at 458m μ , instead of the three peaks of β -carotene at 425, 451, and 482m μ .

Further, the presence of cyclic end groups causes steric hindrance resulting in a shift of the absorption maxima to shorter wavelengths and a loss of fine structure. This is well illustrated by a comparison of the spectra, in the same solvent, of β -carotene and lycopene, both of which possess 11 conjugated double bonds.

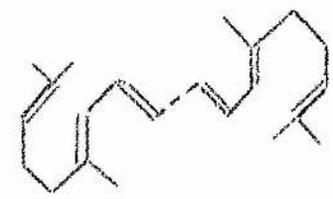


— β-carotene

- - - lycopene



β-carotene



lycopene

In a cyclic carotenoid in which the chromophore does not extend in to the rings, the ratio of the absorption peak at the longest wavelength to that of the central peak, approaches 1. In the case of a cyclic carotenoid in which the chromophore does extend into the rings, this ratio is much less (Trombly and Porter, 1953).

Another cause of spectral change is the formation, intentional or fortuitous, of stereoisomers, which is discussed in the next section.

SECTION /

SECTION 3.

STEREOCHEMISTRY OF CAROTENOIDS.

The main contributor to the understanding of the importance of stereoisomerism has been Zechmeister (1962).

It has been found that all-trans carotenoids can be converted, under the correct conditions, into a mixture of stereoisomers. A number of cis and polycis isomers have been reported isolated from natural sources, but to varying degrees all extraction procedures expose the all-trans forms of stereomutation, and so such reports must be regarded with a certain scepticism. However, cis isomers do exist in vivo, as it is known that neob retinal must be cis about the 11-12 double bond (Weedon, 1965), before it can combine with scotopsin to form rhodopsin. Also, phytoene, one of the polyene precursors of the carotenes, is known to exist in the cis conformation, and that this is the most stable form (Weedon, 1965).

Weedon (1965) states that from theoretical considerations, lycopene can exist in 1056 possible forms, but steric hinderance reduces this number to 72.

However, only a few isomers are present in any isomeric mixture of an individual carotenoid. Nevertheless, it has been found that sterically hindered forms are capable of existence, and several have been synthesized. Thus the crucial 11-12 cis double bond of neob-retinal falls into this category, providing a nice illustration of Nature's delight in confounding the predictions of scientists!

A number of conditions will favour stereomutation.

These are:-

(a) Heat. Carotenoids vary in their susceptibility to heat induced isomerism. To take two examples; a solution of β -carotene will only have isomerised to the extent of 1-2% at room temperature after 24 hours. With spirilloxanthin the proportion can be as much as 23%. (Jensen, 1962).

(b) Visible Light. All carotenoids are liable to stereomutation when exposed to visible light. It is possible that this mechanism operates in vivo for the conversion of cis to trans isomers.

(c) Light + catalyst. The most commonly employed method for the controlled production of stereoisomeric mixtures is to expose to light a solution of the

carotenoid containing a catalytic amount of iodine. This will receive further attention in the experimental section.

Effects of stereomutation

Due to stereomutation on melting, crystalline carotenoids may exhibit the phenomenon of a double melting point. As far as is known, the all-trans form has the highest melting point.

The geometrical configuration has an effect on the adsorption affinity of the carotenoid, thus allowing stereoisomers to be separated by chromatography.

As was mentioned in the preceding section, one of the most dramatic effects of the presence of cis bonds, is that on the spectrum of a carotenoid. The effect of cis bonds is to progressively shorten the wavelengths of the absorption maxima of an all-trans compound, and there is a fall in the extinction coefficient. Another effect is the appearance in the near ultra-violet of a small "cis" peak. This peak occurs quite consistently at $140 \pm 3 \text{ m}\mu$ less than the absorption peak at the longest wavelength of the all-trans compound. This appears to hold true for

chromophores containing 9, 10, and 11 conjugated double bonds.

Spectra at other wavelengths are useful in the detection of cis isomers. Thus the infra-red spectrum of a 15,15' cis bond has an absorption band at 780cm.^{-1} (Weedon, 1965).

A comparison of the products of an iodine catalysed mixture of isomers will give a good idea not only of the number of detectable isomers formed, but also their type. If the shift of the λ max of the all-trans compound to shorter wavelengths is only about 5-10 μ , then a poly cis or "hindered"-cis isomer is unlikely.

SECTION 4

EXTRACTION AND ANALYTICAL METHODS FOR CAROTENOIDS

Since the carotenoids present in some non-photosynthetic bacteria were examined in detail, (especially those of S. flava), during the investigations reported in this thesis, the methods available for the extraction of carotenoids from bacteria, and the general analytical techniques used in carotenoid research, are reviewed in this section. The specific analytical methods employed in the author's work will be dealt with in more detail in the section devoted to experimental methods.

Extraction methods

Goodwin (1955), in his survey of extraction techniques reported that the accepted method of extracting carotenoids from bacteria, which consisted of first dehydrating the cells with anhydrous Na_2SO_4 , followed by extraction with acetone or ether, did not work with the more polar carotenoids, as for example, the corynexanthin isolated by him from a species of coryneform bacterium (Hodgkiss, Liston, Goodwin and Jamikorn, 1954). The method he used in that instance was to shake the cells in methanol at 60°C . for a few minutes, decant the

methanol, and repeat the process until no further carotenoid could be extracted.

Polgar, Van Neil and Zechmeister (1944) extracted the carotenoids from Rhodospirillum rubrum by first dehydrating the cells with methanol, then extracting the carotenoids with benzene.

Starr and Saperstein (1953) used a mixture of ethanol and benzene to extract the pigments of Coryneform poinsettia.

Rothblat, Ellis and Kritchevsky (1964) extracted the carotenoids from M. lysodeiktus by freezing a centrifuged pellet of the cells with a dry ice/acetone mixture, and plunging the frozen pellet into boiling ethanol for 10 minutes. The cells were centrifuged, and the bacteria extracted twice more.

Nakamura (1936) extracted the pigments from the cells of S. lutea by allowing them to stand overnight in 98% aqueous methanol at room temperature. The extraction was not complete.

Sobin and Stahly (1942) used methanol for extracting a variety of bacteria. The moist bacteria were ground with alundum, and then extracted with methanol which was rapidly brought to the boil for a

21

few minutes. They reported that this was not injurious to the carotenoids.

Saponification

As already mentioned, carotenoids are classed as unsaponifiable, and hence saponification is one of the preliminary procedures in their purification. In some instances, however, they are esterified to fatty acids in their natural condition. Saponification would mask this fact by hydrolysing the ester bonds. Thus unsaponified and saponified extracts must be compared to test for the presence of ester groups. This is relatively easy, as the presence of an ester link lowers the polarity of a xanthophyll, giving it a higher R_f value than the free xanthophyll, in adsorption chromatography.

Chromatography

The form of chromatography principally utilized in the separation and analysis of carotenoids, is adsorption chromatography. It is probably no exaggeration to say that, without the invention and refinement of the technique, the knowledge of carotenes would not have advanced much beyond the point of calling

the fatty orange pigment after the carrots from which it was first isolated. The very name of the technique indicates that its first application was the separation of plant pigments (Tswett, 1903).

As has already been mentioned, the polarity of carotenoids varies widely from the non-polar carotenes to xanthophylls, whose polarity increases with the number of oxygen functions. Carotenes themselves differ from each other in their individual affinities for an adsorbent, depending on their structure and degree of unsaturation. One of the effects of stereoisomerism is to change the adsorption affinity of a carotenoid. Thus, by a judicious choice of adsorbent, it is possible to obtain pure samples of any carotenoid.

Chromatography is applied in a number of ways.

(a) Column chromatography

This was the form of chromatography used by Kuhn and Lederer in their classical researches into carotenoid structure in the early 1930s (Kuhn and Lederer, 1931). A variety of adsorbents can be used, depending on the materials to be separated. In order of increasing adsorption affinity, a few

examples are:-

Starch

Sucrose

CaCO_3

MgCO_3

Al_2O_3 (deactivated)

MgO

Ca(OH)_2

Al_2O_3 (activated)

Silica

The solvents used to develop the chromatogram depend both on the adsorbent and the material to be chromatographed.

Column chromatography remains one of the most effective ways of preparing large amounts of pure carotenoids from natural sources. It is of less use for analytical work and co-chromatography since quite large amounts are required. A considerable length of time is usually required to set up and develop a column, and a great deal of solvent is used. A further disadvantage is that a single run is seldom sufficient to procure completely pure samples.

(b) Thin-layer chromatography

This method is also based mainly on adsorption chromatography and can be considered as an "open-column" technique. The name principally associated with the refinement of thin-layer chromatography (T.L.C.) is that of Stahl, e.g. Stahl, 1956; 1958a; 1958b; 1959; and 1961. From his exhaustive researches, a new highly developed technique has emerged. In brief, the technique consists of spreading onto glass plates a layer of known thickness of the adsorbent, in the form of a slurry in water or some polar solvent such as ethanol. The adsorbent usually contains 5-10% calcium sulphate, which acts as a binder when the plate is allowed to dry, either in the air or in an oven. Before using the plate it is "activated" by heating it in an oven at more than 100°C for about 30 minutes. The time and temperature of the activation are decreed by the particular adsorbent, and the degree of activation required. The materials to be chromatographed are then introduced onto the layer, dissolved in a volatile solvent, either in spots or as a line. The chromatogram is then developed in an appropriate solvent. The development

25

is carried out in a closed tank, pre-equilibrated with the solvent. A number of adsorbents are in general use, but those which yield the best resolution are alumina and silica.

The technique can be adapted for preparative work as well as for analytical, as has been done in the work presented in this thesis.

The advantages of T.L.C. are:

- (a) Its speed.
- (b) High degree of resolution achieved.
- (c) Small quantities of material required.
- (d) The inorganic layer enables charring procedures to be used for the visualisation of the components of a mixture.

Its disadvantages include:

- (a) Low reproducibility unless the conditions are closely controlled.
- (b) The materials chromatographed are exposed to the air on a finely divided base, and susceptible to oxidation. This is important in the case of carotenoids. In an attempt to alleviate this, chromatography tanks have been produced with lids

fitted with cocks for the purpose of gassing out the tanks with nitrogen.

(c) In preparative T.L.C. only small amounts of material can be separated on a single plate.

The factors influencing the behaviour of substances on T.L.C. have been thoroughly investigated by Dallas (Dallas, 1965). They include:-

- (a) Adsorbent activity.
- (b) The thickness of the layer.
- (c) Degree of saturation of the atmosphere within the tank.
- (d) Amount of material spotted onto the plate.
- (e) Ambient temperature.

Unless all these variables are closely controlled, little reproducibility can be achieved. The recording of R_f values can have little meaning, and recourse must always be made to co-chromatography with a known material if identification is to be attempted.

Reproducibility can be improved by the use of an "S-chamber" originally employed by Stahl (Stahl, 1962). This technique was used in the present work, and will receive further consideration in the experimental section.

Preparative T.L.C. is gaining popularity, despite the drawbacks already mentioned. For the purpose of applying the material to the plates, a number of mechanical applicators have been designed and marketed; as for instance that produced by Desaga of Heidelberg (Camlab, Glass Ltd., Cambridge).

The other form of T.L.C. which has found application in carotenoid analysis is based on partition rather than on adsorption. This has been called "reverse phase" chromatography due to its reversing the order of the R_f values pertaining in adsorption chromatography. Such a method employing kieselguhr impregnated with liquid paraffin is described by Egger (Egger, 1962). Other impregnated materials can be used, including triglycerides, which yield a good separation of the polar xanthophylls. The developing solvent in this case is always polar, e.g. methanol. Instead of the situation in adsorption chromatography, where the R_f value of a substance is indirectly proportional to its polarity, in "reverse phase" chromatography the non-polar carotenes are held in solution by the impregnating material, and have low R_f values, while the polar

xanthophylls are carried with the solvent until partition equilibrium is reached.

(c) Paper chromatography

This has a limited, but very useful, application. The papers must be filled with some adsorbent material such as kieselguhr, alumina or silica. The use of kieselguhr-filled papers is due to the Jensens (Jensen and Jensen, 1959). The method used is that of circular chromatography suggested by Rutter (Rutter, 1948). It is applicable to a wide range of polarities, and the solvents used are different proportions of acetone in light petroleum. It is particularly useful for -

- (a) the rapid separation of stereoisomers which can be eluted and their spectra compared;
- (b) following the progress of chemical reactions.

Its two great advantages are:-

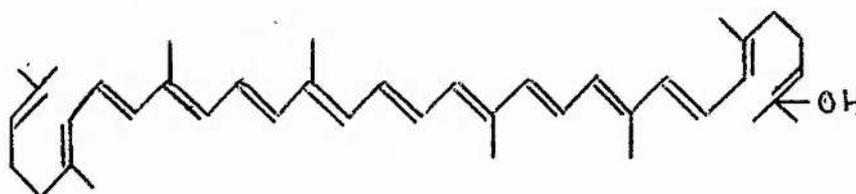
- (a) the small quantities required;
- (b) the speed of development - approximately 15 minutes.

Kieselguhr-filled papers are generally insufficiently adsorbent for the efficient separation of carotenes, but it has been found that the silica-filled papers are excellent for this purpose.

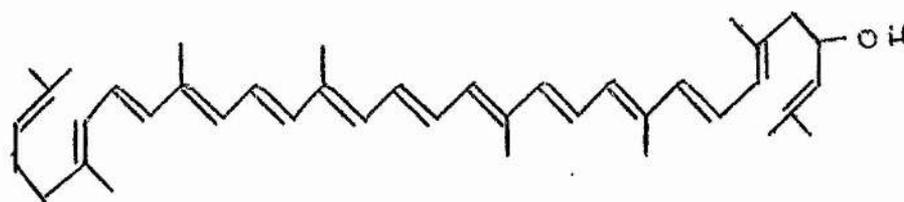
Identification of an unknown by co-chromatography

Co-chromatography is a useful technique for comparing the identity of an unknown with that of a known carotenoid, but it cannot be taken as a final identification. There are reports of a number of examples of different carotenoids which could not be separated by co-chromatography,

e.g. Rhodopin



Lycoranthin



(Goodwin and Land, 1956)

Another possible source of confusion has been the capacity of lipids to occlude one another, giving false impressions on chromatography.

Partition ratio

Chromatography will give a general indication

of the polarity and the nature of a carotenoid, but this can be given a more exact expression by finding its partition ratio between immiscible solvents. Those which have been mostly used are light petroleum (B.P. 60-80°C) and 95% or 85% aqueous methanol. If this test is done under carefully controlled conditions with a pure carotenoid it can give a better indication of polarity than the R_f value from T.L.C. The partition ratios of a large number of carotenoids were documented by Petracek and Zechmeister (Petracek and Zechmeister, 1956), and these can be used as a source of comparison.

Spectral analysis of carotenoids

Spectral methods of analysis are particularly useful, and they have the great advantage of being non-destructive.

(a) Visible and U.V. Spectrum

This has already been thoroughly dealt with in the section on "General Properties".

(b) Infra-red Spectrum

One of the most common substituted groups encountered in carotenoids is the hydroxyl group, and this is not only easily detected by I.R. spectroscopy,

but the nature of the group can also be found.

Tertiary hydroxyl groups have an absorption band of medium intensity around $1,140 \text{ cm.}^{-1}$, while secondary hydroxyl groups have a band at $1,030 \text{ cm.}^{-1}$.

All carotenoids exhibit an absorption close to 970 cm.^{-1} , which is due to the C-H out of plane deformations of the trans-CH=CH- (Lunde and Zechmeister, 1955). This absorption does not interfere with the detection of the C-H out of plane deformations of aryl groups, at 800 cm.^{-1} . (Yamaguchi, 1957, 1958).

Conjugated esters have stretching frequencies at $1,690 \text{ cm.}^{-1}$ (Lunde and Zechmeister, 1955), while conjugated aldehydes and ketones have bands at $1,650-1,670 \text{ cm.}^{-1}$ (Warren and Weedon, 1958).

The intensity of the carbonyl band in the I.R. and in the visible light absorption spectra of conjugated aldehydes is greater than that in the spectra of conjugated ketones.

The detection of end groups by N.M.R. spectroscopy.

The use of this method was pioneered by Barber, Davis, Jackman and Weedon (1960). It has found great

use in the establishment of the end groups of such substances as spirilloxanthin, spheroidenone, spheroidenene, and of some of the colourless polyenes, and other precursors of the carotenoids; e.g. phytoene, phytofluene, ξ -carotene and neurosporene (Barber, Jackman, and Weedon, 1959; Barber, Jackman, Warren, and Weedon, 1961; Davis, Jackman, Siddons and Weedon, 1961; Jackman and Jensen, 1961; Cholnoky, Szabolcs, Cooper and Weedon, 1963).

One of the difficulties of the method is that the majority of the machines in operation at the moment require about 20 mgs. of material for good resolution. In the case of bacterial extracts in particular, this is an almost impossible quantity of which to prepare a pure sample.

The method has a particular use in the location of methoxy groups, e.g. in spirilloxanthin.

All natural carotenoids have methyl groups attached to either oxygen atoms or to fully substituted carbon atoms. There is therefore no spin-spin coupling of the methyl protons with the protons of adjacent atoms, and the different methyl groups give rise to single peaks which are readily distinguished from absorption due to methylene groups, if any are present.

The characteristic end-groups of lycopene, β -carotene,

capsorubin, astacene, spirilloxanthin, aurochrome, isorenieratene, and renierapurpurin can all be easily identified. The methyl groups attached to oxygen are strongly deshielded and give rise to bands at lower fields than those of the carbon methyls. (Barber et al., 1960).

The N.M.R. spectra of all carotenoids show strong absorption in the region 7.95 - 8.15 due to the in-chain methyls. This may provide a convenient standard for the estimation of the number of methyl groups represented by other bands.

The presence of other groups may be identified by their characteristic peaks in N.M.R. spectra. Thus when an acetate grouping is present, more than one peak is recorded in the 7.95 - 8.15 region. Aldehydic protons are uniquely deshielded, and, in conjugated polyene aldehydes, give rise to a band at 0.45 - 0.60.

Mass Spectrometry

This technique is now gaining popularity, but due to the cost and the limited distribution of the machines, little systematic work has as yet been done with carotenoids. Peaks at certain mass numbers are characteristic of

carotenoids, for instance peaks at M-92 and/or M-106, which are found for all carotenoids, and which indicate chain splitting, and cyclisation, to form toluene and xylene. Similarly in the case of a hydroxylated carotenoid a peak might be expected at M-18, which would indicate that water was splitting off the molecule under the influence of the ion beam (Jensen, 1967). The great virtue of the method is that it is possible to deduce an exact empirical formula for a compound by mass measurement of the parent ion with a minutely small quantity of material, (20 μ gm. will suffice). The disadvantage is that a high degree of purity is required, and with the small quantities usually isolated this is not always easy.

Chemical Methods of Analysis

Although the general structure of carotenes was proposed in the early 1930's by Karrer, it was not finally confirmed until β -carotene was synthesised about 20 years later (Isler and Schudel, 1963).

The classical techniques of analysis are not always easy to apply due to the frequent failure to obtain a perfectly pure compound, and are now being replaced by

spectral techniques whenever possible. Oxygen functions are often most easily detected by the various spectral techniques already mentioned, but chemical methods should not be scorned. By conventional acetylation, it is possible to detect primary and secondary hydroxyl groups, and by following the reaction by chromatography one can tell how numerous are the groups undergoing acetylation. If, from the polarity of a carotenoid, the presence of a hydroxyl group is suspected, and is not susceptible to acetylation, then it may be a tertiary hydroxyl group. This may be confirmed by the formation of a silane derivative.

Degree of unsaturation

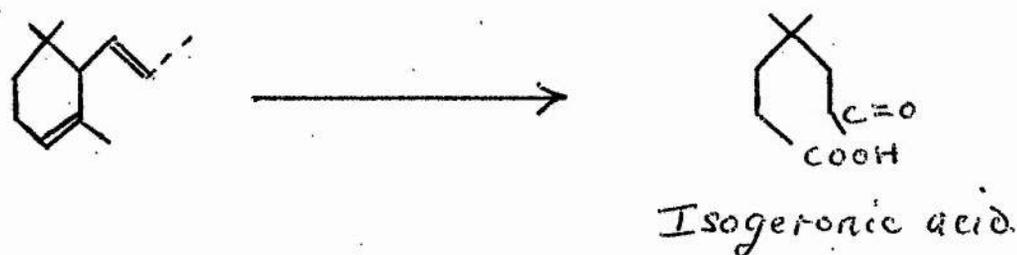
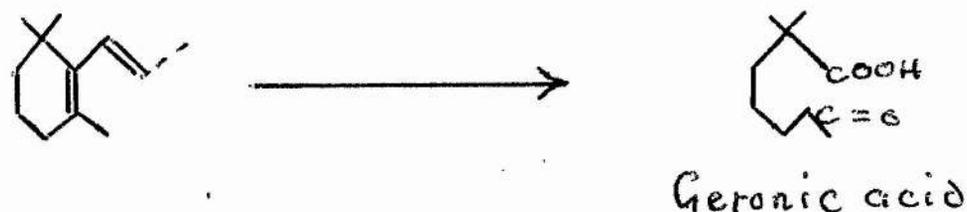
The degree of unsaturation may be found by the micro-hydrogenation method of Kuhn and Müller (1934), but faulty estimates may be encountered due to the tendency of aryl groups, epoxy groups, and carboxyl groups to be reduced. (Yamaguchi, 1957, 1958; Karrer and Jucker, 1945; Barber, Davis, Jackman and Weedon, 1960).

Oxidation

A useful test for the detection of isopropylidene end groups is ozonolysis (Kuhn and Roth, 1932). A

34

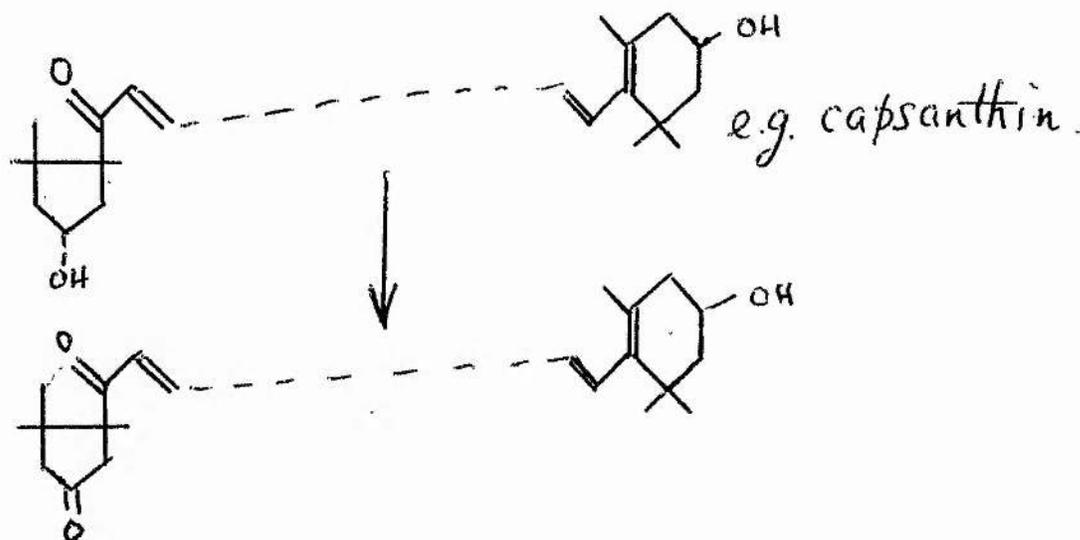
molecule of acetone is yielded by each isopropylidene end group, but the test must be done quantitatively, as acetone has been yielded by compounds which have no such groups, e.g. vitamin A, and spirilloxanthin. Ozonolysis of a carotenoid containing cyclic end groups yields the following:



Oxidation by any of these techniques yields information regarding the position of some oxygen functions. Thus, oxidation of zeaxanthin results only in the formation of *αα* dimethyl malonic acid and *αα* dimethyl succinic acid. No *αα* dimethyl glutamic acid is formed. This indicates that the hydroxyl substituents are on C3 and C3'. The products of these reactions are best tested for by

either paper or gas-liquid chromatography.

Oxidation studies on capsanthin, capcorubin, and kryptocapsin provide the first clue that a new class of carotenoids containing a 5-membered ring had been found. Cyclopentanone was recognised by its characteristic I.R. absorption.



Estimation of the side chain methyl groups

(Kuhn and Roth, 1933)

This involves the oxidation of the carotenoid with chromic acid and measuring the amount of acetic acid produced. Acetic acid is yielded both by the "in-chain" and end of chain methyls, but methyl groups in saturated environments do not react easily. (Karrer and Jucker, 1948).

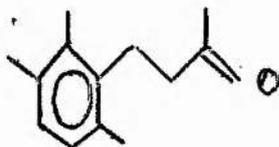
38

Controlled oxidation of β -carotene and zeaxanthin with mono-perphthalic acid gives the mono- or the di-epoxy derivatives. This reaction is general for β -end groups in other carotenoids. In the presence of mineral acids, the epoxides are rapidly converted into isomeric furanoid oxides with a further shortening of the chromophore. This fact can be used as a test if the presence of 5,6 epoxides is suspected.

Oxidation mediated by the useful reagent N-bromo-succinimide can take two forms: either dehydrogenation or addition of oxygen, oxygenation.

Dehydrogenation

Many polyenes react with N-bromo-succinimide in an inert solvent to yield allylic bromides which lose HBr, thus causing dehydrogenation. Lycopene, under these conditions, gives both 3,4,-3',4'; bisdehydrolycope and 3,4,-dehydrolycopene, both of which have been found in nature (Karrer and Rutschmann, 1945; Winterstein, Studer and Rüegg, 1960). Under the correct conditions β -ionone yields 3,4-dehydro- β -ionone or the aromatic compound, -

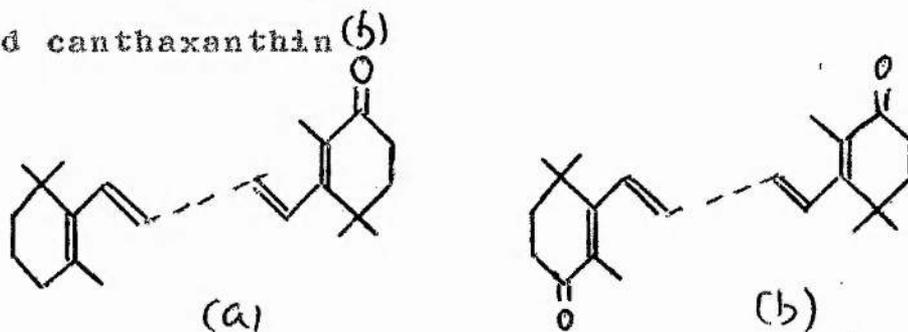


(Henbest, 1951), with a ring system of the type found

in isorenieratene and chlorobactene. (Karrer and Ochsner, 1948; Buchi, Seitz and Jeger, 1949). With the natural hydrolycopene precursors, e.g. phytoene, a fraction can be separated in each case which is chromatographically indistinguishable from the corresponding more highly saturated member of the series (Zechmeister and Koe, 1954). However, it seems possible that some of these products may be isomers differing in the location of the chromophore.

Oxygenation

Among the hydrocarbons and oxygenated compounds resulting from the treatment of β -carotene with N-bromo-succinimide in chloroform with 1% alcohol are the two naturally occurring carotenoids echinenone (a) and canthaxanthin (b)



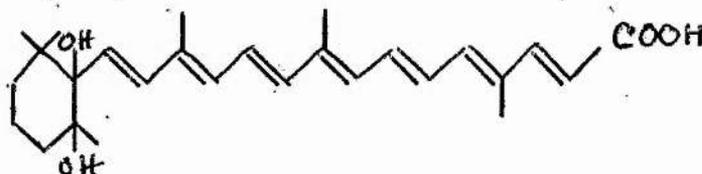
(Petracek and Zechmeister, 1956).

Treatment of β -carotene with N-bromo-succinimide in the presence of glacial acetic acid, yields, after hydrolysis of the crude products, 4,4'-dihydroxy- β -

carotene (Bnteschel and Karrer, 1958). This has not been found in nature, but can be oxidized to the diketone, canthaxanthin (Petracek and Zechmeister, 1956). N-bromo-succinimide is capable of oxidising allylic alcohols to ketones, but other reagents such as quinones are usually superior in this respect (Warren and Weedon, 1958).

Partial Degradation

The first work using carefully controlled chromic acid oxidation of β -carotene was done by Kuhn and Brockman (1935). It established azafrin as a possible degradation product.



Permanganate can also be used for partial degradation. This leaves the cyclic end-groups intact, while chromic acid appears to first open the rings. An interesting point is that α -carotene degrades from the α -end to yield products isomeric

with those produced from β -carotene (Karrer and Jucker, 1948).

Finally, oxidation studies on the aryl carotenoids have given useful information on the structure of these unusual carotenoids (Yamaguchi, 1957, 1958).

Synthesis

The methods of synthesis of carotenoids developed over many years have now reached such a point that they can be used as an integral part of the structural elucidation of compounds, and not merely to confirm an already known structure. This has been demonstrated by the work on the colourless polyenes - phytoene, phytofluene etc. (Davis, Jackman, Siddons and Weedon, 1961), and also the α - and β -zeacarotenes (Rüegg, Schwieter, Ryser, Schudel and Isler, 1961), ζ - and ϵ -carotene (Marchand, Rüegg, Schwieter and Weedon, (in the Press); Karrer and Eugster, 1950; Chapman and Haro, 1963).

Synthesis has also shown the need to revise the structure previously assigned to euglenanone (Cooper, Davis and Price, unpublished observations).

SECTION 5

FUNCTIONS OF CAROTENOIDS

Although this thesis is mainly concerned with carotenoids of the non-photosynthetic bacteria S. flava and S. lutea, so little is at present understood about the functions of carotenoids in any organism, it will be as well to look generally at what is known about carotenoid functions in living tissues, as this might give some clue as to their functions in non-photosynthetic bacteria.

One function which is performed by the brilliant carotenoids present in the petals of flowers and in some fruits is that of the visual stimulation of animals to aid reproduction and the subsequent seed dispersal. This has given rise to the theory that they are essentially by-products, whose existence is maintained by the visual selection of the animals concerned: a suggestion which could be summed up as "the survival of the most meretricious".

The suggestions for the more positive functions of plant carotenoids are as follows:-

1. As auxiliary pigments in photosynthesis.

- 2. In photoresponses: (a) phototropism
(b) phototaxis
- 3. In reproduction, especially in fungi.
- 4. Protection of cells against photodynamic destruction mediated by oxygen and light of the order of intensity of sunlight.

1. Photosynthesis

The first indication that the light energy absorbed by the carotenoids of photosynthetic plants could contribute to the photosynthetic process came from the simple experiments of Engelmann (Engelmann, 1884) who showed that algal filaments exposed to light of the wavelengths absorbed by carotenoids could photosynthesize, and produce oxygen.

This has since been shown to be general for all carotenogenic photosynthetic plants, regardless of their social position in the hierarchy of evolution. The efficiency with which the carotenoids contribute to the process varies widely. According to Dutton and Mannering (1941), the light absorbed by the fucoxanthin of the diatom *Nitzschia* causes, in vivo, almost as much chlorophyll fluorescence as the same quantity

of light absorbed by the chlorophyll itself. In the higher plants and algae the efficiency is thought to be much lower. The exception so far found is that of the fucoxanthin of the brown algae, which is thought to be as efficient an energy trap as in the case of the diatom previously mentioned. In the photosynthetic purple bacteria, the energy transfer is about 50% (Jensen, 1965).

It must be remembered, however, that in this respect, the carotenoid is of secondary importance to the chlorophyll as it has been found that mutant micro-organisms lacking carotenoids can photosynthesize quite adequately (Sager and Zalokar, 1958).

The energy transfer probably takes place by inductive resonance (DuySENS, 1964).

There may be another, as yet unexplored, function of carotenoids, particularly epoxides, in the photosynthesis of phototrophic micro-organisms. Blass, Anderson, and Calvin (1959) have suggested that the reversible removal of the oxygen from carotenoid epoxides may be part of the sequence of oxygen evolution by these cells. This does not apply to the photosynthetic bacteria, which evolve no oxygen, and have not as yet been shown to contain any epoxy carotenoids.

Phototropism and phototaxis

The field is a difficult one in which to work, and the results obtained are very liable to criticism. The difficulties include:-

- (a) Lack of accuracy in the measurement of action spectra.
- (b) The existence "in vivo" of carotenoid/protein complexes, which would have different characteristics of absorption to those of the extracted material.
- (c) If crystalline material were present in the cell, it again would have different absorption characteristics from the extracted material.

It is difficult to generalise about the phenomenon of phototropism in higher plants.

Some evidence of the participation of carotenoids has been found in the case of oats, where there is some similarity between the action spectrum and the absorption of a hexane extract. But it is not without discrepancies.

Phototaxis is defined as the light orientated locomotion of motile organisms. In the case of the phototactic flagellates, it was for long considered that the "stigma" or "eye spot" which, due to its distinctive colour, is thought to contain carotenoids, was the photo-receptor. This has now been disproved by the production

of mutants which lack the "stigma", but which still demonstrate phototaxis. There is some proof that the main receptor wavelengths lie between 400-500 m μ , but nothing is known of the actual nature of that receptor. The photosynthetic purple bacteria have a phototactic response, and it is thought that the action spectrum lies in the regions of both carotenoid and bacteriochlorophyll absorption [Jensen, ("Biosynthesis and Function of Carotenoid Pigments in Micro-organisms", in Ann. Rev. of Microbiol., 19, 48 (1965))].

Reproduction

Apart from the passive role in the attraction of insects and animals, there is no proof that the carotenoids play any part in the reproduction of higher plants, and very little proof that they have any such function in the lower orders.

The theory that there may be a link between sex and carotenogenesis stems from the observations of Chodat and Schopfer (1927) who drew attention to the differential accumulation of carotenoid pigments in the +ve and -ve mating strains of Mucor hiemalis, and concluded that it was an important sexual difference.

Most fungi, however, do not possess any carotenoid at all, and so there cannot be any universal principle drawn from these observations. It might be thought that in this instance, as in so many others, the carotenoids may function as photoreceptors, and indeed, the sexual reproduction in both Puccarium macrosporum and Pyronema confluens has been shown to be photomediated. There is an inconsistency, however, because it has been shown that the photoestimulated production of macrospores can take place in mutants lacking carotenoids (Carlile, 1956; Carlile and Friend, 1956).

It is among the Phycomyces that most of the work linking carotenoids and sex has been done. Despite this, little evidence exists which can be unequivocally said to point to some role of carotenoids in reproduction. In the instance of some Allomyces species, the failure to develop carotenoid appears to be linked with a failure to produce male gametangia.

The evidence for some specific metabolic role in this connection has come principally from studies on the mucoraceous fungi. It was found by Burgeff (1924) that the diffusion of the hormones necessary for the hyphal sexual reaction between the +ve and -ve strains

was accompanied by the accumulation of carotenoid. The phenomenon appears to be restricted to Mucor, Phycomyces, and Choanephora.

Burnett (1965), who reviewed this subject, considered that the accumulation of carotenoid is merely the result of increased metabolism, and that at the present state of knowledge, any direct relationship between sexual reproduction and carotenogenesis is a matter for doubt.

Protection

It has been generally established that carotenoids present in micro-organisms protect the cell against photodynamic destruction by the light energy absorbed by other endogenous pigments, e.g. chlorophyll, porphyrins, flavins etc. This was first established in the case of the carotenoid-less mutant of Rhodospseudomonas spheroides, a photosynthetic micro-organism (Griffiths, Sistrom, Cohen-Bazire and Stanier, 1955; Griffiths and Stanier, 1956; Sistrom, Griffiths and Stanier, 1956; Dworkin, 1958). This mutant, accumulating the colourless polyene phytoene, lacks the coloured carotenoids of the wild type, but has only a slightly diminished production of chlorophyll. It was found to be able to photosynthesise

49

normally under anaerobic conditions, but the admission of air into the culture caused the rapid demise of the cells. The organism could live aerobically in the dark, but the admission of light killed it. This photodynamic destruction, the exact mechanism of which is unknown, requiring both light and oxygen, is accompanied by the destruction of the chlorophyll, but Dworkin (1958) found that death occurred equally well at low temperatures, when the loss of the chlorophyll was minimised, indicating that the disappearance of the chlorophyll was of secondary importance. The action spectrum of photodynamic destruction nevertheless indicates that it is the chlorophyll which acts as the photo-sensitizer. Further, it was found that isolated chromatophores of the carotenoid-free mutant lost, in 10 minutes, their ability to photophosphorylate in the presence of light and air, while the chromatophores of the wild type had retained 63% of their original capacity under the same conditions (Burnett, 1965).

Although in the case already discussed, the depletion of carotenoid was due to mutation, it was found that chemical inhibition by, for example, diphenylamine, was equally effective in exposing the

50

cells to photodynamic destruction. In the case of Rhodospirillum rubrum, it was found that the inhibition of carotenogenesis by diphenylamine caused the bacteria to be killed by photodynamic action under normal conditions of light and air, but that if the cells were washed free of the inhibitor, and resuspended in fresh medium, carotenoids were synthesised, and the organisms became immune to photo-oxidation. This fact, emerging from the work of Cohen-Bazire and Stanier (1958) is a further proof of the function of the carotenoid pigments in photosynthetic bacteria.

There are present in non-photosynthetic bacteria potential photosensitisers such as cytochromes and flavins, and it might be expected that non-photosynthetic bacteria would be liable to photodynamic destruction under the same conditions. This was first shown to be the case with colourless mutants of the normally carotenogenic Corynebacterium poinsettia by Kunisawa and Stanier (1958), and has since been found to be the case with others, e.g. Sarcina lutea Mathews and Sistrom, 1959; Mathews, 1964) and certain other Corynebacterium species (Mathews, 1963; Wright and Rilling, 1963). In each case the carotenoid depletion was produced either by mutation or

by chemical inhibition of carotenogenesis. In the experiments with the Coryneform bacteria an exogenous photosensitiser was required to cause the photodynamic destruction, while the workers with S. lutea reported that at a light intensity of 12,000 f.cs. no such exogenous agent was required. The light intensity employed with the Coryneform bacteria was much less, only 4,000 f.cs. It is possible that the difference in the experimental results lay in the different intensities of light, and not in any difference in the response of the different bacteria.

The light intensity of 12,000 f.cs. was arrived at because it was of the same order as sunlight, and thus reflected more accurately the conditions to which the bacteria were exposed in their natural environment.

The results lead to the conclusion that the pigmentation is a result of natural selection, the pigmented bacteria being more capable of flourishing in conditions where intense light is the general rule. It has been found that the lethal action of light and air is not restricted to non-pigmented strains of normally pigmented bacteria, but is also found with normally colourless species, whose habitats are in

conditions of low light intensities (Rowig and Wyss, 1957).

From a consideration of these factors, it is possible to explain the distribution of carotenogenic bacteria. The majority of aquatic bacteria are pigmented, and they are certainly exposed to light and air, usually growing in shallow lagoons and pools, or for instance, the red halophilic bacteria whose natural habitat is the Dead Sea; and also the Flavobacteria which are known to contain carotenoids (Goodwin, 1952). Another class of bacteria which are exposed to air and intense light, are those asporogeneous bacteria which are normally dispersed by air. This includes the Mycobacteria and the Corynebacteria; both noted for their pigmentation (Mathews and Siström, 1959).

The actual protective action of the carotenoids is unknown, as are the site and agent of the photodynamic killing in these non-photosynthetic bacteria. The possible photosensitisers include the porphyrins and flavins, which absorb light in the same region of the visible spectrum as the carotenoids. This fact led Wright and Rilling (1963) to propose that the carotenoids merely function as a sort of electronic "shelter-belt"

in absorbing the light which would otherwise be absorbed by the photosensitisers, killing the cells. This explanation, however, fails to account for how the carotenoids dispose of the absorbed energy. It is certainly insufficient to account for the protection afforded in these cases in which the photosensitiser is chlorophyll (endogenous) or toluidine blue (exogenous), which absorb light at quite different wavelenghts to carotenoids. Other possible modes of action have been discussed by Calvin (1955); and by Stanier (1960). The ideas put forward include reversible oxidation of the carotenoids during photosynthesis, and the possibility that they play a structural role to the end that the excitation of the chlorophyll molecules does not cause photo-oxidation.

The site of action of the photosensitisation is uncertain, but two factors point to its location in the cell membrane in bacteria. Mathews and Sistrom, (1960) established that both the carotenoids and the possible photosensitisers are located in the cell membranes of S. lutea. Working on the assumption that this was the site of the photodynamic action, they tried to correlate photosensitisation with an increase in

membrane permeability, in a colourless mutant of S. lutea, exposed to intense light and air. Although they failed to find any evidence of this, they did find that the membrane-located enzymes succinic dehydrogenase and diphosphopyridine nucleotide oxidase were almost entirely inactivated under the experimental conditions, a fact which supported the theory that the local of the photosensitisation was the cell membrane.

Finally, it would be interesting to see whether this concept of the function of carotenoids could be extended to higher plant forms. It would certainly afford a logical explanation of the fact that light is known to stimulate carotenoid production in some fungi. The fern Azolla filliculoides develops a deep red colour when heavily insolated, and a similar effect in Potamogeton fluitans is known to be due to the production of rhodoxanthin. These phenomena might be protective responses (Ahrens, 1940). It is thought that this might be a characteristic of higher plants in general (Bond, 1967).

SECTION 6

CELLULAR LOCATION OF BACTERIAL CAROTENOIDS

The organic chemist unravelling the structure of a natural product is most interested in obtaining in a pure condition the material with which he wants to work. His task begins at this point. In the case of the biochemist, the cellular location of the material is of equal or greater importance. From a knowledge of this, he can attempt to elucidate the role of the material in the economy of the cell.

Much evidence exists that carotenoids extracted from many sources are combined with proteins "in vivo". It was, for instance, noted quite early that the pigments (both carotenoids and chlorophylls) from photosynthetic bacteria had different absorption spectra when extracted with organic solvents, than when the bacteria were physically disrupted to produce an aqueous extract. The free pigments would not, of course, be soluble in aqueous media. This was first interpreted by Lubimenko (1922) as indicating that they were complexed to proteins, without giving any specific reasons.

56

The work was extended by Katz and Wassink (1939) and by French (1940), who found that they could isolate water-soluble pigment complexes which had absorption spectra identical to the pigments in undisturbed cells. French further found that the pigment complex could be precipitated with ammonium sulphate, an indication that protein was involved.

Working with the photosynthetic bacterium Rhodospirillum rubrum, Davis, Schachman and Stanier (1952) disrupted the cells by grinding with alumina. After centrifuging down the coarse material and unbroken cells, they were left with a clear coloured solution in water. The coloured fraction of the supernatant was found to have a sedimentation coefficient of 190S, and could be spun down at 21,000G. Electron micrographs of the pellet showed a flattened disc-like structure, of a molecular weight of 30×10^6 . Further evidence that this particle was the site of the photosynthetic activity was obtained from the fact that it was not present in dark grown cells which had not developed pigments. This structure, which they considered analogous to the chloroplasts of higher plants, they called a "chromatophore".

Schachman, Pardee and Stanier (1952) carried out ultra-centrifugal analyses on a number of species of bacteria which had been disrupted by a variety of methods, including -

Sonic oscillation

Grinding with alumina

Lysis with bacteriophage

Vacuum drying

Explosion by the rapid release of gasses.

The results indicated that, regardless of the method of disruption, there was great constancy of the sedimentation pattern for a single species of bacterium, and that there was also a great similarity of the extracts of different types of bacteria tested. One of the species investigated, Micrococcus aureus, was pigmented, and the ultra-centrifugal analysis showed a peak at 10S, but they drew no conclusions from this fact.

Careful kinetic studies on the release of water-soluble pigment complexes from the bacterium Rhodospirillum rubrum, in which the chromatophores were originally said to be present, have indicated that the chromatophores are not discrete organelles, but are part of a much more

extensive membranous structure (Holt, 1964). Thus chromatophores, the final products of membrane disintegration, appear only after much comminution of the cellular contents has taken place. If a mild method of disruption is used, such as osmotic shock, all the pigment is retained within the intact membrane structure, and can be completely centrifuged out at low gravitational fields (20,000G). Because of this fact, it may be doubted that such structures as chromatophores exist at all. Nevertheless, it has been found that when dark-grown photosynthetic bacteria are transferred to the light, new membrane must be laid down, as indicated by the uptake of P^{32} for incorporation in the phospholipids formed in the process. This might point to the formation of some specific structural component, absent in the non-photosynthesising organism, (Mathews, 1966). [N.B. The present trend among workers in this field, however, is to regard the term "chromatophore", (with its implication that the photosynthetic units of the bacterial cells are discrete organelles, which can be isolated in a homogeneous condition), as being too precise, and to use instead the term

"photosynthetic unit" (Jensen, personal communication, 1967).

So much for the photosynthetic bacteria; but it is with non-photosynthetic carotenogenic bacteria that this thesis is mainly concerned. As is the case in the photosynthetic bacteria, it is generally accepted that the carotenoid pigments are located in the protoplast membranes.

Before going any further, the meaning of this term "protoplast membrane" must be as accurately defined as possible in the light of present knowledge.

Fleming (1922) was the first to discover the action of the enzyme lysozyme in destroying the cell walls of certain bacteria, among them, S. lutea, M. lysodeiktus, and B. megaterium, but it was Salton (1952) who proved conclusively that isolated cell walls of M. lysodeiktus acted as a substrate for this enzyme. When this bacterial lysis took place in isotonic solution, the cells ruptured, and the bacteria died. However, Weibull (1953) found that if the lysis took place in 1.0 - 2.0 M. sucrose or 7.5% polyethylene glycol, a spherical "protoplast" was released from the bacterium, much larger in size than the original organism,

6

but retaining all of its metabolic characteristics. For instance, he found that the oxygen uptake of the protoplasts metabolising glucose was identical to that of whole cells. These "protoplasts" were quite stable in hypertonic solution, and could be centrifuged out without rupture. If they were shaken with air, they rapidly lysed. Weibull considered that this might be an effect of oxygen on the membranes, which might have some bearing on the mechanism of photodynamic oxidation disrupting cellular membranes, as Mathews and Siström (1960) attempted to establish for colourless mutants of S. lutea. Weibull (1953) allowed the protoplasts to lyse in hypotonic conditions, and he analysed the resulting suspension ultra-centrifugally. The bacterium was also disrupted by other methods, such as those employed by Schachman, Pardee and Stanier, (1952), and the ultra-centrifugal pattern agreed well with their results. At centrifugal fields between 600G and 14,800G, he found that intact lysed protoplast membranes could be centrifuged down. These lysed membranes, which he called "ghosts", contained

approximately 10% of the dry weight of the intact cells. It was further found that ultrasonic oscillation disrupted the membranes. The resulting fragments could be centrifuged out of suspension only at 105,000G. This result is similar to that found by Holt (1964) for the photosynthetic bacterium Rhodospirillum rubrum. Weibull (1953) gave no indication of whether or not he considered that the membrane could be reduced to a single homogeneous particle. He found evidence that at least some of the ribonuclear material sedimented with the "ghosts", even after treatment of the suspension with ribonuclease. The bulk of the bacterial ribonuclear material was, of course, found to be located in the 40S particle. That this structure was the bacterial ribosome was established by Schachman et al. (1952).

Further evidence for the presence in bacteria of an extensive cytoplasmic membrane system was found in electron micrographs of Rhodospirillum rubrum by Holt (1964) in the work already mentioned, and he quoted the finding of similar structures in A. agilis (Pangborn, Marr and Robrish, 1962). In the Gram positive bacteria a characteristic form of membrane

has been found, and called "mesosomes". These appear to vary widely in form, occurring in *M. lysodeiktu*s in a convoluted "villous" form. Excellent electron micrographs have been obtained illustrating these membranes in *M. radiodurans* (Thornley, Horne, and Glauert, 1965) and *S. manuria* and *S. ventricula* (Holt and Canale-Parola, 1967).

To sum up this work, at the present state of knowledge, there appears to exist in bacteria, a general membrane within the cell wall, which may, in many bacteria, be continuous with a more extensive cytoplasmic organisation. This portion of the cell is variously called the "protoplast membrane", "ghost fraction", "cytoplasmic membrane" or "mesosomes" according to the worker, and the bacterium used. They probably all possess a certain similarity of function and composition, but may differ according to the method of preparation. Due to the complexity of the structure, and the inherent possibilities of error in any analysis by disruption, there is a great difficulty in being more precise.

Where, in all this, does the location of bacterial carotenoids come? As already stated, the photosynthetic

elements in Rhodospirillum rubrum are located in the extensive membrane system. In the case of the non-photosynthetic S. lutea, Mathews and Sistrom (1959) state that all the carotenoids are located in the cytoplasmic membranes, and working with M. lysodeiktus, Gilby, Few and McQuillen (1958) found the same result. These workers went on to carry out a complete analysis of the "protoplast membrane", which they obtained following the methods of Weibull (1953); namely, lysis of the bacterium with lysozyme, followed by centrifugation at 20,000G. They obtained excellent consistency from three different preparations. The membrane was found to constitute approximately 9% of the total dry weight of the bacterium and was composed as follows:

Dry membranes			
<u>Lipid</u>			<u>Non-lipid</u>
28%			72%
	Protein		Carbohydrate
	50%		22%
(Both % of total membrane)			

An analysis of the products of hydrolysis of the protein

component showed that a wide range of amino acids was present in contrast to the paucity of the cell walls in this respect. The sugar moiety was found to be mainly mannose, with some glucose, galactose and hexosamine.

The membranes act as more than merely an osmotic waistcoat to the cell. They contain a large number of enzymes. Stanier, Gunsalus and Gunsalus (1953) found that the entire complement of cytochromes was located in the particulate fraction obtained from bacteria, and which can be identified with the membranes. They found that any oxidation reaction carried out by the membranes was KCN sensitive and that 2,3,5-triphenyl-tetrazolium chloride was reduced by the fraction in the presence of an oxidizable substrate. Visual spectroscopy revealed that there were no absorption bands above 450m μ , but that reduction with sodium hydrosulphite produced bands at 500-550m μ , and at 560-565m μ . These bands were roughly in the position of the $\alpha+\beta$ -bands of cytochromes b and c. Weibull (1953) found the same result with the membranes of B. megaterium, which on reduction, showed peaks of absorption at 530, 555, 600 m μ . Enzymic studies

with membranes from different bacteria, with a variety of substrates, indicated that they were the sites of the respiratory chain (Asnis, Vely and Glick, 1956; Robrish and Marr, 1962; and Marr and Cota-Robles, 1957). Thus, both the respiratory pigments (cytochromes and flavins), which are thought to act as photoreceptors in photodynamic oxidation, and the carotenoids, are located in the protoplast membrane. This is a further indication that this is the site of the photodynamic killing as suggested by Mathews and Siström (1960), and is consistent with the theory that the photoreceptors and the protective carotenoids lie close together, a suggestion occasioned by the fact that the rate of photodynamic killing of a colourless mutant is temperature independent.

As to a specific complex of carotenoid with protein which may be separated as a homogeneous particle from the non-photosynthetic bacteria, it was reported by Saperstein and Starr (1955) that a homogeneous pigmented particle had been isolated from C. poinsettia, by grinding the cells with alumina. They reported a sedimentation value of 35S for this. In view of the later findings

6

about the nature of the membranes, and the fact that the ribosomes have a sedimentation value of 35-40S, it seems unlikely that the particle in question was a carotenoid/protein complex. This does not preclude the existence of such a particle, but on the whole, it would seem that any basic particles would be the result of the comminution of the membranes, and of doubtful homogeneity. They would probably have more components; for instance, lipids and carbohydrates.

SECTION 7

PREVIOUS WORK ON THE CAROTENOIDS OF THE BACTERIA

S. FLAVA AND S. LUTEA

The previous work which has been carried out on the pigments of S. flava and S. lutea has been for the most part inconclusive, due mainly to the difficulty of obtaining sufficient material on which to work.

The first report was the work of Chargaff and Dieryck (1932), who reported the finding of two carotenoids in S. lutea. Based on the result of the partition of the less polar fraction between 90% aqueous methanol and light petroleum (B.P. 60-80°C), they deduced that a carotene was present, to which they gave the name of "sarcinene". In the light of later evidence about partition, it would seem obvious that more polar compounds than pure hydrocarbons would be epiphasic in the system named. It was a bold assumption, on the basis of partition alone, that a hydrocarbon was present, and even more bold to give it a name. The authors considered that the bulk of the material was a xanthophyll. Both reported fractions

had the same absorption spectrum in the visible region of 415, 440, and 462m μ . in light petroleum (B.P. 60-80°C).

In a second paper by one of these workers, (Chargaff, 1933), it is stated that the xanthophyll is partly esterified.

Nakamura (1936), again working with S. lutea, in ignorance of the previous work already mentioned, reported the presence of carotenoids, in particular an esterified xanthophyll. He found that the extract was most soluble in methanol and had little solubility in light petroleum (B.P. 60-80°C). He reported the spectrum in methanol as having peaks at 405, 455, and 468m μ .

Sobin and Stahly (1942) investigated chromatographically the pigments from a wide range of coloured bacteria, from the point of view of taxonomy. From S. lutea they obtained two carotenoid alcohols but found no hydrocarbon. In S. flava they reported the presence of one carotenoid alcohol identical with one of those present in S. lutea. They could find no evidence for the presence of esters.

Takeda and Ohta (1941) separated from S. lutea a carotenoid which they called "sarcinaxanthin". They obtained a crystalline sample which melted at 150°C. On the basis of partition studies, they considered that it contained one hydroxyl group.

Two pigmented coryneform bacteria (A. 1032 and A. 1062) were isolated from the skin of the Arctic cod, and their pigments investigated by Hodgkiss et al. (1954). They found that both organisms contained the same three carotenoids which had identical absorption spectra with peaks at 414, 437, and 467m μ in ether. The least polar of these was identified on the basis of chromatography and spectrum as neoxanthin. The one of medium polarity was said to be sarcinaxanthin by comparison with an extract from S. lutea, and they found a more polar fraction to which they gave the name of "corynexanthin". They ruled out the possibility of

- (a) conjugated keto groups
- (b) carboxyl groups
- (c) epoxy groups.

From a comparison of the chromatographic behaviour of the carotenoids with that of lutein and zeaxanthin,

70

they decided that the pigments must contain more than two hydroxyl groups, and from the absorption spectra, they concluded that the carotenoids might be derivatives of neurosporene. As sarcinaxanthin was reported to be present, the pigments of these organisms were investigated in the course of the present work.

The investigation of the carotenoid pigments of M. lysodeiktus by Rothblat, Ellis and Kritchevsky (1964) indicated the presence of a series of carotenoids differing in polarity, but all possessing the same chromophore. The absorption peaks in methanol of 415, 436 and 466m μ are close to those reported for sarcinaxanthin. They found that at least seven component carotenoids were present. No cis isomers were reported, but a consideration of the R_F values and the spectra makes it seem possible that some may have been present. To judge from the partition ratios between hexane and 95% aqueous methanol, none of the carotenoids isolated were carotenes. The authors discounted the possibility of conjugated ketone groups, carboxyl groups, and epoxy groups, but found some evidence from I.R. spectra that hydroxyl groups

were present. A chromatographic comparison between the pigments of M. lysodeiktus and those of S. flava was made in the work presented here, as the spectral similarities made it appear that there might be also a structural similarity.

EXPERIMENTAL

EXPERIMENTAL SECTION

The bulk of the work reported in this thesis was carried out on the pigments of the bacterium Sarcina flava, Strain No. 7503, supplied by the National Collection of Type Cultures, London. As the main interest in the work undertaken was the carotenoid pigments, and not the bacteria themselves, the preliminary bacterial experiments were merely designed to find the optimum conditions for bacterial growth, to allow the large scale production of bacteria for pigment extraction. Consequently, the experiments are of a superficial type.

The Morphology of the Sarcina bacteria

A check on the morphology of S. flava was made by examination of the bacteria under the light microscope. The bacteria were first stained by the Gram method, as detailed by Meynell and Meynell (1965).

(a) A bacterial smear was "fixed" on a glass microscope slide, by gentle heating.

(b) The smear was inundated with 1% aqueous methyl violet for 30 seconds, and then rinsed with water.

(c) Fixation of the stain with Burke's iodine solution took 1 minute (Burke's iodine solution consists of 2 gm. KI

and 1 gm. I_2 dissolved in 100 ml. of distilled water).

(d) The unfixed methyl violet was washed off with acetone, followed immediately by water.

(e) The bacteria were then counterstained with Ziehl's carbol fuchsin, diluted 1/20 with distilled water, for two minutes.

(Ziehl's carbol fuchsin is constituted as follows:- 1 gm. basic fuchsin is dissolved in 10 ml. of absolute alcohol, which is then added to 100 ml. of 5% w/v aqueous phenol).

The slide with its fixed and stained sample was then blotted dry, and viewed under the oil immersion lens ($\times 400$ magnification) of a light microscope.

The growth of cultures of *S. flava* at different temperatures.

Counting Methods

In order to ascertain the optimum conditions for bacterial growth, cultures were grown on nutrient agar surfaces, as well as in liquid medium.

Bacterial numbers were estimated by the optical densities of suspensions of the bacteria in buffered saline (Oxoid Ltd.) at pH 7.3.

The total amount of light scattered by any suspension increases with the ratio

$$\frac{\text{Particle size}}{\text{wave-length of incidental light}}$$

In the case of a bacterial suspension, the average diameter of the bacterial cells is constant, and the wavelength at which the turbidity is measured is also kept constant. In this case it was 600 μ , a wavelength at which there is no absorption due to pigment.

Thus at low concentrations of bacteria, the Lambert-Beer law holds true,

$$I = I_0 10^{-0.1c}$$

where I_0 is the incident light

I is the transmitted light

e is the extinction coefficient

l the depth of suspension

c the bacterial concentration

$$\log \frac{I_0}{I} = e l c.$$

Thus $\log \frac{I_0}{I}$, the "extinction" or "optical density" of the suspension plotted against c will give a straight line. As this only holds true at low bacterial

concentrations, plots had to be made to find the limits beyond which the method ceased to be valid.

(a) Two sets of measurements were made. In one experiment, the extinction was plotted against the actual number of bacteria per cu. mm. present in a suspension, as counted on a haemocytometer. In the other, a graph was drawn in which the extinction was plotted against the dry weight of bacteria present in a given volume of suspension. The dry weights of the bacteria were obtained as follows,-

The suspensions were placed in clean, weighed centrifuged tubes, the bacteria sedimented at 1970 g., and the supernatant decanted. The cells were then washed with 0.85% saline with 1% formalin added, and finally with distilled water (Meynell and Meynell, 1965), and dried at 105°C. until the weight was constant.

(b) Growth with temperature on nutrient agar

It was decided to trace the growth of a culture of bacteria in terms of the number of bacteria present on a nutrient agar plate over a period of time at 8°C, 15°C, 20°C, 30°C, 34°C, 40°C, 47°C. The cultures were grown on nutrient agar (Oxoid Ltd.) in petri dishes.

Each plate was inoculated with 2 ml. of a 48 hour nutrient broth culture (Oxoid Ltd.) which was spread evenly over the surface of the agar. Two such plates were then incubated in the dark at each of the recorded temperatures.

At intervals, samples of identical areas of the agar surfaces were taken in order to estimate the number of bacteria present. This was achieved by cutting three circles out of the agar with a wide bore cork-borer. The three samples were taken from different areas of the plate, to obtain as general an estimate as possible. The bacteria from each of these samples were then carefully washed off into 50 ml. volumetric flasks, with the buffered saline, and the volume finally made up to the mark. The three suspensions from each plate were then thoroughly shaken, and the extinction of each measured on a "Unicam" S.P.600 spectrophotometer. The measurement was carried out with 2 mm. light path cuvette in order to lessen the effects of secondary scattering. The mean of these three samples taken from each plate was then found, and taken as a measure of bacterial numbers.

The sampling was done over a period of 102 hours at

the following times:

6, 24, 30, 48, 54, 72, 78, 96, 102 hours.

The results were plotted to find the conditions of temperature and time producing the maximum number of bacteria.

(c) Growth in nutrient broth

The effectiveness of this method of following the growth of a bacterial culture was tested by carrying out a similar experiment more conventionally in liquid culture. In this case nutrient broth (Oxoid Ltd.) was made up, filtered through a bacterial filter to remove any particulate contaminants, and placed in 10 ml. aliquots in "Universal" bottles, which were then sterilized at 120°C for 20 minutes in an autoclave. These were then each inoculated with 0.5 ml. of a 24 hour broth culture of the bacterium, and incubated at 18°C, 30°C, 34°C, 37°C, 46°C. At intervals, 0.5 ml. was taken from each, under sterile conditions. The sample was then counted on a Neubaur haemocytometer (x 400) using a white blood cell pipette for dilution. The diluting fluid was M saline, containing 1% methylene blue.

(d) Effect of enrichment of the growth medium with glucose

In an attempt to increase the yield of bacteria, the

effect was tested on the growth of a culture after enrichment of the medium with glucose. Nutrient agar plates were made up with 1%, 2%, 5% glucose added. The inoculation and estimation of growth was made in the manner already described. All the cultures were inoculated at 34°C. in the dark.

Culture of the bacteria

- (a) The bacteria were continually maintained throughout the experiments on nutrient agar (Oxoid Ltd.) and the cultures, which were incubated at 34°C. in the dark, were replated every three days.
- (b) To ensure that there would always be a pure strain of the supplied culture of S. flava, a number of lyophilized samples were prepared at the beginning of the experiments. Several "Universal" bottles containing 10 ml of nutrient broth (Oxoid Ltd.) were autoclaved, and inoculated with samples of the bacterium from a plate culture. These were allowed to grow for 24 hours at the usual temperature. The cultures were then shaken, and 1 ml. samples poured into sterile tubes, the mouths of which were then sealed lightly with sterile cotton wool. The suspensions were frozen solid at -30°C. and

subsequently lyophilized. The tubes were then sealed, and stored at room temperature until required.

Bulk culture of the bacteria

A number of methods of producing large quantities of bacteria were tried.

(a) Using large aluminium dishes

The bacteria were grown on nutrient agar (Oxoid Ltd.) enriched with 5% glucose, in 6" x 5" aluminium foil dishes enclosed in nylon bags. The dishes were sterilized in the nylon bags at 120°C. in an autoclave for 20 minutes, and then the mouths were temporarily sealed with clothes-pegs. The nutrient medium was autoclaved separately, and poured into the aluminium dishes under sterile conditions, and allowed to solidify. The mouths of the bags were then sealed by heat. 10 ml. of inoculum from a 48 hour broth culture were introduced on to the agar through the nylon bag by means of a disposable syringe; the needle of the syringe was replaced after two inoculations, and the syringe itself after six inoculations. The inoculum was spread evenly over the surface of the agar by tilting the plate, and the excess poured off to lie in the bottom of the bag.

The plates were then incubated at 34°C for 48 hours before harvesting.

(b) Using large developing dishes

Large scale cultures on agar surfaces were also grown in sterilized enamel developing dishes. These were autoclaved with aluminium foil covers, which were not removed thereafter, until the cultures were finally harvested. The aluminium foil was merely folded back a little from one corner, to admit first the still-liquid nutrient agar medium, and, after that had solidified, an inoculum of 20 ml. of a 48 hour broth culture of the bacterium. This latter was spread evenly over the agar surface with a heat sterilized glass rod, and the excess broth poured off into lysol. The aluminium foil cover was then carefully resealed, and the plates incubated under the usual conditions.

(c) Bulk liquid culture

As methods involving surface culture of the bacteria did not produce a large yield of bacteria relative to the amount of medium used, an attempt was made to grow the bacteria in large scale liquid culture.

An aluminium milk churn of 8 gallon capacity was adapted as follows:

(1) The interior was coated with an epoxy resin ("Araldite"), which was cured by means of an electric light bulb suspended within the churn. The resin was allowed to harden for seven days prior to use.

(2) A large hole was bored in the lid to admit a rubber bung, through which were passed two 1 cm. bore glass tubes, for inoculating and aerating the culture.

(3) Two holes were bored low in the side of the churn. One admitted a 2.5 K.W. heating element, while the other held a thermometer.

The total convenient capacity of the churn was 40l.

A test culture of 1 litre of nutrient consisting of "Oxoid" nutrient broth enriched with 5% glucose, and containing $1/15$ M phosphate buffer (pH 6.8) (Kolthoff, 1932) was set up as a pilot experiment. This medium, in a 5 litre flask with facilities for aeration, was sterilized for 30 minutes at 120°C , and 1 ml. of an alcoholic solution of methyl red indicator added to check that the production of acidic metabolic end-products by the bacteria did not exceed the buffering capacity of the phosphate buffer. The medium was then inoculated with 100 ml. of 24 hour broth culture of S. flava under sterile conditions, and incubated for 48 hours. The culture was aerated by

means of a Leybold "Minni" pump. The incoming air passed through a filter of sterile glass wool. After 48 hours, the pH of the centrifuged medium was tested with a pH meter, and found to have fallen to pH 6.5. This was taken as being within safe limits, and consequently the pilot experiment was stepped up to the full 40 l. scale.

First, all the rubber tubes and bungs incorporated in the vessel were washed with alcohol to clean and sterilize them.

The quantities of nutrient medium required were weighed out, dissolved in hot tap water, and transferred to the churn, which was then topped up to the full 40 litres. 5 litres of this were removed to act as an inoculum for the whole, and sterilized separately in a 10 litre flask, inoculated with 100 ml. of a 24 hour broth culture and incubated at 34°C for 24 hours, with brisk aeration.

Meanwhile the main bulk of the culture medium was sterilized by boiling with the heating element; the open ends of the glass tubes being covered with cotton-wool-filled beakers as filters. A collar of cotton-wool

was fitted round the loose-fitting lid also as an air filter. The large bulk of liquid required 2-3 hours to boil. A glass tube filled with glass wool to act as a final air filter had been dry sterilized at 160°C for 6 hours.

The cotton-wool collar around the neck of the churn was now replaced by an air-tight seal of sterile moulding clay. The glass wool air filter was fitted to the air inlet, while an Edwards rotary vacuum pump was attached to the air outlet, and air was drawn through the medium to aid cooling. The line between the pump and the churn had inserted in it a water-vapour trap, consisting of a flask packed with absorbent silica. This was to ensure that the water-vapour drawn from the warm medium did not cause the pump to seize up.

When the medium had reached a temperature of 36°C, the pump was switched off, the air filter removed, and the outlet from the 5 litre culture attached. The pump was switched on and the 5 litre inoculum drawn into the main bulk. The empty flask was disconnected, a little silicon anti-foam added, and the air filter refitted. The churn was then placed in a cupboard maintained at a

temperature of 34°C and the culture allowed to grow for 48 hours. The constant aeration ensured good mixing of the medium.

Finally, the bacteria were harvested on the continuous head of the M.S.B. "High Speed 18" centrifuge.

Extraction of the pigments

A number of methods of disrupting the bacteria were assayed, in combination with a variety of solvents for the extraction of the pigments. The results of these purely qualitative tests are recorded in the "Results" section.

Many of the methods yielded partial extractions, but for quantitative work this was not sufficient. The method finally adopted was as follows:-

The harvested bacteria were placed in 50 ml. centrifuge tubes and washed with 0.85% saline. They were then suspended in three times their own volume of "Analar" methanol. The bacterial suspensions were subjected to 5 mins. of ultrasonic vibration in order to rupture the cell walls. The methanol was rapidly brought to the boil, by placing the tubes in a boiling water bath. Immediately the methanol had boiled, the tubes were taken out of the bath, and cooled before being centrifuged at 1970 g. for 2 mins. The clear yellow supernatant was decanted to be stored in the dark under an atmosphere of nitrogen. Two further similar extractions left the bacterial remnants entirely white. The three extracts were bulked, and stored under

nitrogen at -30°C until required.

This method of pigment extraction was necessarily rigorous to ensure the complete extraction of the pigments. It did, however, expose the carotenoids to the possibility of stereomutation. Besides the fact that the methanol was boiled, the use of ultrasound carries with it considerable potential hazards. Hughes and Cunningham (1963) stated that ultrasonic disintegration produces locally temperatures of 10,000 K., and pressures of 10^6 atmospheres.

To ensure that the adopted method of pigment extraction was not causing the formation of artefacts, a method which exposed the pigments to much less severe conditions was tried, and chromatographic comparisons made between the pigments extracted by both methods.

Unfired porcelain was ground in a ball mill and passed through a $76\ \mu$ sieve (Endcotts, London) to obtain reasonable homogeneity. This powder was mixed with an approximately equal quantity of washed bacteria, in a 50 ml. centrifuge tube. 20 ml. of methanol were then added, and the bacteria disrupted by grinding with a Potter homogeniser, the pestle of which had been turned to fit the tube. The procedure was carried out in a bath of ice-water. After grinding for 5 minutes, the

tube was centrifuged at 1970 g., and the yellow supernatant of methanol decanted. After the extraction had been repeated three times, the bacterial remnants were still quite yellow, but little further pigment could be extracted. The pigment extraction was finally stored at -30°C under nitrogen until required.

Purification procedures

Using methanol as the solvent meant that many lipids other than the desired carotenoids were extracted. Before the final stage of chromatography, a number of preliminary purification steps were carried out.

(a) Lipid precipitation

The precipitation procedure used was according to the method of Blessein (1962). To the methanolic extract an equal volume of diethyl ether was added. The diethyl ether had been prepared by allowing it to stand with sodium wire to dry it, and it was distilled over LiAlH_4 immediately prior to use, to destroy any peroxides which might have been formed. It was then kept in a dark-painted bottle, as light aids the formation of peroxides.

The addition of the ether to the methanol solution caused the immediate formation of white precipitate of

lipid, which flocculated, and was centrifuged off. The diethyl ether was then evaporated off on a rotary evaporator under reduced pressure at 35°C.

(b) Saponification

The next stage of the purification procedure was that of saponification of the total extract in order to separate the unsaponifiable carotenoids from the saponifiable lipid contaminants. Of the several methods listed in the literature, the one adopted was that mentioned by B.C.L. Weedon in "Chemistry and Biochemistry of Plant Pigments" (1965).

The volume of the methanol extract was measured, and sufficient KOH was weighed out to make a final concentration of 10% w/v with reference to the methanol. The KOH was dissolved in a minimal volume of water. When this had cooled, it was added to the methanol extract, which was gently agitated overnight, under nitrogen, in a covered flask at room temperature.

After about 16 hours, the saponification mixture was transferred in 50 ml. aliquots into a 500 ml. separating funnel, and 50 ml. of diethyl ether added. The unsaponified material, including the carotenoids, was driven into the diethyl ether layer by the addition

of 300 ml. of water and 10 ml. of saturated brine solution to break any emulsion which might form. The aqueous hypophase was discarded and the ether extract retained. The phase separation was repeated with the whole of the saponification mixture, and the combined ether extracts washed repeatedly with water, until the washings were neutral to "Universal" indicator paper.

The ether extract was taken to dryness at reduced pressure on a rotary evaporator, the water remaining being evaporated by the repeated addition of small volumes of methanol.

Finally, the extract was taken up in methanol for storage.

To ensure that the pigments were not alkali labile, the spectrum in the visible and ultraviolet regions of the extracts in methanol before and after saponification were compared. Chromatographic comparisons before and after saponification were also made to check that no isomers had been formed, and to investigate the presence of esters of carotenoids. The quantity of carotenoid before and after saponification was estimated spectrophotometrically in order to find the recovery from saponification.

Experiments to determine the loss in absorbance of a pigment solution with time, under a variety of conditions.

From the moment of extraction of carotenoids from tissues, solutions of the pigments, which are exposed to the effects of light and oxygen, decrease quite rapidly in their absorbance at the λ max. A certain amount of exposure to the deleterious effects of these agents is inevitable in most extraction procedures. In order to obtain an estimate of the loss involved, and if possible to alleviate it, the following experiment were carried out.

(a) The loss of absorbance of a carotene solution under various conditions

A light petroleum* solution of pure crystalline β -carotene (Sigma Chemical Co., St. Louis, U.S.A.) was made up. It had an initial absorbance of 0.379. 10 ml of this solution were pipetted into each of five glass "Quick-fit" 50 ml. flasks. The flasks were disposed as follows:-

* Unless otherwise stated the term "light petroleum" will refer to that fraction boiling between 60-80°C.

- 91
1. Control. Flushed out with nitrogen, sealed, and placed in a refrigerator at 4°C in the dark.
 2. Exposed to sunlight, unsealed, at room temperature.
 3. Flushed out with nitrogen, sealed, and exposed to sunlight at room temperature.
 4. Placed unsealed in the dark at room temperature.
 5. Placed unsealed in the refrigerator at 4°C.

At noted intervals over a period of 30 hours, the volume of each sample was restored to 10 ml. to compensate for evaporation, and the absorbance at 450 m μ measured with a "Unicam" S.P.600 spectrophotometer.

(b) Effects of sampling

It was thought that the actual sampling of the solutions maintained in an atmosphere of nitrogen might negate the effect of the inert atmosphere. To investigate this, a further experiment was tried, in which the loss of absorbance in sunlight of three identical solutions of β -carotene in light petroleum was followed; two under an atmosphere of nitrogen, only one of which was sampled at hourly intervals, while the other remained sealed for six hours; the third sample was open to the air, and also sampled at hourly intervals.

92

(c) The effect of an anti-oxidant on carotene degradation

To prevent the loss of carotenoid due to the photo-induced oxidation, α -tocopherol was tried as an anti-oxidant.

A 1% solution of α -tocopherol was made up in light petroleum. Test solutions were set up as follows:-

1. Control. 10 ml. of α -carotene solution in light petroleum (1.5 μ g/ml.).
2. Control. 10 ml. of β -carotene solution in methanol (1.5 μ g/ml.).
3. 9 ml. of α -carotene solution in light petroleum plus 1 ml. of α -tocopherol solution.
4. 9 ml. of β -carotene solution in methanol plus 1 ml. of α -tocopherol solution.

These solutions were placed in 50 ml. "Quick-fit" flasks open to air and sunlight at room temperature.

At intervals over a period of time, the volume was restored to 10 ml., and the absorbance of the solutions at 450 m μ measured. The absorbance of those carotene solutions to which solutions of α -tocopherol had been added, were corrected for dilution.

The experiment with the solution of α -carotene was carried out over a period of 6 hours; that with the solution of β -carotene over a period of 32 hours.

95

Variation in pigment production by S. flava with variations of (a) time of growth; (b) temperature of growth; (c) pH of the medium; (d) enrichment of the nutrient medium with different concentrations of glucose.

(a) Variation of pigment production with time of growth at 34°C.

It was found that the optimum quantity of bacteria for quantitative pigment extraction was that amount which could be supported on a petri-dish of "Oxoid" nutrient agar.

The dishes were inoculated with a 48-hour broth culture as already described. Incubating at 34°C, the plates were harvested at the following times:-

24 hrs., 30 hrs., 48 hrs., 54 hrs., 72 hrs., 96 hrs., 102 hrs., 120 hrs., 192 hrs.

The yellow layer of bacteria was scraped off the surface of the agar, with a spatula, and transferred to 10 ml. of methanol in a clean, weighed, glass centrifuge tube, 50 ml. capacity. The extraction procedure already described was carried out, and the white bacterial remnants dried to a constant weight at 105°C.

The combined methanol extractions were transferred to either a 25 ml. or a 50 ml. volumetric flask, which was made up to the mark with methanol. The amount of pigment extracted was calculated by finding the extinction of the methanol solution at 440 m μ , the λ max. of the carotenoids in that solvent. The extinction coefficient of the carotenoids was taken to be 3000 (see page/36).

(b) Variation of pigmentation with temperature of bacterial growth.

Nutrient agar plate cultures were inoculated and incubated at 8°C., 14°C., 20°C., 25°C., 40°C. The pigment production of those grown at 25°C. and 40°C. was estimated at 18 hrs., 24 hrs., 42 hrs., 48 hrs., 66 hrs., 72 hrs., 90 hrs., 96 hrs. At the other temperatures, the pigment production was measured only at one time, viz. 48 hours after inoculation. The extractions and estimations were carried out as before.

(c) The variation of pigmentation with pH of medium.

Nutrient agar (Oxoid Ltd.) was prepared and the pH adjusted with various volumes of 0.1N HCl, to obtain acid pH values, and 0.1N NaOH to obtain alkaline pH values. The final pH was tested with a pH meter, and the agar

9

finally autoclaved. Large aluminium dishes in nylon bags were used to culture the bacteria. The pH values at which the bacteria were grown were as follows:-

~~5.2~~, 5.2, 6.8, 7.8, 8.0, 9.0, 10.4.

The plates were inoculated with 48-hour broth culture and after 48 hours at 34°C., the bacteria were harvested, and the pigment extracted, each dish culture being halved in order to obtain an average for that particular pH. The pigment per unit dried bacterial remnants was found in the usual manner.

(d) Variations on pigment production with the enrichment of the medium with different concentrations of glucose

Nutrient agar plates were made up, enriched with 1%, 2% and 5% (w/v) glucose, and the bacteria inoculated and incubated for 48 hours at 34°C. in the dark before an estimate was made of the pigment production.

Final purification of the carotenoid fractions.

Phase separation

By far the most speedy of the methods of pigment resolution is the phase separation technique. Goodwin (1955) advocates the use of this method in conjunction with chromatography in the separation of carotenoids of different polarities. Many previous authors have based the characterisation of carotenoids on their partition behaviour (Chargaff and Dieryck, 1932; Sobin and Stahly, 1942).

In order to see whether this method could be conveniently employed in routine preparative work, phase separations of the total saponified extract were carried out between methanol, containing different concentrations of water, as the hypophase, and light petroleum, as the epiphase. The method followed was essentially that described by Petracek and Zechmeister (1956).

Solutions of 98%, 95%, 90% and 85% aqueous methanol were made up, and each was equilibrated against light petroleum by shaking the two phases together in a separating funnel, allowing them to separate, and then

running each off separately. A quantity of the total saponified bacterial extract was then dissolved in 10 ml. of the desired aqueous methanol. The absorbance of the solution was measured at 440 m μ on a "Unicam" S.P.600 spectrophotometer, to find the quantity of pigment present. This solution was shaken with an equal volume of light petroleum, which had been saturated with the aqueous methanol, and a partition of the carotenoids effected. The phases were separated, and each washed twice with 5 ml. of fresh solvent of the other phase. The washings were combined, and the new extinction of the methanol solution found. From this the partition ratio was calculated.

Both phases were taken to dryness on a rotary evaporator, at reduced pressure at 35°C., and the carotenoids taken up in a small volume of ether, for the chromatographic comparison of the phases.

Column chromatography

Following the report of Rothblat et al. (1964), who resolved the carotenoid pigments of M. lysodeiktus by column chromatography, Ca CO₃ was tried as an absorbent. 40 gm. of Ca CO₃ were activated for three hours at 140°C. and then dry-packed in a water-jacketed column with the aid of a little suction, and allowed to equilibrate overnight with light petroleum (B.P. 60-80°C). Throughout the experiment, cold water was run through the jacket to prevent the evaporation of the volatile solvent which would have disrupted the packing of the column.

A sample of the total saponified extract from S. flava was dissolved in 10 ml. of light petroleum and added to the top of the column with a pipette. A disc of filter paper, which had been pressed gently down on the top of the absorbent, prevented this action disturbing the packing of the column. A jacket of black plastic, with an observation slit, was placed around the column to prevent light-induced isomerisation and degradation of the carotenoids. The column was then developed, and the pigment fractions eluted, with eluants of increasing polarity.

The eluants were:-

1. Light petroleum (B.P. 60-80°C).
2. " " " /Acetone; 95/5 v/v.
3. " " " / " ; 90/10 "
4. " " " / " ; 80/20 "
5. " " " / " ; 70/30 "
6. " " " / " ; 60/40 "
7. " " " / " ; 50/50 "
8. " " " / " ; 40/60 "

The final fractions still remaining on the column were then eluted with methanol.

The eluants were passed through the column under a small pressure of nitrogen. The nitrogen line contained a manometer of mercury in order to check the pressure applied.

The effluent from the column was collected on a fraction collector (Central Ignition Co., Ltd., London), activated by a 5 ml. siphon. The pigment in each tube was estimated spectrophotometrically on a "Unicam" S.P.600 Spectrophotometer, and a graph drawn of the results. The tubes containing each fraction were combined, the pigments taken to dryness, and taken up

in a little methanol. Samples of each fraction were applied to silica gel G (Merck) coated thin-layer chromatography plates, to monitor the results. The thin-layer plates were developed in a solvent consisting of benzene/methanol/acetic acid: 87/11/2 v/v/v (Rothblat et al., 1964).

Thin-layer chromatography (T.L.C.)

Great use was made throughout this work of the technique of thin-layer chromatography. The method has already been discussed in general terms in the introduction. For the most part, the principle involved is that of adsorption chromatography.

Most of the experimental chromatograms were run on thick glass plates 20 x 20 cm. in area, on which an adsorbent layer of precise thickness was spread by means of one of the commercially available spreaders. More detail of this will be given later in the section.

However, in trial experiments carried out to find either the most useful adsorbent, or the best solvent to resolve a mixture, it was found convenient to carry out T.L.C. on a micro scale. This was achieved by spreading the layers on microscope slides. This micro-technique combined a conservation of adsorbent and solvent, with great speed, as a chromatogram on this scale required only 10-15 minutes to develop.

The technique used was as follows:-

A slurry of the appropriate adsorbent was made up in water, and thoroughly stirred to ensure complete

homogeneity. This slurry was poured into a clean petri dish. Into it were laid microscope slides which had been cleaned in ethanol containing 1% HCl. The slides were thoroughly coated with the slurry, removed, the undersides of the slides wiped clean, and finally dried and activated in an oven at an appropriate temperature.

It was found that 10 gm. of adsorbent were sufficient to prepare 6-8 slides. Of course it is obvious that the thickness of the layer could not be controlled by this method of preparation.

After the layers had been activated, the material to be chromatographed was applied as a line and the plate developed in a small closed vessel. 5 ml. of solvent were usually sufficient.

Using this technique, experiments were carried out to assess the relative qualities of a variety of adsorbents, and to investigate the effect on them of different times and temperatures of activation.

The adsorbents used were:-

1. Silica Gel G (Merck)
2. Alumina
3. MgO
4. CaCO₃

It was found necessary to add 5% CaSO_4 to the MgO and CaCO_3 to act as a binder for the layer. Slurries of these materials with water were made up in the following proportions:-

Silica Gel G/water : 1/2 w/v

Alumina/water : 1/1 w/v

$\text{MgO} + 5\% \text{CaSO}_4$ /water: 1.0/2.5 w/v

$\text{CaCO}_3 + 5\% \text{CaSO}_4$ /water: 1/2 w/v

The coated slides were placed in slide racks, and dried and activated at 100°C . and 160°C . for periods of $\frac{1}{2}$ hour, 1 hour, 2 hours, and 3 hours. After activation, the racks were removed from the ovens and cooled in a desiccator.

The standard dyes of Sudan Yellow and p-aminoazobenzene, dissolved in light petroleum (1% solution w/v), were then applied to the layers as a line by means of an "E-mil" 0.025 ml. micro-pipette (H.J. Elliot Ltd., Glamorgan). While the dyes were being applied to the layers, a glass plate covered all but the area where the dyes were applied. This served the dual purpose of maintaining the activation of

the adsorbent, which would otherwise have been deactivated by the water vapour from the atmosphere and the breath of the chromatographer, and also of providing a convenient straight edge for application of the line of dye. The micro-chromatograms were then developed with carbon tetrachloride, until the solvent front was near the top of the slides, which were then removed, the position of the front marked, and R_f values calculated.

The use of mixed adsorbents.

The main failing of Silica Gel G was the slowness of the development of a chromatogram of this material. With a view to using silica as an adsorbent for column chromatography, it was hoped to speed up the running of a chromatogram by the addition to the silica gel G of kieselguhr and "Hyflo". Microscopic slides were prepared with the following adsorbents -

- 1. Silica Gel G.
- 2. " + "Hyflo"; 1/1 (w/w)
- 3. " + " 1/2 (w/w)
- 4. " + kieselguhr; 1/1 (w/w)

The test dyes Sudan Yellow and p-aminoazobenzene were again run with carbon tetrachloride, noting the time required, the R_F values, and the resolution obtained for each adsorbent.

Thin-layer chromatography (2)

From the results of the experiments on a microscale, it was decided to use T.L.C. with Silica Gel G (Merck) as an adsorbent, both for analytical and preparative chromatograms.

Silica Gel G (Merck) consists of silicon dioxide especially prepared for T.L.C. containing about 13% CaSO_4 as a binder. The particle size of the adsorbent varies between 5-25 μ .

For analytical experiments, layers of 250 μ were used, but for the preparative work much thicker layers were prepared, as will be discussed in the section on preparative T.L.C. The glass plates used to support these layers were usually the standard 20 x 20 cm. square plates, but on some occasions 20 x 5 cm. plates were more convenient.

The apparatus used was that supplied by the firm of Desaga (Heidelberg, Germany), which consisted of a plastic base, on which plate glass plates of an accurately controlled thickness were placed, and an adjustable spreader of the type invented by Stahl (1958). The plastic base held a maximum of five 20 x 20 cm.

plates. These plates were carefully washed, and rinsed with distilled water. They were placed on the base, which had been moistened with a few drops of water. A little pressure on the plates spread out the water, and the plates were firmly held to the base by air pressure. The surface of the plates was wiped with paper towels soaked with acetone, which dried the plates, and ensured that no hydrophobic contaminants were present to prevent the formation of perfect layers.

30 gm. of Silica Gel G were weighed out and 60 ml. of distilled water slowly added, with stirring, to form a homogeneous slurry. It was found that the addition of a few drops of acetone lowered the surface tension sufficiently to prevent the formation of bubbles which would have marred the layers.

The spreader was placed at the end of the first plate, and the gap set to the desired thickness of layer. The slurry was poured into the trough of the spreader, the operating lever moved through 180° to open the gap, and the spreader moved steadily along the five plates. The plates were allowed to dry

slightly before being placed in a rack and activated at 105°C. for 1 hour. If they were not to be used immediately, they were stored in a large desiccator to preserve the activation of the adsorbent layer.

In analytical chromatography, the material to be chromatographed, dissolved in a volatile solvent such as light petroleum or diethyl ether, was spotted on to the layer with the aid of a micro-pipette.

This operation had to be carried out with some care to avoid breaking the adsorbent layer. The use of the plastic template supplied by Desaga (Heidelberg, Germany) was essential from three aspects,-

1. It enabled a line to be drawn through the silica near the top of the plate to ensure an even front and an accurate distance of development.
2. The fragile layer was protected while application of the material was in progress.
3. The spots of material could be applied along a straight line at a definite distance from the bottom of the plate. Before the spots of material were applied to the plate, a narrow strip of adsorbent was removed from the edges of the plate to prevent

edge effects. The spots were applied, and the solvent allowed to evaporate.

The plates were placed in special T.L.C. chromatography tanks, the walls of which had been lined with chromatography paper to ensure a constant degree of saturation of the atmosphere with solvent vapour (Randerath, 1966). The foot of each plate was dipped into the solvent which then ascended the adsorbent layer by capillary attraction, developing the chromatogram. While the development was in progress, the tanks were covered with black cloths to exclude the light.

(A development which was introduced after many chromatograms had already been run, was the use of standard dyes as markers. This made it possible to quote R_x values, which are more constant than R_f values in T.L.C. The dyes used were Sudan Yellow and p-aminoazobenzene).

When the development of the chromatograms was complete, the plates were removed from the tanks, and quickly dried with a gentle current of nitrogen. The resolution was then examined by a variety of techniques.

1. In the case of carotenoids, which were visible, the position of the fractions could be immediately marked.
2. Examination of the plates under ultraviolet light, either at 254 m μ or 356 m μ , exposed certain colourless materials, which either absorbed or fluoresced. Some chromatograms were run using as adsorbent Silica Gel G F₂₅₄ (Merck), which fluoresces under ultraviolet light of this wave-length, showing up any material which is strongly adsorbent.
3. Carotenoids could be stained, and their precursors made visible, by spraying them with a saturated solution of antimony trichloride in chloroform. 25 gm. of SbCl₃ were dissolved in 75 gm. of alcohol-free chloroform. The chromatogram was sprayed (in a fume cupboard) and heated at 110°C. for 10 minutes, then examined in daylight and under ultraviolet light. The stain is due to Carr and Price (1926).
4. Iodine vapour. This was found to be an excellent method for revealing the presence of colourless lipids. Iodine crystals in a flask were sublimated by heating the flask and the vapour directed at the layer (Randerath, 1966). The lipids took up the iodine, and showed up as

brown spots.

5. Charring. A solution of 25% aqueous H_2SO_4 (v/v) was sprayed on to the plates which were then placed in an oven at $160^{\circ}C$. for 10 minutes. If this method was used after iodine vapour, the lipids were stained a variety of colours (Randerath, 1966).

6. Rhodamine by Wagner, Horhammer and Wolff (1961). When the plates were prepared, rhodamine was added to the slurry in 0.05% w/v solution relative to the volume of the slurry. The rhodamine does not move in non-polar solvents. Lipids fluoresce red on a pink background under ultraviolet light.

Methods of recording the results of chromatography

Once all the detectable compounds had been marked, a number of methods existed of obtaining a permanent record of the chromatogram.

One was simply to take a tracing of the chromatogram. This was rapid and convenient, but gave no idea of the relative amounts of material represented by the spots.

Another technique was to obtain a photographic record of the visible compounds on the plate by use of light-sensitive "Azoflex" paper (Kodak Ltd.). The paper was taken from its light-proof packet, and placed on a flat surface with the sensitive side uppermost. On top of this was laid the chromatogram, with the adsorbent layer downwards. The paper with the chromatogram on top of it was exposed to the light of a 3 K.W. quartz iodine lamp for intervals of 40-90 seconds at a distance of 3 feet. The "Azoflex" paper was bleached with the exception of those spots which absorbed the light. The paper was then immediately passed through a bath of developer, and a permanent record of the visible compounds obtained. Other staining procedures, such as iodine vapour, or charring with sulphuric acid, could then be

used to reveal colourless fractions, and the photographic procedure repeated.

The third method, which was little used, consisted of preserving the whole chromatogram by "fixing" the adsorbent layer with a plastic material "Neatan" (Merck). A margin was first taken off the edges of the layer to leave a clean edge. The layer was sprayed with "Neatan", which was dried in an oven at 100°C. for ten minutes. The plastic-coated layer was then floated off the glass plate, dried, and filed.

Reproducible R_f values in T.L.C.

In his publication on the conditions necessary for the production of reproducible R_f values in T.L.C., Dallas (1965) states that unless these conditions are rigidly applied, the results obtained from the technique are variable, but that, with care, excellent reproducibility may be obtained. Consequently, in this work his suggestions were closely adhered to.

1. Adsorbent. Throughout the work, the adsorbent used was Silica Gel G (Merck) as this is a standard commercial preparation. However, despite this, the R_f values tended to vary from one batch of material to another, and so all the chromatograms in the series were made from the same batch of adsorbent.
2. Thickness of the adsorbent layer. This was maintained at 250 μ for all the experiments on reproducibility, as it had been found that the R_f values depended on the thickness of the layer (Randerath, 1966).
3. Activity of the adsorbent. As it had been found that the moisture content of the adsorbent, and thus its activity, was of cardinal importance in deciding the R_f values obtained, it was standardised by the following

procedure:- After the layers had been dried and activated in the normal manner, they were allowed to equilibrate in an atmosphere of 58% relative humidity in a closed chromatography tank, over a saturated solution of NaBr in water, for 16 hours. When they were taken out for use, they were immediately covered with a glass plate, which remained in position while the loading of the plates continued. The glass plate was held in position with a strong paper clip.

4. Use of the S- chamber. To ensure a good liquid-vapour equilibrium, the S- chamber was used. In design this was similar to that described by Davies (1963). A piece of 3 mm. diameter glass rod was bent to fit three sides of a 20 x 20 cm. T.L.C. plate to act as a divider. This divider was ground with carborundum dust on a glass plate to ensure a good fit with the glass plate. It was then stuck to a 20 x 20 cm glass plate with "Talurit" (Cable Covers Ltd.). This adhesive was not affected by the organic solvents used. In use, this S- chamber was clipped firmly to the plate on which the chromatogram was being run. To test that the seal between the S- chamber and the chromatogram was

air-tight, the S- chamber was clipped to a 20 x 20 cm. glass plate, the space between them filled with water, and the plates inverted. No water leaked from the apparatus, indicating a good fit.

When the S- chamber was to be used, a margin was carefully removed from all the edges of the layer of adsorbent. The layer was then equilibrated in an atmosphere of known humidity as already described. After the plate had been loaded with the material to be chromatographed, the protective glass plate was removed, and the S- chamber immediately placed over the layer, and held in place with six strong paper clips of the "fold-back" type. When these clips had been folded back, the entire S-chamber assembly could be placed in a T.L.C. tank of normal dimensions, which had been pre-equilibrated with the desired solvent. This procedure was considered superior to the method described by Davies (1963), whereby the S-chamber stood in a trough partially open to the atmosphere, and from which the solvent was likely to evaporate, thus changing the polarity of a mixed solvent while the chromatogram was developing.

The S-chamber was allowed to stand over the solvent

for 15 minutes until the small enclosed space was saturated with solvent vapour, then more solvent was added to the tank until it reached the layer, and the chromatogram was developed.

5. To ensure that there was a constant liquid/solid ratio throughout the whole chromatogram, the solvent was allowed to "overrun" for 15 minutes. If the chromatogram was allowed to run until the solvent front was some arbitrary distance from the top of the layer, and viewed by transmitted light, it was noticed that the transparency (and thus the liquid/solid ratio) fell off towards the front. It was found by Dallas (1965) that if the solvent was permitted to rise until it reached the top of the layer, and the plate left in the solvent, two things happened. Firstly, the transparency rapidly became uniform, and, secondly, that the R_f values increased slightly. In fact, the materials chromatographed continued to move slightly until an even distribution of liquid had been reached throughout the adsorbent layer. This process was called "overrunning".

6. Other factors which were kept constant to ensure reproducibility were:-

(a) Temperature at 15-16°C. in a constant temperature cabinet.

(b) The amount of material applied on to the plate in a single spot was kept reasonably constant.

The solvent front was allowed to travel a distance of 14 cm. from the origin to make the maximum use of the 20 cm. plates.

In order to increase the resolution, "double-development" was used on occasion. After the chromatogram had been developed for the first time, it was dried with a stream of nitrogen, and developed for a second time. This increased the resolution of materials with similar R_f values and low R_f values, but the results obtained were not so constant as from single development.

Still on a purely analytical scale, other techniques involving T.L.C. were:-

1. "Reverse-phase" chromatography

The method employed was that mentioned by Randerath (1966). The adsorbent layer used was kieselguhr, which was spread in 300 μ layers and dried in the usual way, and then dipped in a 7% (v/v) solution of olive oil in light petroleum (B.P. 100-140°C). A small area of kieselguhr at the bottom of the plate was left unimpregnated. The light petroleum was allowed to evaporate at room temperature for 24 hours. The samples were then spotted on the unimpregnated portion of the layer and the chromatogram developed with methanol.

2. "Wedge-strip" method

The technique can be considered to be an adaption of circular chromatography. Its main advantage is the excellent separation of materials with similar R_f values. The method depends on the development of the chromatogram being partially radial as well as vertical. This has the effect of spreading out the chromatographed material into narrow bands, yielding excellent resolution.

In practice, a template of the required shape, cut

from transparent plastic, was placed on the adsorbent layer, and outlined with a sharp-pointed instrument, leaving only a small intact portion of the layer at the tip of the "wedge", through which the ascending solvent flowed. It was at the tip of the "wedge" that the spot of sample was applied. The bottom of the plate was placed in solvent, and the chromatogram developed in the usual way.

The technique was particularly useful in comparing unsaponified and saponified extracts of the bacteria.

Solvents

The solvents used for analytical chromatography varied with the material to be chromatographed and the adsorbent used.

The solvent employed for much of the analytical work and in particular the reproducible chromatography, and the comparisons of the carotenoid pigments from different bacteria was a mixture of benzene, methanol, acetic acid in the proportions 87/11/2 (v/v). This solvent was originally employed by Rothblat et al. (1964) in their investigation of the pigments of M. lysodeiktus.

This polar solvent was excellent for the resolution of all the carotenoid fractions.

Other less polar solvents employed were:-

- (a) Light petroleum (b.p. 60-80°C)/ acetone 95/5 (v/v).
- (b) Ether/light petroleum: 70/30 (v/v).

The use of lipid standards other than carotenoids, in the T.L.C. of S. flava extracts.

(a) Co-chromatography with steroids

In the belief that the saponified methanolic extract of S. flava contained a steroid, thin-layer chromatograms were run on 250 μ layers of Silica Gel G comparing S. flava extracts with a number of commercially available steroids. The solvent used was diethyl ether/light petroleum: 70/30 (v/v).

The steroid standards which were used were:

1. Deoxycorticosterone
2. Testosterone propionate
3. Corticosterone
4. Hydrocortisone
5. Cholesterol

After the development, the plates were sprayed,

and the lipids rendered visible, with antimony trichloride in chloroform.

(b) Effects of lipid contaminants on T.L.C. of carotenoids

Another use to which lipid standards were put was in testing the effect of large quantities of lipid contaminants on the T.L.C. of carotenoids.

Mixed chromatograms of saponified extracts of S. flava were run on 250 μ Silica Gel G layers with large amounts of cholesterol and lecithin. The solvent used was benzene/methanol/acetic acid: 87/11/2 (v/v). (Rothblat et al., 1964). The developed chromatograms were recorded visually and after staining with iodine vapour. The chromatograms were run in S-chamber as well as in the normal chromatography tank.

Preparative T.L.C.

In order to make effective use of preparative T.L.C., it is essential to have a rapid and efficient method of applying large quantities of material to the adsorbent layers. In these experiments, the commercial applicator produced by Desaga (Heidelberg, Germany) was used. With some adaptation and much practice, this

produced excellent results.

From the results of the initial experiments it was decided to use Silica Gel G (Merck) as the adsorbent.

(a) Correct thickness of layer

To find the optimum thickness of the layer, and the maximum quantity of carotenoid which could be resolved on a single chromatogram, different quantities of carotenoids extracted from tomatoes were chromatographed on Silica Gel G layers of 300 μ , 500 μ , 1 mm., 2 mm., thickness.

(b) Choice of solvent

The solvent used in the analytical work, viz. benzene/methanol/acetic acid: 87/11/2 (v/v) (Rothblat et al., 1964) had certain disadvantages for preparative work, which will be discussed in the results section. Consequently, a solvent with similar polarity, but without these failings, was sought. Recourse was made to the technique of T.L.C. on microscope slides, employing the standard dyes, Sudan Yellow and p-aminoazobenzene. The solvents tried were the following mixtures of methanol in chloroform:

Chloroform

"	/methanol	9/1 (v/v)
"	"	5/1 (")
"	"	4/1 (")
"	"	1/1 (")
"	"	4/1 (")

The solvent eventually used for the initial resolution into main fractions of the total pigment extract was chloroform/methanol: 9/1 (v/v). (The chloroform was freshly distilled before use).

In order to find out whether better results were obtained using T.L.C., tanks, which were lined or unlined with chromatography paper, chromatograms were run under both these conditions, and the relative resolutions noted

The thickness of layer of Silica Gel G finally decided upon was 500 μ . Layers of this thickness were prepared in the normal way on 20 x 20 cm. glass plates, and dried. They were pre-run in chloroform/methanol: 9/1 (v/v) to wash any contaminating lipid material to the top of the layer, which was then scraped off the plate. The plates were dried and reactivated at 100°C. for 1 hour.

A quantity of the methanolic solution of the partially purified bacterial extract was taken from the deep freeze

and the amount of carotenoid present estimated spectrophotometrically. The solution was then taken to dryness on a rotary evaporator, and taken up in a suitable small volume of diethyl ether which had been purified as already described. The volume of ether was such that 2 ml. contained the maximum amount of pigment which could be conveniently resolved on a single plate. The concentrated ether solution was centrifuged at 1970 g. for 5 minutes to ensure that no undissolved material was present which would have blocked the syringe of the mechanical applicator. 2 ml. of the solution were transferred with the aid of a pipette into the syringe of the Desaga applicator, and applied as a thin line to a prepared 500 μ Silica Gel G plate under a gentle pressure of nitrogen. The operation was done in subdued light as much as possible. The plate was developed with a chloroform/methanol: 9/1 (v/v) solvent in a normal unlined T.L.C. tank. It was found that 5 20 x 20 cm. plates, held apart with rubber spacers, could be easily accommodated in a tank. While the chromatograms were being run, the tank was covered with a black cloth to exclude the light

When development was complete, the plates were dried with a stream of nitrogen, and the silica strips with carotenoid fractions were scraped off the plate with a spatula into 50 ml. glass centrifuge tubes containing 5 ml. of methanol. The methanol was stirred, and the carotenoid eluted. The tubes were centrifuged at 1970 g. for 5 minutes and the coloured methanol solution decanted. The silica was then washed with successive 5 ml. portions of methanol until no further carotenoid could be eluted. The washings were bulked, and the quantity of carotenoid present in each fraction estimated spectrophotometrically. In this way the relative proportions of the fractions could be found, and also the recovery of carotenoid from the plate estimated.

Paper chromatography

The form of paper chromatography used was that originally reported for carotenoids by Jensen and Jensen (1959), employing kieselguhr-filled papers (No. 287, Schleicher and Schüll, Dassel, W. Germany). As already described in the introduction, the method was particularly useful for the separation of isomers of carotenoids after the experimental induction of isomeric mixtures by light, catalysed by iodine, and also to follow the progress of the formation of derivatives of the carotenoids.

The papers were prepared by cutting out a wick, which extended to the centre of the paper, and which dipped into the solvent to develop the chromatogram in a circular manner.

The carotenoid, dissolved in acetone or diethyl ether, was spotted with a capillary tube on to the circumference of a pencilled circle around the end of the wick in the centre of the paper, and dried with a continuous stream of nitrogen directed from beneath the paper. In this way more than one carotenoid could be run on the paper for the purpose of comparison, the

paper being divided into sectors by slits to separate the development of individual spots.

The paper was placed in a petri dish with a little solvent in it, the rigidity of the paper preventing it dipping indiscriminately into the solvent. Only the folded-down wick was in contact with the solvent, which consisted of various mixtures of acetone in light petroleum. The actual solvents used will be described in the relevant sections.

Development took about 15 minutes, and the positions of the solvent front and the coloured carotenoid bands marked for the purpose of measuring the R_f value of the fractions.

The segments of paper with carotenoid could then be cut out, wedged firmly into pasteur pipettes, and the carotenoid eluted with 0.5 ml. of acetone or methanol.

Examination of the eluted carotenoids by spectroscopy on a "Unicam" S.P.800 automatic spectrophotometer ("Unica Instruments Ltd., Cambridge) then revealed the absorption spectrum and the quantity of material eluted.

It was found that the kieselguhr-filled papers were insufficiently adsorbent for the resolution of the

1

components of the least polar fraction of the carotenoids, and the colourless non-polar materials. For this purpose silica-filled chromatography paper (Whatman, S.G.81) with light petroleum as solvent was found to be excellent. It was used both in circular chromatography using the circular paper chromatography tank, for analytical purposes, and in simple descending chromatography on 2" strips for preparative work.

The use of carotenoids extracted from tomatoes for
experimental purposes and for the preparation of
standards

In order to try out techniques of isolation of carotenoids, a readily available source of large quantities of carotenoids was sought. Tomatoes provided this source.

A number of tomatoes were homogenised in a mixture of light petroleum/acetone 1/1 (v/v) in a "Waring" blender. 100 ml. of the solvent mixture were used for each tomato. The extract was centrifuged, to separate the two phases. It was found that the solid matter remained in the aqueous hypophase, and that the pigmented epiphase could be easily decanted off. The light petroleum solution was dried over anhydrous sodium sulphate, saponified, and reduced in volume in a rotary evaporator at 35°C, and subjected to column chromatography with two adsorbents.

1. Alumina

220 gm. of alumina (Brockman Grade 3) formed a column 33 x 3 cm. in length in a water-jacketed glass

column. This was equilibrated with light petroleum. A total of 5-6 mg. of carotenoid, estimated on the quantity of lycopene present (this constituting the largest proportion of the component carotenoids), was introduced on to the top of the column in 10 ml. of light petroleum. The solvents used to develop this column were:-

1. light petroleum
2. 1 - 10% acetone in light petroleum
3. 50% acetone in light petroleum.

A total of six fractions were collected from the column. Samples of these fractions were taken to dryness and their absorption spectra read on a "Unicam" S.P.800 spectrophotometer. Some of the fractions were further chromatographed by T.L.C. on alumina and/or Silica Gel G (Merck).

Fractions 2 and 3 were chromatographed by preparative T. L. C. on alumina with light petroleum.

2. Silica Gel G/Hyflo: 1/1 (w/w)

50 gm. of the mixture when packed in a 1.5 cm. column produced an adsorbent column 15 cm. in length. 2.5 mg. of carotenoid from tomatoes were chromatographed

on this adsorbent in the following solvents:-

1. light petroleum
2. 1 - 20% acetone in light petroleum
3. 30% benzene in acetone.

Crystallization

A portion of the solution of lycopene obtained from tomatoes was concentrated in light petroleum to a small volume (c. 0.5 ml.), and transferred to a centrifuge tube. It was cooled slowly in a refrigerator at 4°C for 2 hours, then transferred to a deep-freeze at -30°C. After 24 hours, needle-shaped crystals of lycopene had crystallized out of solution. These crystals were sedimented by centrifugation, and washed twice with cold methanol. The crystals were then dried under vacuum. Their shape was examined by microscopy.

The purity of the crystals was checked by dissolving some of them in light petroleum and examining the solution spectrophotometrically, and also by chromatography on Silica Gel G (Merck), using light petroleum as solvent.

The use of Rhodamine 6 G and Silica Gel G F₂₅₄ to locate colourless lipids

Thin-layer chromatograms were run with tomato

carotenoids in order to test the effectiveness of two methods of locating colourless lipids:-

- (a) impregnation of the adsorbent layer with 0.05% (w/v) Rhodamine 6 G,
- (b) Silica Gel G with added fluorescent indicator, (Silica Gel G F₂₅₄), which causes the layer to fluoresce when it is viewed under ultraviolet light of wavelength 254 mμ.

The preparation of carotene standards from tomatoes

This was combined with the experiments already mentioned, concerned with finding the optimum thickness of Silica Gel G layers for preparative chromatography.

Freshly extracted tomato carotenoids in light petroleum were partitioned with 95% aqueous methanol. The epiphase containing the carotenes was dried over anhydrous sodium sulphate, and reduced to a small volume. The quantity of carotenoid present was again estimated on the basis of the lycopene present.

Samples containing different quantities of carotene were loaded onto Silica Gel G (Merck) plates of different

13

layer thickness with the applicator as described. The solvent used was light petroleum. Double and triple development was used on occasion to increase the resolution of the component carotenes. Alumina layers were also used.

After development, the individual carotenes were scraped off the plates and eluted into light petroleum. The carotenes were examined spectrophotometrically to identify them, and to estimate the quantity recovered. Identification was also aided by a consideration of the R_f values of the fractions.

They were then concentrated to a small volume, and stored under nitrogen for use as standards to compare with the carotenes extracted from the bacterium S. flava.

The controlled deactivation of Alumina

Tomato carotenoids were used to test the efficacy of Alumina for the resolution of the least polar carotene ϵ -, α -, β -carotene. It was found that activated Alumina layers exposed to the air rapidly lost their activity. It was decided to find whether or not an optimum activity could be achieved by the controlled deactivation of fully activated plates.

An alumina (Merck) layer, 500 μ in thickness, was prepared, and activated at 100°C for 1 hour. On taking it out of the oven it was immediately covered with a glass plate. This plate was gradually moved across the alumina layer throughout a period of 40 minutes, progressively exposing sections of the plate for different periods of time. The end result was a plate on which strips of alumina had been exposed to the air for 40 minutes, 30 minutes, 20 minutes, 10 minutes, 0 minutes. The plate was then loaded with tomato carotenes in light petroleum by means of the mechanical applicator. α - and β -carotene (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) were used as standards, and the plate was developed in light petroleum.

Analysis of the carotenoids

1. General methods employed.

(a) Quantitative estimations

Quantitative estimations on the carotenoids were carried out spectrophotometrically according to the usual method in a cuvette with 1 cm. light path (Gillam and Stern, 1955). The amount of carotenoid present in a

solution was calculated from the equation:-

$$w = \frac{D \times v \times 10}{E_{1\text{ cm.}}^{1\%}}$$

where w = amount of material in solution in mg.
 D = optical density of the solution at the wavelength of maximum absorption
 v = volume of the solution
 E = optical density of a 1 cm. thick layer of a 1% (w/v) solution of the material measured at the wavelength of maximum absorption.

As the quantity of the carotenoids isolated from the bacteria did not permit the empirical finding of the exact $E_{1\text{ cm.}}^{1\%}$ for the individual pigments of S. flava, it was taken as 3000 throughout the work. This figure was based on the $E_{1\text{ cm.}}^{1\%}$ for neurosporene (2990 in light petroleum), and β -carotene (2890 in light petroleum), the spectra of which most nearly resemble those of the carotenoids from S. flava (Isler and Schudel, 1963).

As the quantities of the carotenoids estimated in the course of the work were mainly relative values, either for finding the yield from a particular experiment

1

or to find the proportions of fractions, an absolute measurement was not of great importance.

(b) Spectroscopy

Visual and ultraviolet

Finding the absorption spectrum of the individual carotenoids in the visual and ultraviolet region of the spectrum was an essential process in their characterisation.

It was carried out in a solvent such as spectro-quality hexane (B.D.H.) or Analar methanol. The spectra were measured in silica cuvettes of 1 cm. light path. Where the concentration of the carotenoid permitted, the cells used had a capacity of 4 ml. Often, however, the measurement had to be done in a small volume, as, for instance, when the carotenoid had been eluted from a paper chromatographic separation. In this case a micro-cuvette was used which had a capacity of only 0.4 ml.

The spectrophotometric measurements were made initially on an S.P.500 or S.P.600 spectrophotometer ("Unicam" Instruments Ltd., Cambridge), and, when it became available, S.P.800, made by the same firm.

The machines were occasionally checked for accuracy with a holmium filter.

The deductions which could be made from the position and form of the absorption peaks have been well detailed in the introduction, and need not be repeated here.

Mass spectrometry

The mass spectrometry reported here was only made possible by the generous collaboration of Associated Electrical Industries Ltd., Manchester. The work was actually carried out by Dr. J. R. Chapman of their Consultative Laboratory. He was also of great help in the interpretation of the results.

The sample was dissolved in the minimum volume of methylene dichloride, and the solution spotted on to a ceramic direct-insertion probe, which was inserted through a vacuum lock into the ion chamber of an A.E.I. "M.S.9" mass spectrometer. The sample was heated by contact with the walls of the ion chamber to achieve a reasonable rate of evaporation, and the mass spectrum was scanned with the resolving power set at 1000. The temperature of the ion chamber was approximately 250°C.

Masses of the peaks were then measured very accurately at a resolving power of 12,000.

From the results, the molecular weights of the main fractions were found, and their empirical formulae calculated.

Infra-red spectroscopy

I.R. spectroscopy of those carotenoid fractions which were obtained in sufficient quantity was carried out on a "Unicam" S.P.200G.I.R. spectrophotometer. ("Unicam" Instruments Ltd., Cambridge). The spectra were read with the carotenoids either in solution in spectroquality carbon tetrachloride and carbon disulphide (Merck), in cells with NaCl windows, and 1 mm. "Teflon" spacers, or incorporated in KBr discs.

The quantity of KBr which was used for a single disc was approximately 100-125 mgm. The carotenoid, dissolved in chloroform, was dropped on to the KBr, and the chloroform evaporated off by gently grinding the powder in an agate mortar. The dry material was transferred to an agate ball mill, and ground for 5 minutes. The intimately mixed powder was then pressed

in an evacuable die by means of a hydraulic press.

The powder was transferred to the bore of the die (R.I.I.C. which was then evacuated with a high vacuum pump for 18 minutes, and 10 tons pressure then applied for 20 minutes. The pressure and the vacuum were released, and the pressed disc carefully removed, and placed in a disc holder. The spectrum was scanned over the full range of 2.5 - 14 microns.

(c) Tests for the presence of epoxy and aldehyde groups

All the polar carotenoids were subjected to the purely qualitative test outlined by Karrer and Jucker (195 in which the addition of concentrated HCl to an ethereal solution of a carotenoid containing an epoxy or aldehyde group(s) causes the formation of a blue colour.

A somewhat more exact test for the presence of 5-6 epoxy groups is the observation of a spectral shift of 20-25 m μ to shorter wavelengths by the formation under acid catalysis of a furanoid 5-8 epoxy formation. (Curl and Bailey, 1954)(Jungalwala and Cama, 1962).

The carotenoid was dissolved in methanol; to 3 ml. of solution in a 1 cm. light path cuvette, one drop of a

solution of 0.05 N HCl in methanol was added. The λ max. was read before and after the addition of the acid/methanol, and again after 10 minutes.

(d) Partition ratio

The partition ratios between light petroleum and 95% aqueous methanol were found for all the main fractions. The method used was essentially that of Petracek and Zechmeister (1956). Each phase was first shaken with the other. The carotenoid, dissolved in 5 ml. of one phase, was partitioned with 5 ml. of the other phase in a 10 ml. measuring cylinder, to check for any change in volume of the mixture. The amount of carotenoid present in one of the phases was estimated before and after partition. From this the distribution of the fraction between the phases, and thus the partition ratio, was calculated.

(e) Iodine isomerisation

The method is based on the observations of Zechmeister and Polgar (1943). To a solution of the fraction in acetone, a drop of iodine solution in light petroleum was added: (10 μ gm. ml.). The carotenoid

14

solution was exposed to weak sunlight for 2-3 hours, under an atmosphere of nitrogen. At intervals throughout the period, the course of the isomerisation was followed by chromatographing samples of the solution on circular kieselguhr-filled chromatography paper (Schleicher and Schüll, Dassel, Germany), using as solvent appropriate mixtures of acetone in light petroleum. When an equilibrium had been reached, a larger sample of the isomeric mixture was chromatographed in the same manner, the R_f values of the component isomers measured, the fractions cut out, eluted with methanol, and their spectra and relative proportions measured.

(f) Acetylation

The technique used in this case was the modification suggested by Jensen and Jensen (1962) of the original method of Kuhn and Sørensen (1938). A sample of the chromatographically pure carotenoid was taken to dryness on a rotary evaporator, and further dried under high vacuum for 30 minutes. The carotenoid was dissolved in 1 ml. of pyridine, which had been dried over NaOH

pellets and redistilled. 0.1 - 0.2 ml. acetic anhydride was added and the reaction allowed to proceed at room temperature, in the dark, under an atmosphere of nitrogen. Samples were withdrawn at regular intervals in order to follow the course of the reaction over a period of 24 hours. Circular paper chromatography on kieselguhr-filled papers were used for this purpose. It was found that the reaction was complete after 24 hours. The formation of intermediates and the final product was easily followed by this means. The final ester was recovered into diethyl ether, which was thoroughly washed with water to remove the pyridine. Spectrophotometric examination was used to check that no degradation had taken place during the reaction, and to estimate the yield.

(g) Formation of a silane from an unesterified tertiary hydroxyl group

Only primary and secondary hydroxyl groups are susceptible to acetylation according to the method described. If the presence of a tertiary hydroxyl group was suspected, it was assayed for by the formation of a silane after esterification had eliminated any

primary and secondary hydroxyl groups. (Jensen, 1966). A sample of the dry esterified carotenoid was dissolved in 0.5 ml. dry pyridine and 0.2 ml. hexamethyldisilane and 0.1 ml. trimethyl chlorosilane added. The reaction proceeded at room temperature in an atmosphere of nitrogen. After one hour, carbon tetrachloride was added to the solution which was then taken to dryness. The carbon tetrachloride removed the excess silane. The product was dissolved in methanol, and examined by chromatography on kieselguhr-filled paper as before. The presence of a product other than the original ester indicated the presence of a tertiary hydroxyl group.

(h) Reduction of esters with LiAlH_4 (Goodwin, 1956)

A small quantity of LiAlH_4 was suspended in dry ether, and the suspension filtered through glass wool to remove the larger particles. The filtered suspension was added to an ethereal solution of the ester in a separating funnel. After a short time the reaction was terminated by adding wet ether to the solution. The addition of water destroyed any excess of LiAlH_4 remaining. The products were studied by chromatography

on kieselguhr-filled papers. The appearance of products other than the ester or the original carotenoid, indicated that reducible groups were present.

(i) Oxidation with nickle peroxide (Jensen, 1966)

The carotenoid was dissolved in dry ether and nickle peroxide was added in the ratio 5 mg. nickle peroxide to 1 mg. sample. The reaction occurred spontaneously at room temperature under nitrogen. Samples were taken at intervals, and the course of the reaction followed chromatographically as before. The test was specific for the presence of allylic hydroxyl groups.

(j) Methylation (Metcalfe and Schmidt, 1961)

A sample of the fraction under test was dried as for acetylation, and dissolved in moisture-free methanol. 0.5 ml. of methanolic boron trifluoride was then added, and the reaction mixture refluxed for 2-3 minutes. The product was recovered into ether and examined chromatographically and spectrophotometrically.

Characterisation and analysis of the individual
carotenoid fractions isolated from S. flava.

Fraction 1.

This was the least polar of the fractions. It was isolated in such small quantity that no chemical tests could be tried on it. The fraction was chromatographed on Whatman S.G.81 silica impregnated paper with standard carotenes isolated from tomatoes. These were - phytoene, phytofluene, α -carotene, β -carotene, lycopene, ξ -carotene, and neurosporene. The solvent used was light petroleum. Excellent results were obtained by the use of circular chromatography. The results were examined by visible and ultraviolet light.

The main bulk of the fraction was chromatographed on strips of the same silica impregnated paper in the same solvent. The components were cut out, eluted, and the spectrum of each found, in the visible region.

Fraction 2.

Spectral measurements were made in the ultraviolet, visible, and infra-red regions of the spectrum. The fraction was subjected to mass spectrometry. All the

1

chemical tests mentioned were carried out with the exception of Test (1) (methylation).

Fraction 3.

The same analytical tests were carried out as mentioned above for Fraction 2.

A comparison was made of this fraction with a carotenoid possessing an identical absorption spectrum, which had been isolated from the bacterium Flavobacterium dehydrogenans by Dr. O. B. Weeks of New Mexico State University. The two compounds gave identical results on chemical examination by acetylation, oxidation, and iodine catalysed isomerisation, and neither the original compounds or their derivatives could be separated by chromatography on kieselguhr-filled paper. This work was done in Dr. S.L. Jensen's laboratory in Trondheim, Norway, under her supervision.

Fraction 4.

Spectral measurements were made in the ultraviolet, visible and infra-red regions of the spectrum. All the previously mentioned chemical tests were assayed on this fraction.

Comparison of the carotenoid pigments from S. flava with those from other pigmented micro-organisms.

The carotenoid pigments were extracted from large quantities of the following bacteria:-

<u>Name</u>	<u>Source</u>
<u>S. lutea</u>	National Type Culture Collection (No.196)
<u>M. lysodeiktus</u>	" " " " (No.2065)
<u>Coryneform species</u>	Torry Research Station, Aberdeen (No.1036)
<u>M. radiodurans</u>	Dr. B.E.B. Moseley, Molteno Inst., Camb.
<u>S. aurantiaca</u>	American Type Culture Collection, Maryland U.S.A.

The conditions of culture of the bacteria were as follows

<u>Medium</u>	<u>Temp.</u>	<u>Time of growth</u>
<u>S. lutea</u> : "Oxoid" nut. agar.	25°C	72 hrs.
<u>M. lysodeiktus</u> " " "	34°C	48 hrs.
<u>Coryneform species</u> : Blood agar	15°C	72 hrs.
<u>M. radiodurans</u> :	34°C	48 hrs.
"Oxoid" tryptone 0.5%		
Glucose 0.1%		
Yeast Ext. (Difco) 0.3%		
Aspartic Acid 0.2%		
"Oxoid" agar No.3 2.0%		

Adjusted to pH 7.2 with N NaOH

<u>S. aurantiaca</u> : "Oxoid" nut. agar.	34°C	72 hrs.
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14

The bacteria were harvested, washed, and the pigments extracted and saponified as described for S. flava. Comparisons of the carotenoid pigments from the various sources were made by T.L.C. on 250 μ layers of Silica Gel G (Merck), using benzene/ethanol/acetic acid: 87/11/2 (v/v).

Carotenoids from tomatoes were also compared with those from S. flava. The chromatograms were recorded in visible light, and after staining with iodine vapour and 25% aqueous H_2SO_4 .

In addition to these qualitative comparisons, a quantitative comparison of pigment production was made between S. flava and S. lutea grown at 34°C. in the dark for 48 hours, on nutrient agar. In each case the amount of pigment extracted, estimated spectrophotometrically, was related to the weight of dry bacterial remnants after extraction.

EXPERIMENTS WITH THE PROTOPLAST MEMBRANE OF S. FLAVA

It was decided to prepare the protoplast membranes after the manner described by Weibull (1952), and by Gilby, Few, and McQuillien (1958).

The bacteria were grown in bulk on nutrient agar in developing dishes at 34°C in the dark. After harvesting the distilled water, the suspension was filtered through glass wool to remove any solid nutrient medium, which might affect the analyses carried out at a later stage. Examination of the suspension under the microscope confirmed that no extraneous matter was present. The bacteria were then washed three times with 0.85% saline and 1% formalin, and finally with distilled water, and stored at 4°C for a few days until sufficient bacteria had been harvested for the experiments.

Lysis of the bacteria

To find out the minimum time required for the lysis of a given suspension of bacteria in buffer solution, the following experiment was carried out.

Drying a sample of the bacteria by lyophilisation, it was found that 75% of the wet weight was due to water

and from this it was possible to estimate the dry weight of newly harvested bacteria, when suspending them for lysis with lysozyme.

A suspension of the bacteria containing an estimated 10 mg. dry weight of bacteria/ml., was made up in Sørensen's sodium citrate buffer, pH 6.8 (Kolhoff, 1932). Crystalline lysozyme (Sigma Chemical Co., St. Louis, Missouri, U.S.A.) was dissolved in this suspension to a final concentration of 100 μ gm./ml.

The suspension was incubated at 25°C, and the course of lysis followed by the fall in optical density at 600 m μ . At the end of six hours the concentration of the lysozyme was increased to 150 μ gm./ml., to investigate any further lysis.

Isolation and purification of the protoplast membranes

The minimum length of time finally arrived at for the complete lysis at 25°C of a suspension of bacteria containing approximately 10 mg. dry weight/ml. in buffer containing 100 μ gm. lysozyme/ml., was 1 hour. The citrate buffer, pH 6.8, contained 0.025M MgSO₄ to stabilize the protoplast membranes (Weibull, 1956).

After the period allowed for lysis, a small quantity of D.N.A.-ase was added to the suspension to lower the

viscosity, and the absorption spectrum found, so as to follow the changes in components produced by the differential centrifugation. The suspension was transferred to 50 ml. plastic centrifuge tubes, and centrifuged at 600-1000 g. for 15 minutes. The centrifuge was maintained at a temperature of 4°C, and the suspension thereafter maintained at this temperature, to prevent enzymic degradation. This centrifugation produced a diffuse yellow pellet, which was tested, microscopically and by plating out on nutrient agar, for the presence of entire bacteria. No evidence of whole or viable bacteria could be found, and so the pellet and supernatant were recombined, and centrifuged at 15,000 g. for 15 minutes. The clear colourless supernatant was removed by suction to avoid disturbing the pellet. The pellet was washed thrice with cold buffer, and dialysed against running tap water for 24 hours, then against distilled water in the cold room at 4°C. Finally, the material was centrifuged at 15,000 g., most of the supernatant removed, and the pellet lyophilised for analysis.

Analysis of the protoplast membranes

(a) In order to obtain a general indication of the composition of the entire bacteria, so that the analysis of the membranes could be related to it, a quantity of thoroughly washed whole bacteria was lyophilised, and samples taken for estimation of total nitrogen, phosphorous and carotenoid, as well as for the estimation of the relative proportions of lipid and non-lipid.

Nitrogen estimation

This was done by the micro-Kjeldahl technique. Weighed samples of lyophilised bacteria were digested with 2 ml. nitrogen-free, concentrated H_2SO_4 , in Kjeldahl flasks, until the acid was colourless. The digest was carefully washed into the bulb of the micro-Kjeldahl apparatus, with freshly distilled water. 40% KOH was added until the digest was alkaline, and steam passed through the mixture to distill off the ammonia gas, which was collected, dissolved in the condensed water, in 10 ml. of 2% (w/v) boric acid containing mixed indicator (Ma and Zuazaga, 1942).

The dissolved ammonia was titrated with standardised N/10 HCl, and the nitrogen present in the digested sample estimated.

Phosphorus estimation

The estimations were made according to the method of Allen (1940), which is based on a colorimetric procedure.

The reagents used were as follows:-

1. Ammonium molybdate solution. An 8.3% solution of the A.R. salt (B.D.H.) in distilled water.
2. 10N sulphuric acid (A.R.)
3. Amidol reagent. 1 gm. of amidol and 40 gm. of sodium bisulphite dissolved in 100 ml. of distilled water. This solution could only be retained for five days, stored in the dark.

Method. With the aid of a standard solution in distilled water, containing 1 mg. of phosphorus/ml., a standard curve of phosphorus concentration against colour was obtained. Different quantities of the diluted standard solution were pipetted into 25 ml. volumetric flasks, and to each was added first 1.25 ml. of the 10N sulphuric acid, then 2 ml. of the amidol reagent, and 1 ml. of the ammonium molybdate solution. Distilled water up to 25 ml. was added, and after mixing, the blue colour was allowed to develop for 20 minutes, at the end of which

1

period the extinction of the solutions at 540 m μ was found on an S.P.500 spectrophotometer (Unicam Ltd.). From these results, a standard curve was drawn, to which the later estimations of unknown quantities of phosphorus were related. Great care was taken to ensure that all the glassware used in the estimations was scrupulously clean.

When phosphorus estimations were made on bacteria and bacterial extracts, a weighed quantity of the material was digested in micro-Kjeldahl flasks with 1.25 ml. of 10N sulphuric acid until the digestion mixture was clear. Digestion overnight usually secured this. This digest was then carefully washed into a 25 ml. volumetric flask with distilled water, and the phosphorus estimated in the manner described.

Carotenoid content

The lyophilised bacteria were rehydrated overnight with a little water, and the carotenoids extracted with absolute methanol in the usual manner. The methanol solution was made up to 25 ml. in a volumetric flask, and the amount of carotenoid extracted estimated spectrophotometrically at 450 m μ .

Extraction of the lipids from entire bacteria

Weighed samples of lyophilised bacteria were rehydrated overnight and the lipids extracted with chloroform/methanol 4/1 (v/v) (Few, 1955). Ultrasound was used to disrupt the cells, and the lipids were extracted on a boiling water bath. The solutions of lipids were transferred to clean, weighed, round-bottomed flasks, and the solvent was taken off on a rotary evaporator. The drying process was completed in a desiccator, under reduced pressure, until a constant weight was reached.

The non-lipid material remaining was dried at 100°C until the weight was constant.

The nitrogen and phosphorus content of both fractions was estimated.

(b) To check the report that the entire complement of carotenoid in non-photosynthetic carotenogenic bacteria is located in the protoplast membrane system (Mathews and Siström, 1959 (S. lutea); Gilby, Few and McQuillen, 1958 (M. lysodeiktu)), the membranes prepared from a known weight of lyophilised bacteria were suspended in a known volume of buffer, and

15

disintegrated with ultrasound to render the suspension translucent. The amount of carotenoid present was estimated spectrophotometrically, correcting for the light dispersion due to the turbidity of the suspension. From this, the carotenoid present in the membrane fraction could be related to the amount present in the entire bacteria.

(c) In order to find the proportion of the weight of lyophilised bacteria represented by the membrane fraction, four weighed samples of dry bacteria were soaked in the citrate buffer for twelve hours, and the protoplast membranes isolated as described. They were then lyophilised and weighed.

Preparation and testing of a water-soluble fraction
of the protoplast membranes

In order to test the hypothesis that there might exist a specific interaction between the carotenoids and a protein (or proteins), forming particles which could be isolated from the bacterial protoplast membranes in a homogeneous condition, a method was devised to reduce these membranes to water-soluble fragments.

The following synthetic detergents were tested as a means of producing a water-soluble extract from the protoplast membranes:-

1. Tween 20 (B.D.H.)
2. Manoxyl O.T. (B.D.H.)
3. E.D.T.A. (disodium salt) (Light and Co.)
4. Cetyl trimethyl ammonium bromide (B.D.H.)
5. Tergitol 7 (B.D.H.)
6. Cetyl trimethyl pyridinium chloride (B.D.H.)
7. Crill S.6 (Croda Ltd.)

Protoplast membranes were prepared as already described, and suspended in water. Aliquots of identical volume were diluted to 100 ml., and the

listed synthetic detergents added in concentrations varying from 0.1% to 1%. The suspensions were stirred at room temperature for 24 hours, and then centrifuged at 40,000 g. for 30 minutes. The supernatants of each sample were made up to the same volume, and a measure of the quantity of pigmented material remaining in solution obtained by finding the optical density at 444 m μ . Crill S.6 appeared to yield the best results, and was employed for the following experiments.

The full method of preparing the soluble fraction is outlined in the scheme, Fig. E1.

The following tests were done on the fraction S 2, which contained the bulk of the pigmented material.

(a) The spectrum of the yellow, water-soluble material was compared with that of the entire protoplast membranes

(b) Precipitation

1. Ammonium sulphate was added to 10 ml. volumes of S 2 in increasing concentrations, until no further precipitation occurred. The concentration at which full precipitation occurred was noted.

Figure E 1

Method of preparation of a soluble extract of the
protoplast membranes

Bacterial cells suspended in phosphate buffer,
pH 7.0. Lysozyme added and lysis of the cells
allowed for one hour at 25°C. Lysate finally
centrifuged at 15,000 g. for 15 minutes.

Almost clear supernatant.

Yellow pellet resuspended
in 1% Crill S.6 solution
in water, and shaken over-
night. Finally centrifuged
at 40,000 g.

Coloured supernatant (S2).

Pellet re-extracted as
above.

Coloured supernatant,
added to S2.

Pellet of unextractable
material.

16

2. Trichloroacetic acid. To an aliquot of S2, 0.5 ml. of 10% T.C.A. was added, and the resulting precipitate centrifuged out of suspension at 1970 g.

3. Two volumes of acetone were added to 10 ml. of S2. The colour of the precipitate was noted, and extraction with ether was carried out by shaking the two phases together in a separating funnel. The spectrum of the ethereal solution was read. The extracted material was then saponified, and chromatographed on kieselguhr-filled paper, to identify the carotenoids extracted.

(c) Boiling for 10 minutes followed by ether extraction.

(d) Extraction of the carotenoids after disruption of the protein present in the solubilized protoplast membranes.

1. 20 ml. of ether saturated with water were shaken in a separating funnel with an equal volume of S2, and the ether examined for extracted carotenoids.

2. 20 ml. of S2 were made 4M with respect to urea, and shaken with ether as above, to investigate if this H-bond breaker could release the carotenoid.

3. Two drops of thioglycollic acid were added to 20 ml. of S2, and the fraction again shaken with ether.

4. A number of aliquots of the fraction were taken, and the pH adjusted with 0.1N HCl and 0.1N NaOH to obtain a range of pH from pH 3 - pH 11. From each aliquot a known volume was taken and the above tests tried.

Effects of light and air on the carotenoid in
solubilized protoplast membranes

As the results of the previous experiments seemed to indicate an intimate relationship between the carotenoids and their environment in the protoplast membrane, it was investigated whether or not the carotenoids in the water-soluble fraction S2 were affected by light in the same manner as were free carotenoids in organic solution.

(a) Two suspensions of S2 were made up in 1/15M phosphate buffer, pH 7.0 (Kolthoff, 1932). The effective concentration of carotenoid was estimated spectrophotometrically, and found to be 0.48 $\mu\text{gm./ml.}$ (The suspension had to be diluted x 3 with buffer for spectroscopy.) A crystal of thymol was added to both suspensions to prevent bacterial growth. A solution of commercial, crystalline α -carotene (Sigma Ltd.) was made up in light petroleum in exactly the same concentration, viz. 0.48 $\mu\text{gm./ml.}$

One of the suspensions of S2 was exposed to sunlight, and stirred vigorously with a magnetic

stirrer. The other suspension was gassed out with nitrogen, sealed and placed in the dark. The α -carotene solution was exposed to sunlight and air.

At hourly intervals, samples of the two suspensions were taken, diluted $\times 3$ with buffer, and the optical density of both read at 600 $m\mu$ and 445 $m\mu$. The optical density at the longer wavelength gave an indication of the turbidity, while that at 445 $m\mu$ measured the amount of carotenoid present. Any decrease in the ratio

$$\frac{\text{optical density at 445 } m\mu}{\text{optical density at 600 } m\mu}$$

was taken to indicate a differential loss of carotenoid. The optical density of the α -carotene solution was measured also at hourly intervals in the manner previously described.

The experiment was continued for 3 hours, until darkness intervened.

During the night, the suspension of S2 which had been exposed to the sunlight was continuously illuminated by the light from a fluorescent bulb which yielded about half the light intensity of sunlight.

On the second day the experiment was continued for a further three hours in bright sunlight, and the final concentration of carotenoid estimated in both suspensions.

(b) Iodine was dissolved in methanol (10 μ gm./ml.) and a few drops added to 20 ml. of the suspension which was gassed out with nitrogen, and left exposed to sunlight for two hours. The spectrum was finally read over the whole of visible region with a "Unicam" S.P.800 spectrophotometer, after suitable dilution, to see if the formation of any isomers could be detected.

Thin-layer chromatography

Using Silica Gel G (Merck) as an adsorbent, layers were prepared on microscope slides, in order to find the most effective solvent. The solvents tested were:-

1. n-butanol/acetone/acetic acid/5% NH_2OH /water;
4.5/1.5/1/1/2 (v/v)
2. n-butanol/acetic acid/ NH_2OH : 5.5/3/1.5 (v/v)
3. n-butanol/acetic acid/5% NH_2OH /water
6/1/1/2 (v/v)
4. n-butanol/acetic acid/pyridine/water
15/3/10/12 (v/v)
5. n-butanol/acetic acid/water: 70/12/25 (v/v).

(Marini-Beltolo, 1964).

After development, the position of the spots was noted by visible light and after spraying with ninhydrin.

The solvent system finally decided upon was No.5, and the scale of the T.L.C. was increased to 500 μ layers on 20 x 20 cm. plates. These were strip loaded with S2, dried with a current of cool

air, and developed. Location of the spots was effected by

1. Visibility in daylight
2. Visibility in ultraviolet light
3. Spraying with ninhydrin.

Gel Filtration (Molecular-sieve Chromatography)

Gel filtration methods depend on the relative abilities of molecules of different dimensions to penetrate a suitable stationary phase.

The important contribution of Porath and Flodin (1959) to this technique was to introduce artificially cross-linked dextrans as the stationary phase for molecular sieve chromatography. The amount of cross-linking can be controlled in the manufacture, and the resulting water-insoluble polymers characterised by their water regains. The water regain is inversely related to the degree of cross-linking.

10

Carefully standardised dextrans are available from A.B. Pharmacia, Uppsala, Sweden, under the trade name of "Sephadex". This material was used in the present work. Five grades are available, and are characterised in terms of the molecular weights of soluble dextrans which are just excluded from the gel particles.

Preparation of the gel columns

Each column was packed in a vertical glass tube (2.4 cm. internal diameter), across the base of which was a sintered glass disc to support the gel. "Sephadex" gel filtration media G.100 and G.200 were suspended in 0.05M phosphate buffer pH 7.0, and allowed to swell for 2-3 days.

The columns were prepared by pouring a thin slurry of gel particles in buffer solution into the vertical tube, already partly filled with buffer. The addition of the gel was continued until a bed height of 30 cm. was obtained, and then a solvent reservoir was connected to the top of the column. A flow of buffer was maintained at a rate of

approximately 15-20 ml./hour for 18 hours, using a D.C.L. pump.

A sample of S2 was first concentrated to a small volume by evaporation from a dialysis tube in a stream of cold air. The pump was switched off, excess buffer removed from the top of the column, and 1 ml. of the concentrated material added to the column. When this had entered the column, more buffer was added and the pump reconnected. The effluent was collected on a fraction collector fitted with a 2 ml. siphon.

The following separations were attempted:-

1. S2 on G.100
2. S2 on G.200
3. F₁, the coloured fraction from filtration of S2 on G.100, was run on G.200.

The fractions collected in each case were submitted to the Folin-Lowry test and the absorption at 750 m μ measured on a spectrophotometer.

Folin-Lowry method of protein estimation

Solutions

1. Sodium carbonate solution: 20 gm. Na_2CO_3 + 0.5 gm. sodium tartarate + 100 ml. of N NaOH made up to one litre.
2. Copper sulphate solution: 1 gm. $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in one litre of water.
3. 45 ml. of 1. + 5 ml. of 2.
4. One volume of Folin's reagent diluted with two volumes of water. (This solution was made up freshly for each estimation).

Method

0.5 ml. of sample + 2.5 ml. of solution 3. were shaken together and allowed to stand for 10 minutes. 0.25 ml. of solution 4. were added and mixed. The sample was then allowed to stand for 30 minutes for the colour to develop before measuring the absorption, at 750 m μ , against a reference of 2.4 ml. solution 1. + 0.25 ml. of solution 4. + 0.5 ml. of buffer.

Electrophoresis in free buffer film

Electrophoresis, the separation of similar materials by their differential movement under the influence of an electrical field, can be divided into two classes - heterogeneous and homogeneous.

The system in which the material to be analysed, dissolved in a buffer of appropriate pH, moves on some supporting medium, be it paper, or some form of gel, is heterogeneous. The technique has the disadvantages that it can be employed only for analysis, or preparative work on a very small scale.

In homogeneous electrophoresis there is no supporting medium, and the electrophoretic separation takes place entirely in the buffer. For this reason it is called "electrophoresis in free buffer film". The fact that there is no supporting medium eliminates the complications of adsorption and molecular sieving which might be attendant on the heterogeneous forms. The other great advantages of the technique are: (a) that it can be used continuously, and thus forms an excellent preparative method, and (b) that large particles, such as whole cells and cell organelles,

can be separated by this method.

The technique was developed by Professor Grass and Dr. Hannig of Munich, and the machine used in this instance was manufactured by Dr. Bender and Dr. Hebein of Karlsruhe, Munich and Zurich.

The general principle behind the apparatus is to allow the material under analysis to flow in a fine stream through a flowing buffer, which moves vertically downwards across an electrical field. It is essential that the flow of material should be vertical, as large particles tend to sediment under gravity at an appreciable rate, and in a horizontal arrangement, this would mean that they would soon cease to move with the buffer.

Charged particles are deflected from their vertical path by the influence of the electrical field. The angle of deflection is governed by the speed of flow of the buffer and by the electrophoretic mobility of the particles. These two factors tend to oppose each other in effect, with the result that, by a judicious manipulation of the two factors, any deflection can be achieved.

The actual separating chamber consists of two glass plates, 0.5 mm. apart in a vertical position, between which the buffer flows. The buffer is pumped at a carefully controlled rate through six tubes, which enter the top of the chamber. The sample is pumped continually into the descending buffer film, and enters the chamber near the top. The actual point of entry depends on the charge of the material and the separation desired.

The fractions are collected at the bottom of the chamber through 50 small-bore tubes. The buffer must be pumped out of the chamber at the same rate at which it is pumped in.

The actual conditions pertaining in the experiment were:-

Voltage: 2500 volts

Current: 90 milliamps

Rate of pumping of the buffer: 45 ml./hour

Rate of pumping of the sample: 3 ml./hour

The sample used was in each case dialysed against the relevant buffer for 12 hours.

The buffers tried were:

- | | | |
|----|----------------|--------|
| 1. | Tris-citrate | pH 7.2 |
| 2. | Sodium-acetate | pH 3.9 |
| 3. | " " | pH 4.1 |
| 4. | " " | pH 8.6 |
| 5. | Phosphate | pH 6.0 |
| 6. | " | pH 7.0 |

The fractions were collected in 50 test tubes in each case, and the fractions monitored for the presence of protein by the Folin-Lowry reaction.

Effects of light on a culture of S. flava

A further experiment was tried, which cannot be easily included in a particular section, the results of which, however, are of interest.

It was decided to find the effects on a culture of S. flava growing in liquid medium, of exposure to light.

A series of "Universal" bottles was set up with exactly 14 ml. of nutrient broth in each. These were sterilised at 120°C for 15 minutes, and each inoculated with 1 ml. of a 24 hour broth culture of dark-grown S. flava.

Half of the bottles were wrapped in silver paper to exclude the light. All were then placed in a water-bath at 34°C, fitted with a reciprocating shaker, and exposed to the light from a "Photoflood" tungsten bulb (Philips), two feet distant.

At intervals of up to five days, duplicate cultures grown in the light and the dark were removed from the water bath. First, 1 ml. was taken from each, diluted to 3 ml. with isotonic saline, and an

estimate of the bacterial numbers obtained by measuring the optical density of the suspension at 600 m μ . The bacteria were harvested from the remaining 14 ml. of broth by centrifugation, washed with citrate buffer, pH 6.8, and finally suspended in 0.5 ml. of this buffer. A small quantity of lysozyme (Sigma Ltd.) was added, and the bacteria allowed to lyse for 1 hour. At the end of this time the suspension was rendered translucent by 1 minute of ultrasonic treatment. The spectrum of this suspension was then found on an automatic spectrophotometer (Unicam Ltd.). From the curves obtained, an estimate of the amount of carotenoid pigment present was made. No absolute measurement, but a comparison of the carotenoid contents of the light- and dark-grown bacteria, could be derived.

R E S U L T S

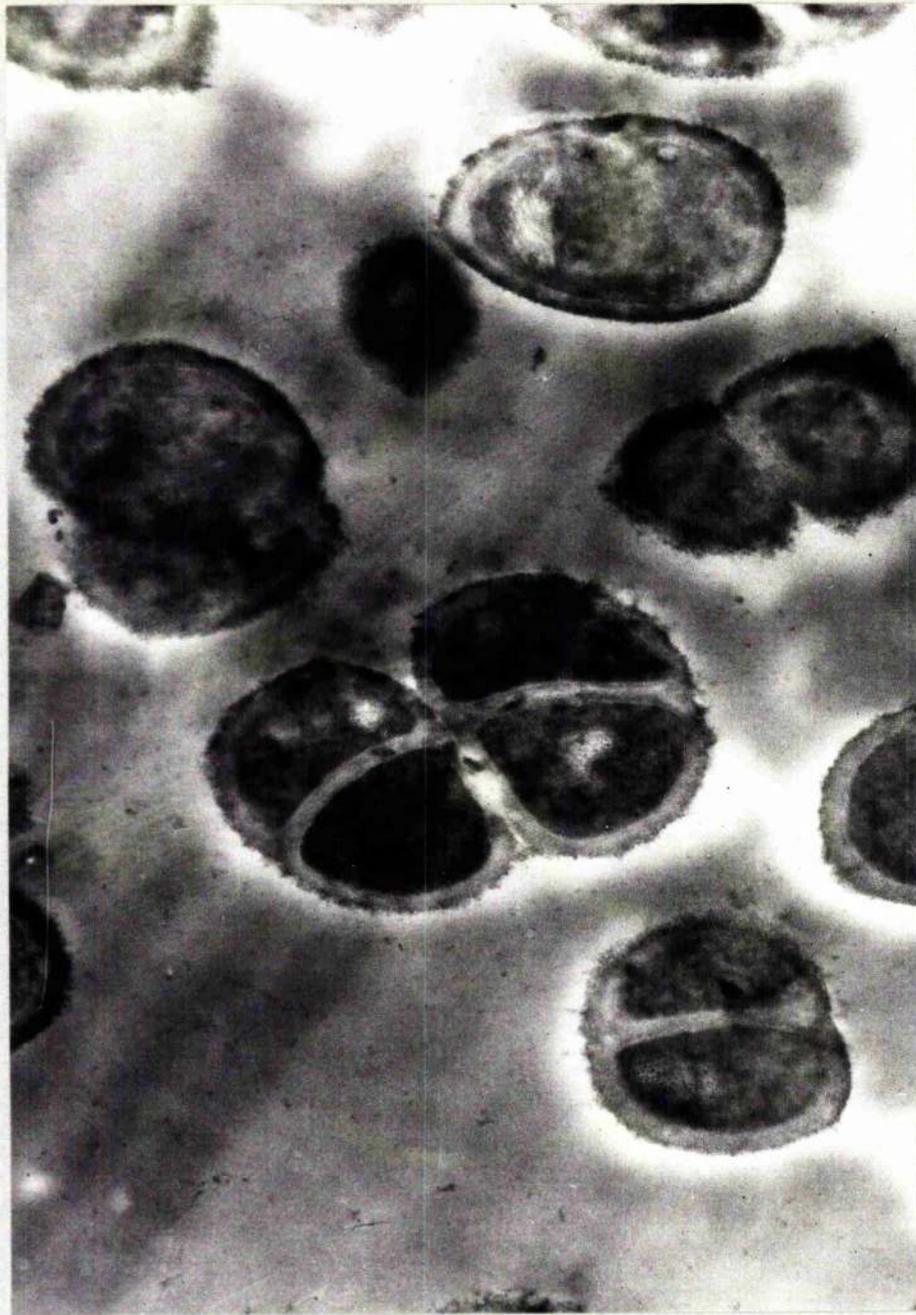


Fig. 1 - Photomicrograph of thin sections of *S. flava* (mag. x 20,000) showing the characteristic tetrad formation (Courtesy of Dr. D. Thirkell).

R E S U L T S

The morphology of the Sarcinae

Although all of the bacteria investigated were nominally Gram positive, it was found that they could be Gram negative, especially if the samples were taken from ageing cultures.

The most common combination of the bacteria was in tetrads, and possibly clumps of eight. Pairs of bacteria were also a common occurrence. There was no evidence that the bacteria formed large irregular clumps, as do the Micrococci. (Fig. 1)

The growth of cultures of S. flava at different temperatures

Counting Methods

(a) In the first plot of the optical density of a suspension of the bacteria against the actual number of bacteria/cu.mm. the inaccuracies inherent in the counting of large numbers of bacteria on a haemocytometer meant that there was a considerable spread of values. Nevertheless, taking into consideration an experimental error of 10%, a straight line could be drawn through the points on the graph, up to the

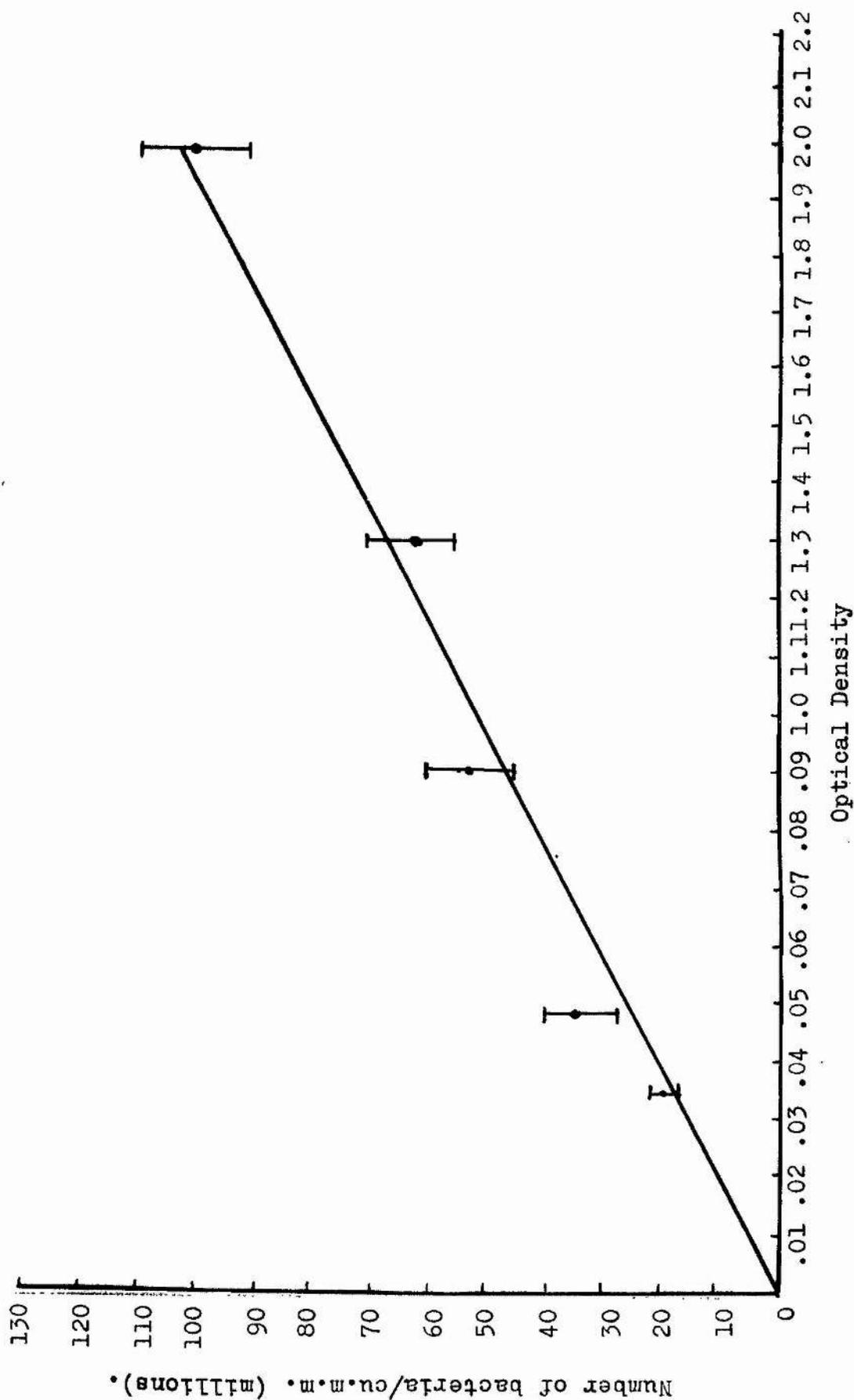
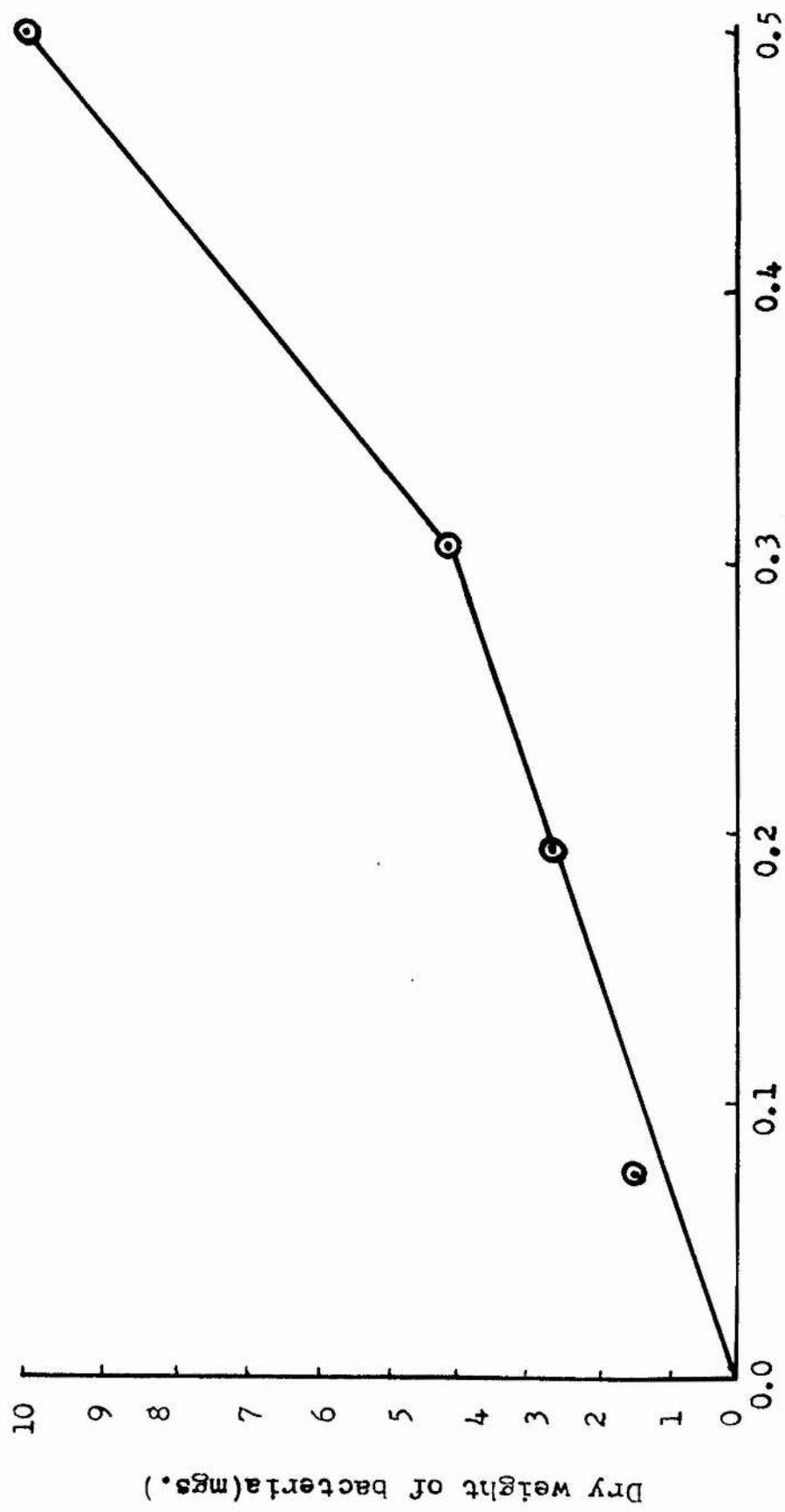


Fig. 2. Relationship between light dispersion and the numbers of bacteria in a suspension.



Optical density.

Fig. 3. Relationship between light dispersion and the dry weight of bacteria in a given volume of suspension.

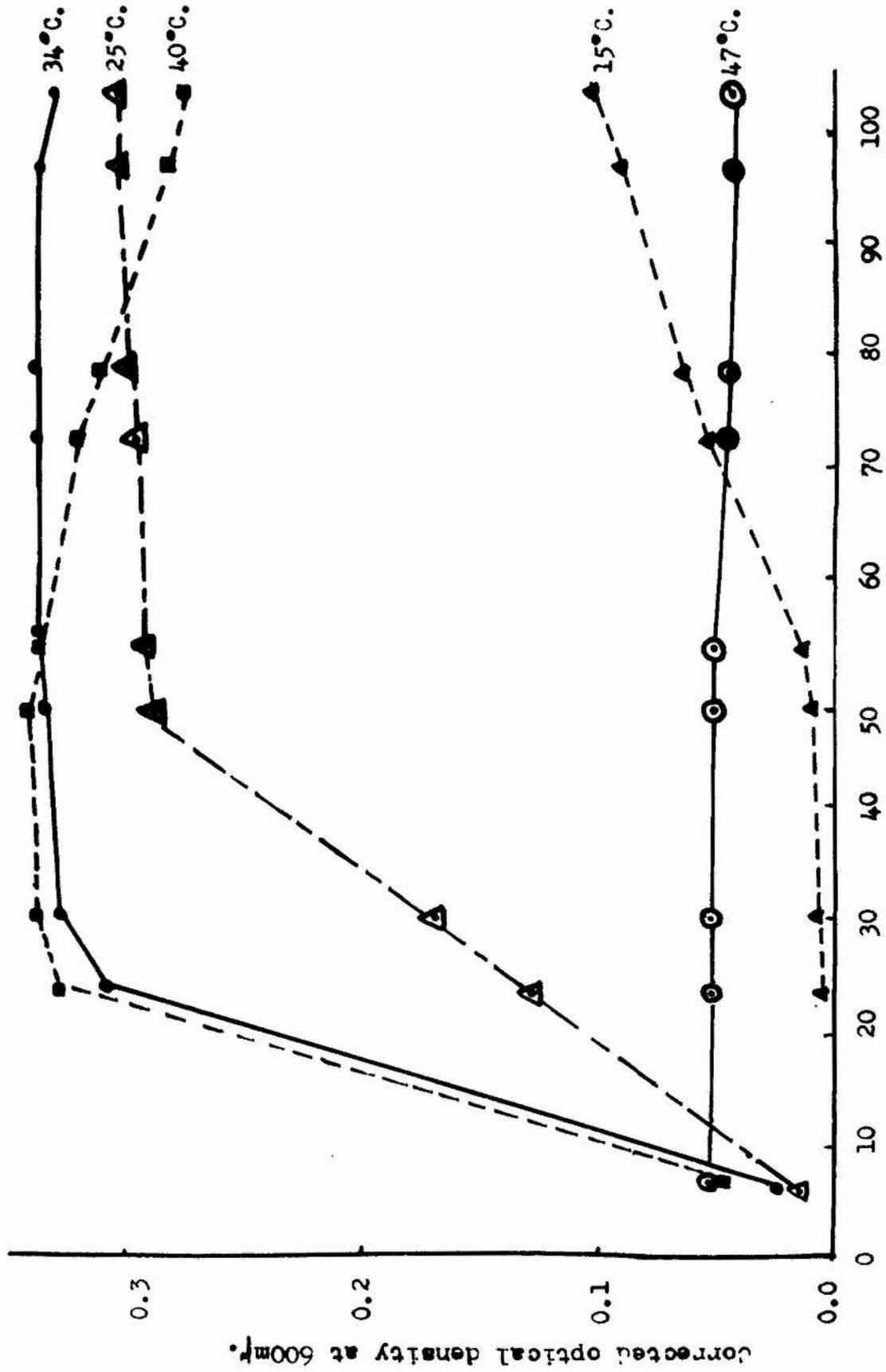


Fig. 4. Growth curves of *S. flava* cultured on nutrient agar at different temperatures.

particular maximum optical density, which in this case was 0.200 (Fig. 2).

The results of the second experiment confirmed this. In this case, the error involved in weighing dried samples of the bacteria was much less, and it was found that the concentration of a suspension of bacteria could be accurately reflected by the optical density of the suspension up to a limiting optical density of 0.300. When the concentration of bacteria was such that the optical density exceeded this value, factors such as secondary scattering meant that the optical density was no longer an accurate measurement of the concentration of bacteria in suspension (Fig. 3). Thus it was ensured that in all further estimations of bacterial numbers by this method, the suspensions were diluted until the optical density lay within the limits of accuracy.

(b) Growth with temperature on nutrient agar

The results of the experiments to trace the growth of cultures of S. flava on nutrient agar at different temperatures over a period of 102 hours are presented in the form of a graph (Fig. 4).

The results indicated that the optimum temperature for growth was 34°C., and that the maximum yield was

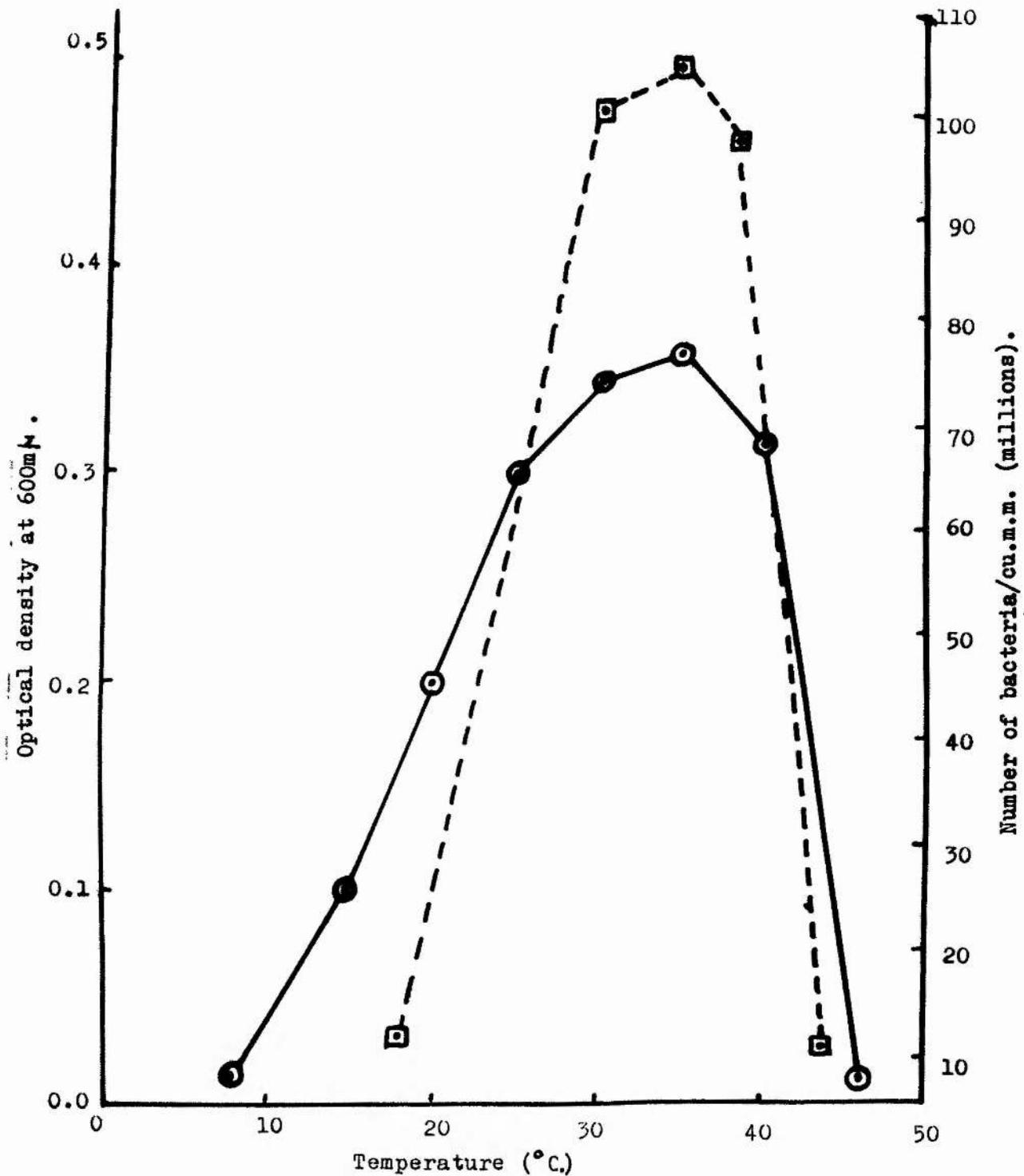


Fig. 5. Growth of cultures of *S. flava* with temperature. Comparison of results obtained on solid and in liquid medium.

- Growth on nutrient agar estimated by the optical density of a suspension of the bacteria.
- Growth in nutrient broth estimated by counting the cells on a haemocytometer.

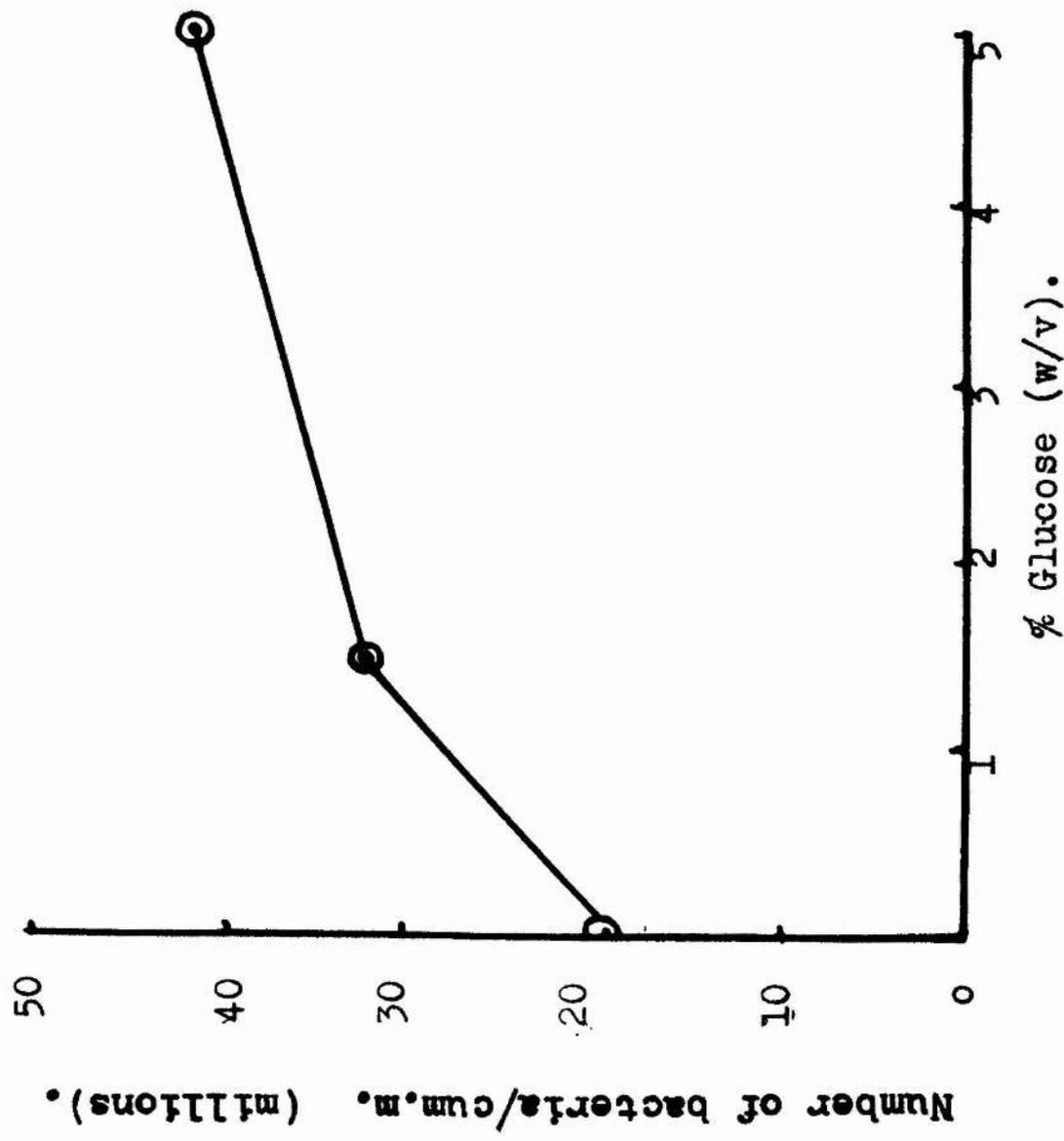


Fig. 6. The effect of addition of glucose on the growth of *S. flava* on nutrient agar.

obtained after 48 hours of growth at this temperature.

(c) Growth in nutrient broth

The results of the experiment to check the validity of the technique used to estimate bacterial numbers growing on a nutrient agar surface are presented graphically in Fig. 5. The results verify those obtained previously by the optical density method, namely, that the optimum temperature for growth of S. flava was 34°C in broth as well as on nutrient agar plates.

(d) Effect of enrichment of growth medium with glucose

The bacterial yield was increased by 100% when 5% (w/v) glucose was added to the medium. This is demonstrated by the graph (Fig. 6).

Culture of the bacteria

(a) It was found that both S. flava and S. lutea could be maintained uncontaminated for an indefinite period of time by continual replating of the bacteria at three-day intervals on nutrient agar, and incubating the cultures at 34°C. in the dark. There was no sign of any decrease of pigmentation with time.

(b) Lyophilized samples of S. flava were used twice in the course of the experiments to re-establish a culture. It was found that the bacteria so treated grew and pigmented normally on nutrient agar, after an extended lag period.

Bulk culture of the bacteria

(a) Using large aluminium dishes

The method whereby the bacteria were grown on nutrient agar (Oxoid Ltd.) in aluminium dishes enclosed in nylon bags yielded an excellent growth of well pigmented bacteria, but involved a great deal of work in setting up, and in harvesting the bacteria. It was, however, used extensively.

(b) Using large developing dishes

The growth of the bacteria on large nutrient agar surfaces in developing dishes proved to be excellent. The method suffered from the handicap that if a contaminant were introduced, then much of the culture was lost. The number of suitable developing dishes available was severely limited.

(c) Bulk liquid culture

Growing the bacteria in large scale liquid culture

1

proved to be the most successful from the point of view of the actual yield of bacteria. Approximately 150 gm. of wet bacteria were harvested from 40 litres of culture medium. It was found, however, that the pigmentation of these bacteria was much reduced. This was the deciding factor in discontinuing the method.

Extraction of the pigments

A summary of the methods which were tried on a purely qualitative basis is given in Table 1.

The extreme polarity of the bulk of the carotenoid pigments meant that only the most polar solvents, such as methanol, used in association with ultrasound and brief heating, were completely successful. Although it would have been better to avoid it if possible, heat for a very short time was found necessary if quantitative extractions were to be made.

A chromatographic comparison of the carotenoid extracted by the rigorous and more mild methods exposed no qualitative difference between the two extracts. Thus for the remainder of the experimental work the method involving the use of ultrasound and boiling methanol was employed for the extraction of the carotenoids.

TABLE 1

Qualitative comparison of techniques for the extraction of the bacterial carotenoids.

No.	Method	Result	Comment
1	<u>Grinding:</u> Bacteria, suspended in acetone, were ground with acid-washed sand and centrifuged	-ve	Virtually no pigment extracted
2	<u>Autolysis:</u> Bacteria were suspended in distilled water for 48 hrs. at 34°C. Extraction with acetone was attempted.	-ve	No autolysis took place
3	Bacteria in acetone were subjected to ultrasonic oscillation for 5 mins. at room temperature, and the remnants centrifuged.	+	Very little pigment extracted
4	Bacteria saponified for 12 hrs. in the dark with N methanolic KOH.	++	Some pigment extracted. Remains still yellow.
5	Bacteria suspended in N methanolic KOH, subjected to ultrasonics for 5 mins., and then saponified at room temperature, under N ₂ for 12 hrs.	++	" "
6	Bacteria suspended in methanol and subjected to ultrasonic oscillation for 5 mins.	+++	Moderate extraction.

7	Lysis of bacteria suspended in distilled water by egg-white lysozyme for 48 hours at 34°C., followed by acetone extraction.	+++	Much pigment not extracted. Pigment still bound to protein
8	Bacteria suspended in methanol and subjected to ultrasonics (2 mins.) and the homogenate boiled for 3 mins.	+++++	Bacterial remnants white.

TABLE 1

Purification procedures

(a) Lipid precipitation

The actual chemical nature of the lipids precipitated by the Blessin procedure (1962) was not fully investigated, but chromatograms of the bacterial extract before and after ether precipitation indicated that, before the precipitation, there was a mass of highly polar lipid material which did not move even in the polar solvent of benzene/methanol/acetic acid: 87/11/2 (v/v), and which appeared to have the effect of occluding much of the chromatographically mobile material. Ether precipitation removed much of this lipid material.

(b) Saponification

The spectrum of the total carotenoid extract before and after saponification showed the same three peaks at 414, 439, 468 m μ in methanol in the visible region of the spectrum. This was taken to indicate that no structural degradation had taken place during saponification.

Thin-layer chromatographic comparisons of the saponified and unsaponified extracts were made on 250 μ thick layers of Silica Gel G (Merck). To investigate the presence in the unsaponified extract of any carotenoid

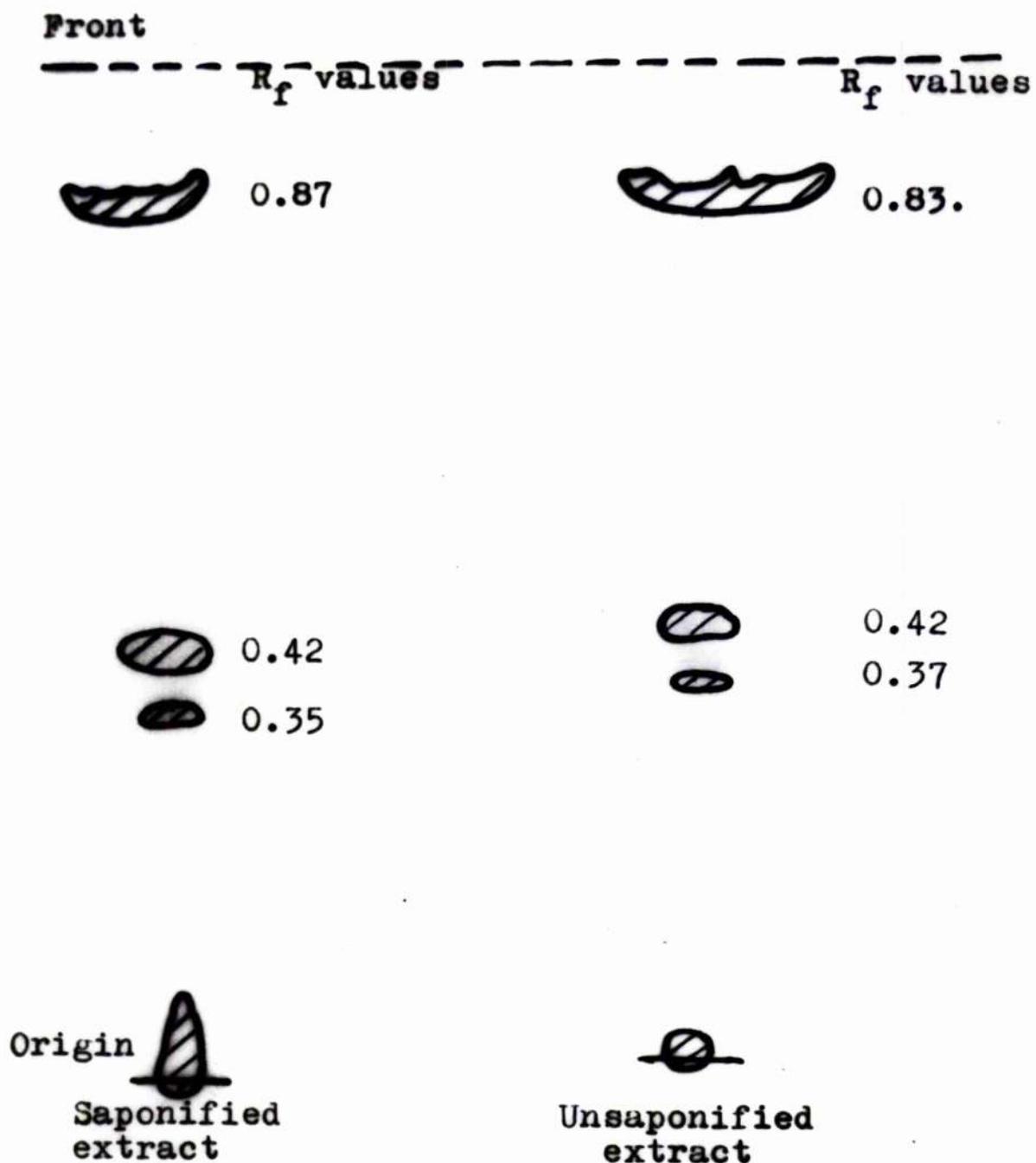


Fig. 7. Chromatographic comparison of saponified and unsaponified methanolic extracts of *S. flava*. T.L. chromatogram on Silica Gel G(Merck)(250 μ): Solvent; ether/light petroleum; 70/30; (v/v). Spots marked are visible carotenoids.

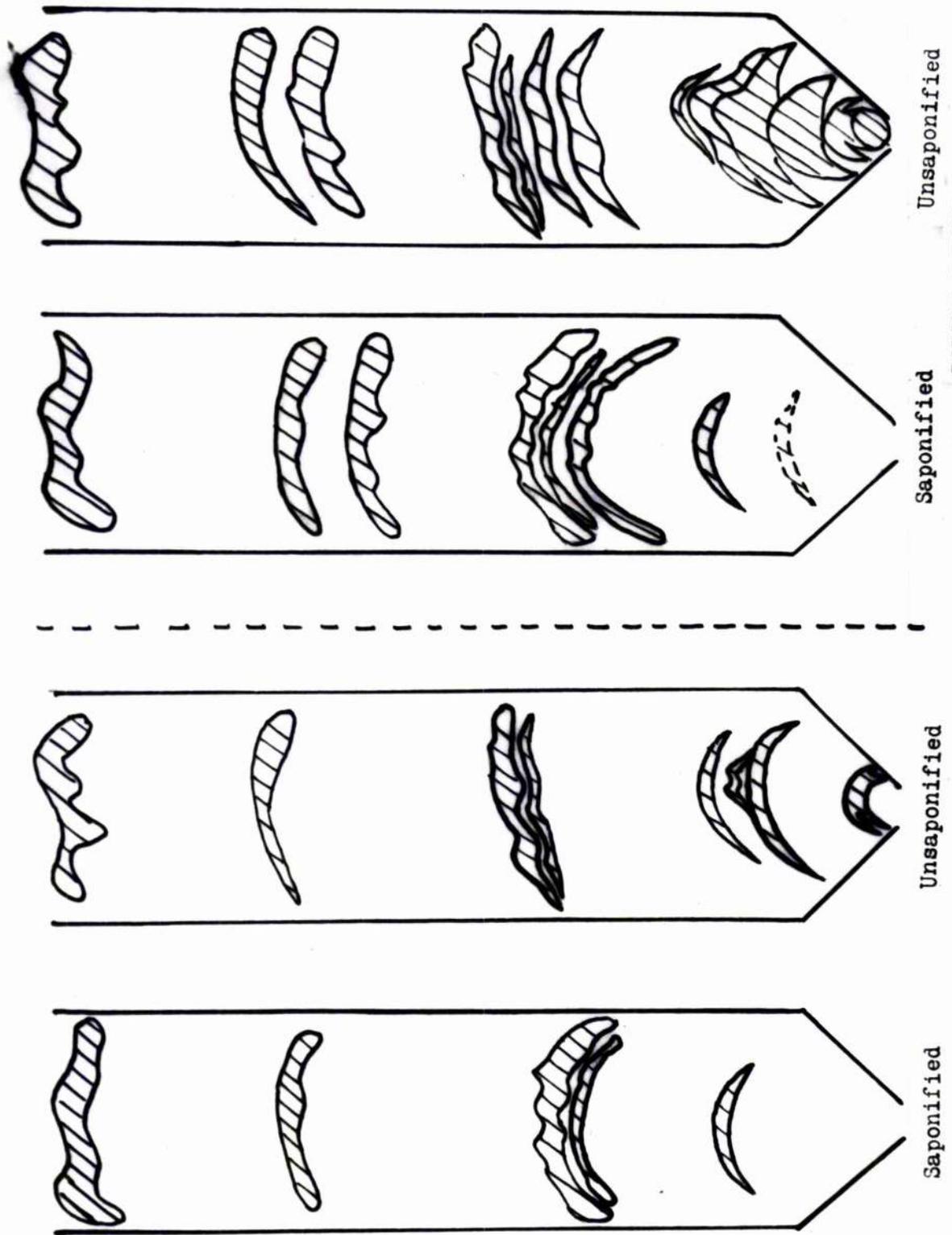


FIG. 8. Comparison by T.L.C. on Silica Gel G ("wedge-strip" method) of saponified and unsaponified lipid extracts of *S. flava*. Solvent: Benzene/methanol/acetic acid; 87/11/2; (v/v/v).

1

esters, the non-polar solvent of ether/light petroleum: 70/30 (v/v) was used. The results of such a chromatogram is shown in Fig. 7. There was no evidence of carotenoid esters in the unsaponified bacterial extract.

Another point of interest for comparison between the saponified and unsaponified extracts was the apparent occurrence in the unsaponified extracts of a number of highly polar carotenoids, which did not appear in the saponified material. The results of a typical chromatographic comparison between the extracts on Silica Gel G (Merck) by the wedge-strip method is shown on Fig. 8. When the chromatograms were stained with iodine vapour and 25% aqueous sulphuric acid, it became apparent that the supposed extra carotenoids were being carried on the fronts of large amounts of saponifiable lipids, which, of course, were no longer present after saponification. It was supposed that this phenomenon was due to the occlusion of the carotenoids by the saponifiable lipids.

To test this theory, saponified extracts were co-chromatographed with large quantities of known lipids. These experiments will be described at a later stage.

The experiments showed the necessity of saponifying the total extract from the bacteria before proceeding with

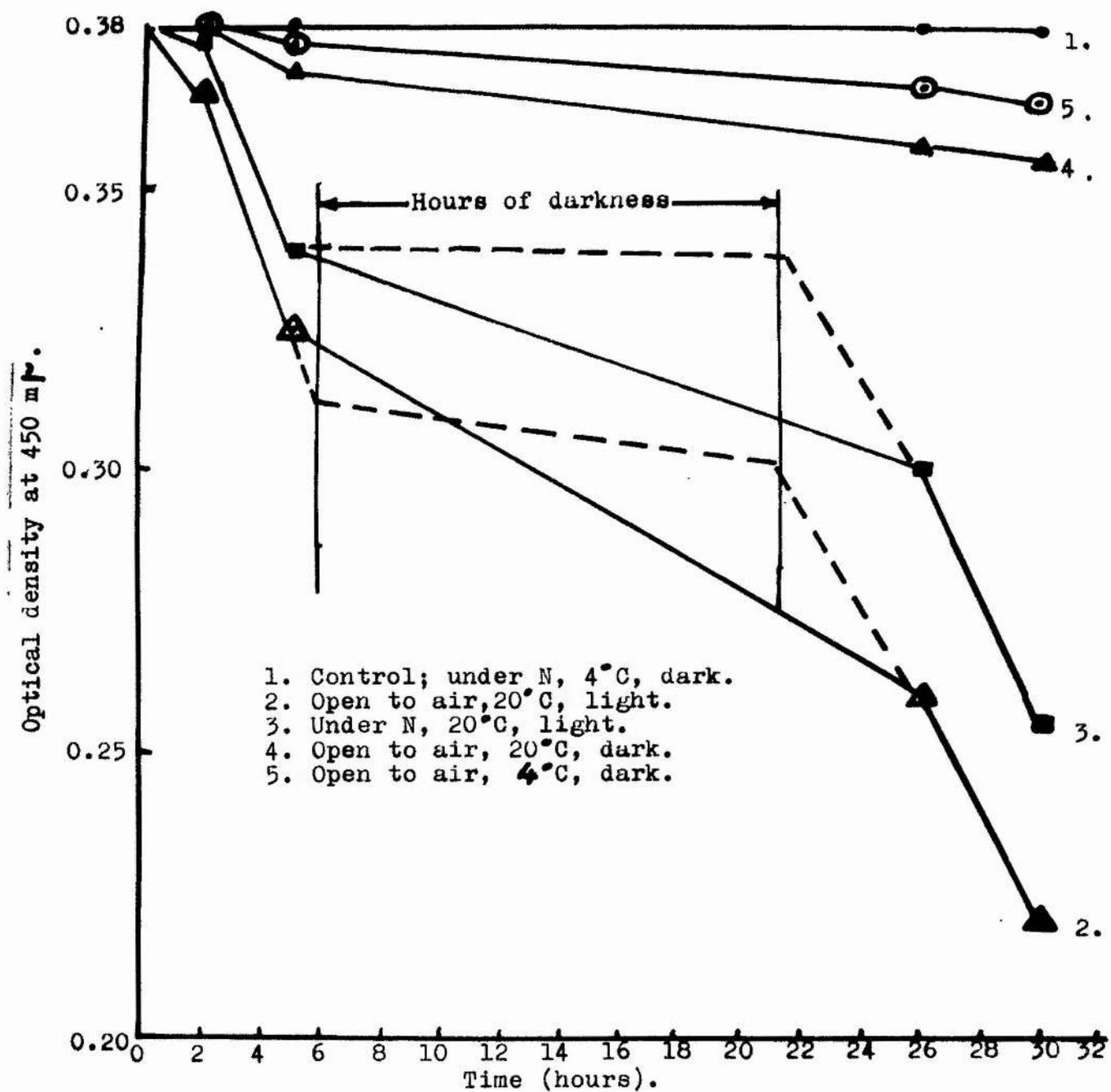


Fig. 9. Loss of absorbance at 450m μ of a solution in light petroleum of β -carotene under a variety of conditions.

the final purification of the carotenoids.

The estimation of the recovery of the carotenoids after saponification, indicated that 98% of the original carotenoid had been recovered into the ether layer.

Experiments to determine the loss of absorbance of a carotenoid solution with time, under a variety of conditions

(a) The loss of absorbance of a carotene solution under various conditions.

The results of the first experiment are given in the form of a graph (Fig. 9), which illustrates well the effect of light and oxygen on a solution of a carotene. It was evident that the effect of sunlight was the more important of the two agents causing the loss of absorbance of carotene as the solution exposed to the light in a sealed vessel, under an atmosphere of nitrogen, lost absorbance to almost the same extent as the solution in the light and open to the air.

On the other hand, the solutions in the dark, open to the air, whether at room temperature or at 4°C., lost absorbance to only a small extent.

By finding the approximate hours of darkness and assuming a roughly constant loss of absorbance, it was possible to extrapolate back the graphs of the solutions

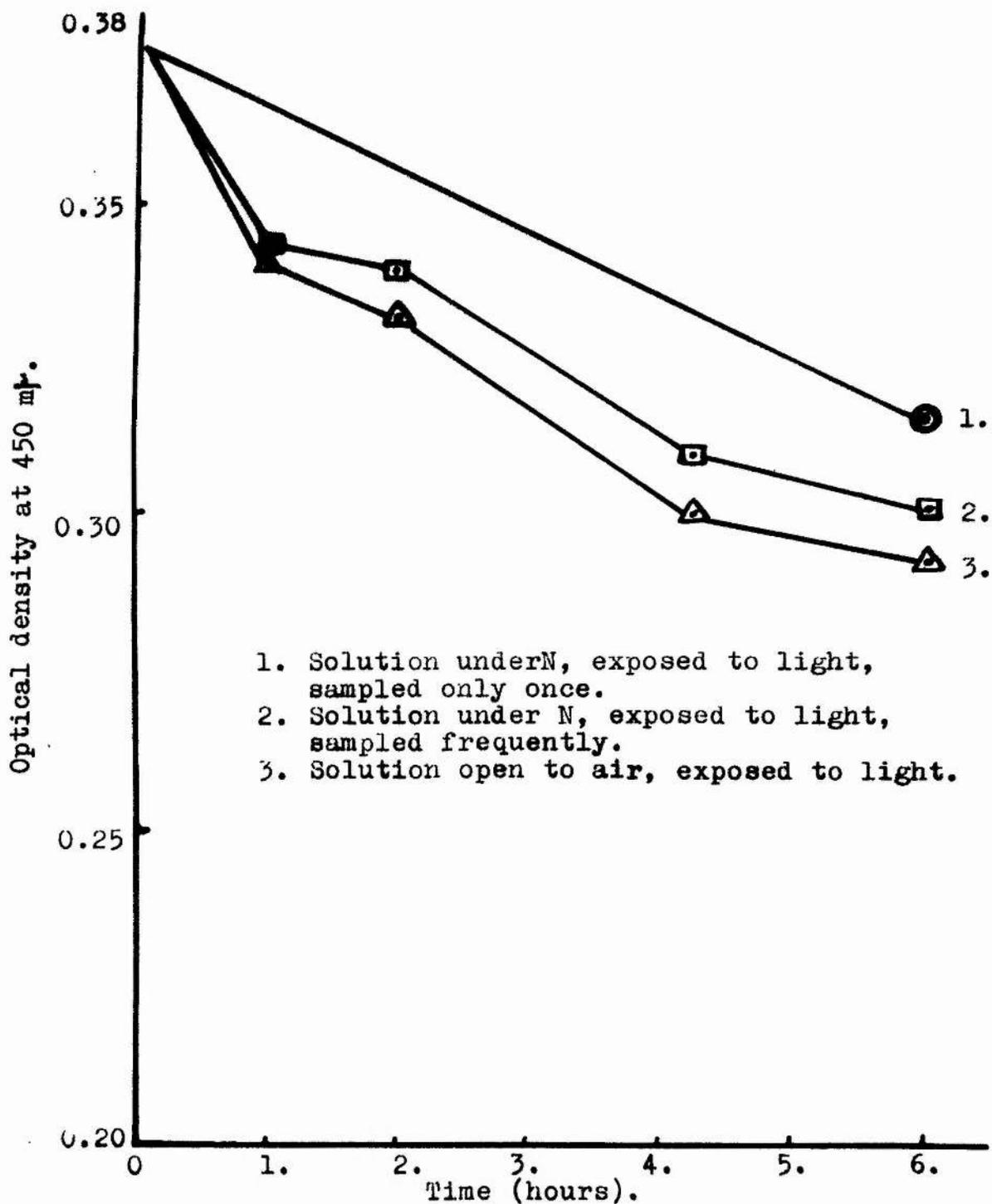


Fig. 10. Experiment to test the effectiveness of an inert atmosphere in preventing the light-induced loss of absorbance of a solution in light petroleum of β -carotene.

exposed to the sunlight. From this it was found that little or no loss of absorbance took place during the hours of darkness.

It can be seen that sunlight rapidly reduced the amount of carotene estimable by spectrophotometry, and that this took place even in an atmosphere of nitrogen.

(b) Effects of sampling

The results are shown in Fig. 10, and show that the solution under nitrogen which remained sealed throughout the experiment, lost almost as much absorbance as the solutions which had been continually sampled.

After six hours the absorbance of the solutions had fallen by the following amounts:-

<u>Conditions</u>	<u>% loss of absorbance</u>
1. Air and light	24%
2. Nitrogen and light (sampled continually)	21%
3. Nitrogen and light (sealed)	17%

It was concluded that a solution of a carotene, even in an inert atmosphere, was liable to light-induced degradation.

(c) The effect of anti-oxidant on carotene degradation

The effect of the addition of α -tocopherol on the

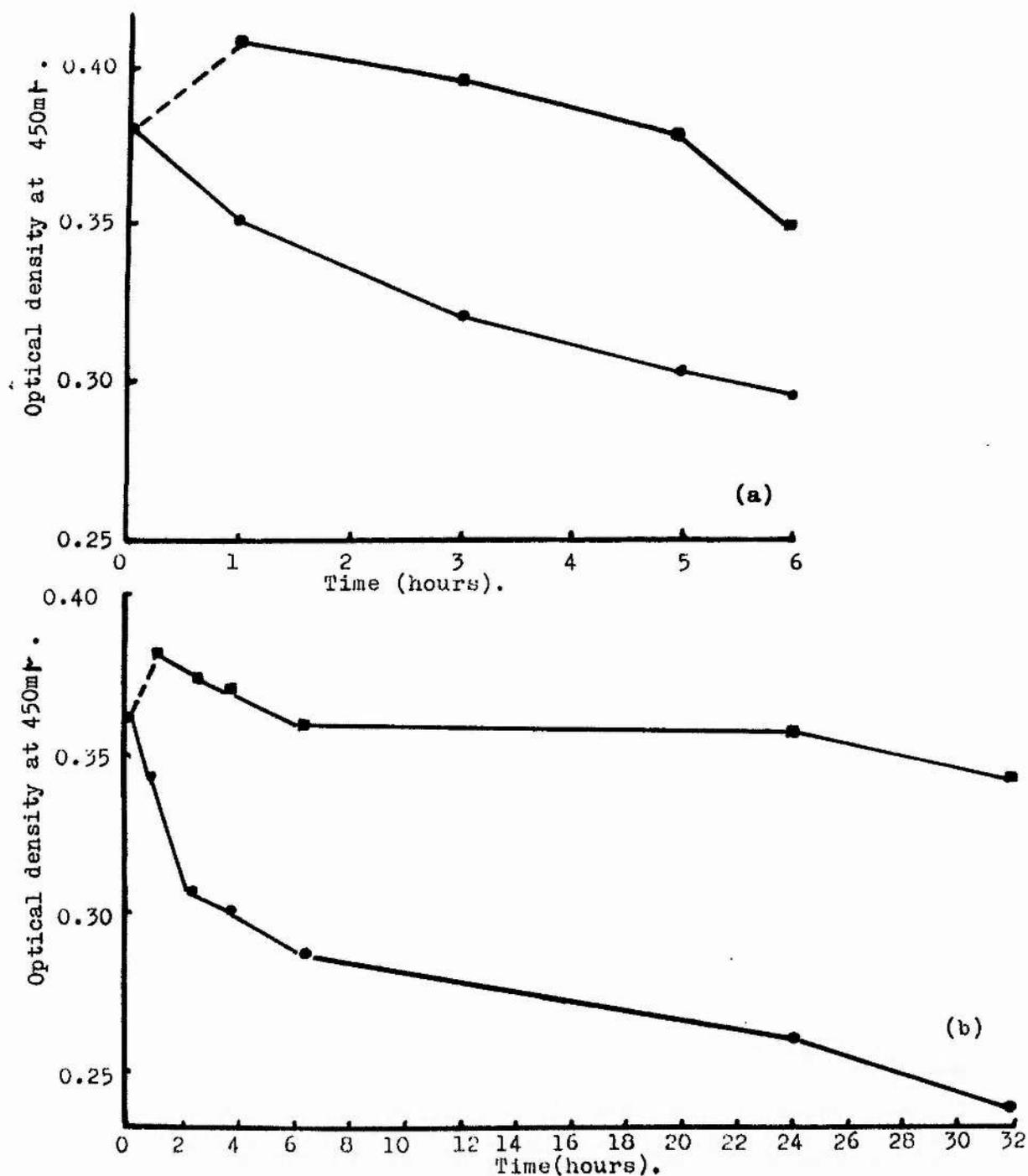


Fig. 11. Effects of the addition of 0.1% (w/v) α -tocopherol on the light induced loss of absorbance of carotene solutions. (a) α -carotene in light petroleum; (b) β -carotene in methanol.

■—■ Solutions containing 0.1% (w/v) α -tocopherol.
 ●—● " not " " " " "

light-induced loss of absorbance of a solution of carotene, was quite dramatic. Fig. 11a represents the results obtained with solutions of α -carotene of identical concentration, in light petroleum, with and without the addition of 0.1% α -tocopherol.

The relative loss of absorbance after six hours was:

	<u>% loss of absorbance</u>
1. Control: α -carotene in light petroleum	21%
2. α -carotene in light petroleum + 0.1% α -tocopherol	8%

Fig. 11b shows the results of a similar experiment with solutions of β -carotene in methanol. The concentration of the β -carotene solution was identical to that of α -carotene solution in light petroleum, namely, 1.5 $\mu\text{gm./ml.}$ The experiment was carried out over a period of 32 hours.

The loss of absorbance of the β -carotene solutions after six hours was:-

	<u>% loss of absorbance</u>
1. Control: β -carotene in methanol.	22%
2. β -carotene in methanol + 0.1% α -tocopherol	2%

The experiments show that:

1. The two commercially prepared crystalline carotenes, α - and β -, in identical concentration lost absorbancy by the same amount over a period of six hours under identical conditions, namely, exposed to the light and air.
2. That the loss of absorbance was independent of the solvents used, methanol and light petroleum.
3. That the addition of 0.1% α -tocopherol prevented this loss of absorption almost completely.

One wholly inexplicable finding was, that after the addition to the solutions of the α -tocopherol, the absorbance of the α - and β -carotene actually increased by about 5% before slowly falling.

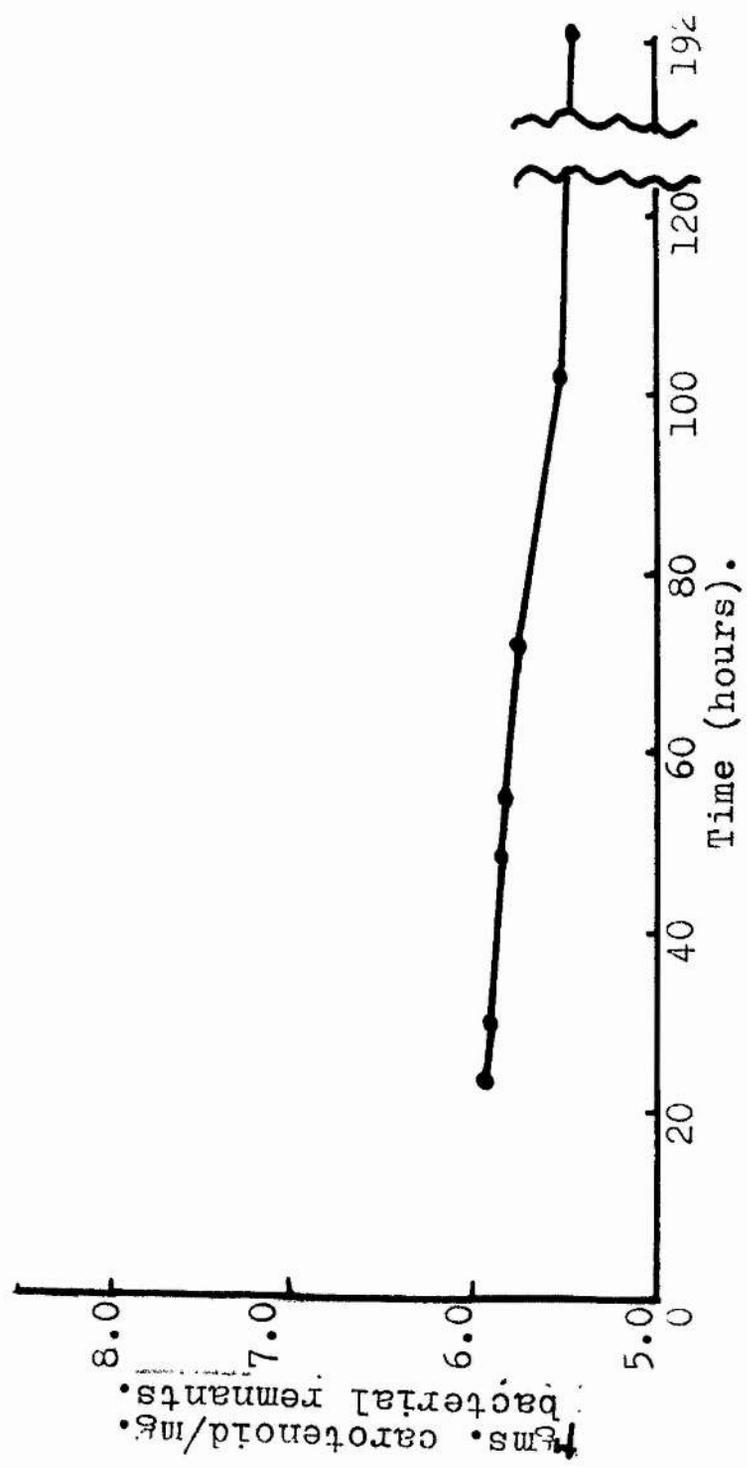


Fig. 12. Carotenoid production of *S. flava* at 34°C, in the dark, over a period of eight days.

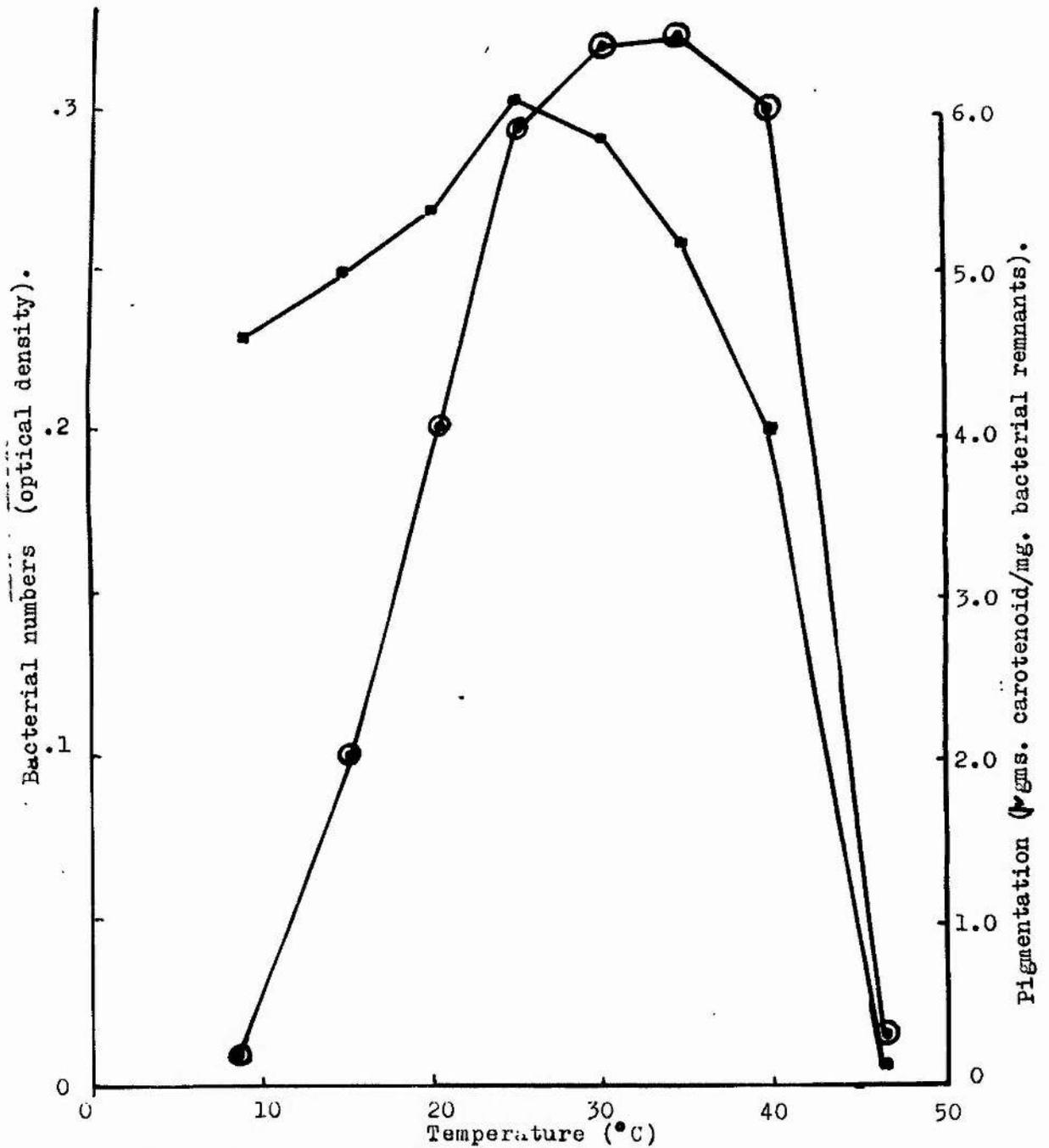


Fig. 13. Variation of growth and carotenoid production of *S. flava* with temperature.

- Carotenoid production.
- Numbers of bacteria measured by the optical density of a suspension.

Variations of pigment production of *S. flava* with variations of (a) time of growth; (b) temperature of growth; (c) pH of the medium; (d) enrichment of the nutrient medium with different concentrations of glucose.

(a) The results are presented graphically in Fig. 12.

From such time of growth as there were sufficient bacteria to be harvested, and the pigment extracted, the pigmentation per milligram of dried extracted bacterial remnants remained constant, even after the culture had ceased to multiply, and the bulk of the bacteria were presumably dead. This would indicate that the carotenoids were not destroyed on the death of the bacterium if the cells remained intact.

(b) The graph of the results of pigmentation with temperature plotted along with the growth of a culture with temperature (Fig. 13) indicates that the temperatures of maximum pigmentation and maximum growth do not coincide the former being at 25°C., the latter at the higher temperature of 34°C.

(c) The results of the variation of pigmentation with pH

11

indicate that, within the pH range which supports bacterial growth (pH 5.0 - pH 10.2), there was a fall of about 20% in pigmentation with increasing alkalinity. The level remained constant in the range pH 5.0 - 7.5 after which a dramatic drop occurred.

(d) It was found that increasing concentrations of glucose, while greatly increasing the growth of a culture, had no effect on the level of pigmentation, which remained quite constant.

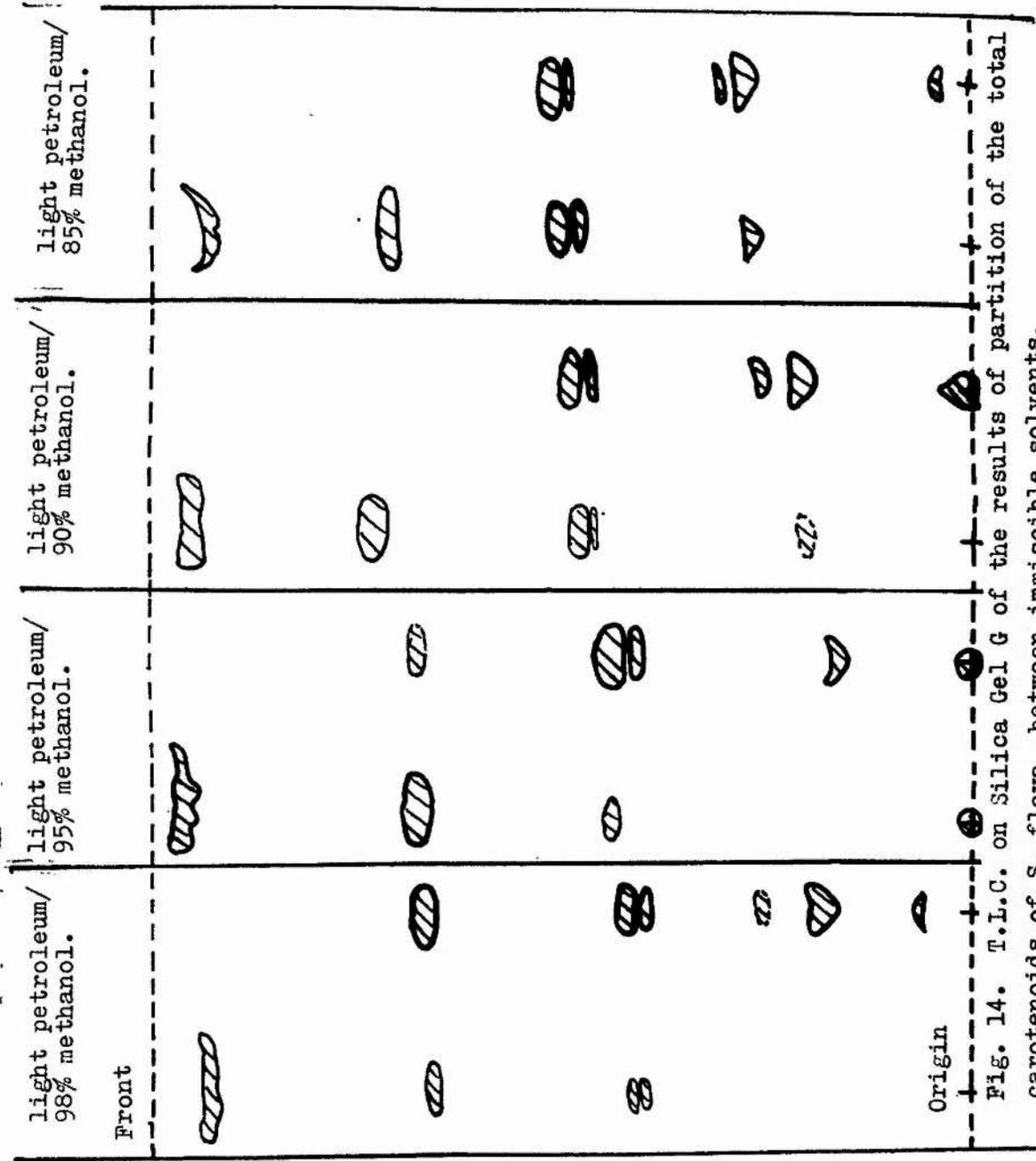


Fig. 14. T.L.C. on Silica Gel G of the results of partition of the total carotenoids of *S. flava* between immiscible solvents.
Solvent: Benzene/methanol/acetic acid; 87/11/2; (v/v/v).

Final purification of the carotenoid fractions

The partition coefficients between light petroleum and methanol with various proportions of water of the total carotenoid content of S. flava after saponification, are given below in Table 2.

Table 2

Partition ratios of total carotenoid extract of S. flava between light petroleum and aqueous methanol.

<u>Hypophase</u>	<u>Partition coefficient</u> $\left[\frac{\text{light pet.}}{\text{methanol}} \right]$
1. 98% methanol	1/20
2. 95% methanol	1/12
3. 90% methanol	1/6
4. 85% methanol	1/4

The results show the extremely high polarity of the bulk of the carotenoid pigments; a fact later borne out by the more detailed studies.

The chromatographic studies carried out on the carotenoids separated by these partitions are shown in Fig. 14, along with the solvents used to develop the chromatograms. The chromatograms show that the method is far too imprecise for preparative work because complete separation of the carotenoid fractions cannot be achieved by this technique.

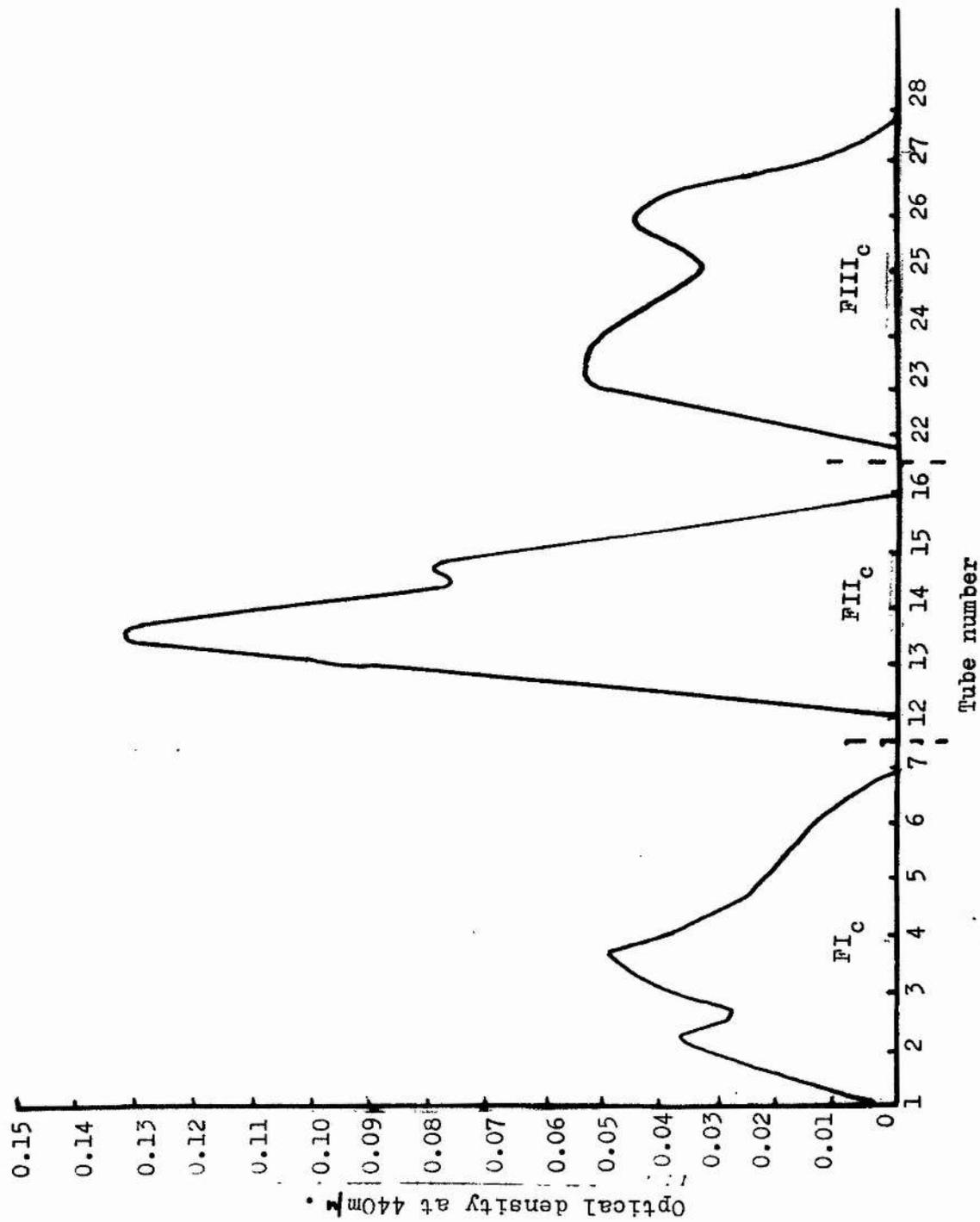


Fig. 15. Column chromatographic separation of the carotenoids of *S. flava* on CaCO_3 .

Column chromatography

The resolution obtained by chromatography of the carotenoids of S. flava on CaCO_3 is indicated in the graph (Fig. 15) in which the absorbance of the aliquots of eluant containing pigment is plotted against the increasing polarity of the solvents used to elute them.

The fractionation achieved was monitored by T.L.C. on Silica Gel G. (Merck). (Fig. 16). This chromatogram indicates that two coloured fractions were eluted with light petroleum. These were identified with Fractions 1 and 2 under the final separation achieved by preparative T.L.C. In the column chromatography, however, they were not fully separated, and constituted a single fraction (Fraction I_c). Fraction II_c eluted from the column of CaCO_3 with 5% acetone in light petroleum, made up the bulk of the pigment eluted from the column, and was identical to Fraction 3, under the preparative T.L.C. classification. The most polar carotenoid material proved difficult to elute. As it was known that this in fact constituted the largest single fraction of the total carotenoid extracted from the bacterium, much of it must have remained on the column. The thin-layer chromatogram of Fractions III_c, IV_c, and V_c eluted from the column, sho

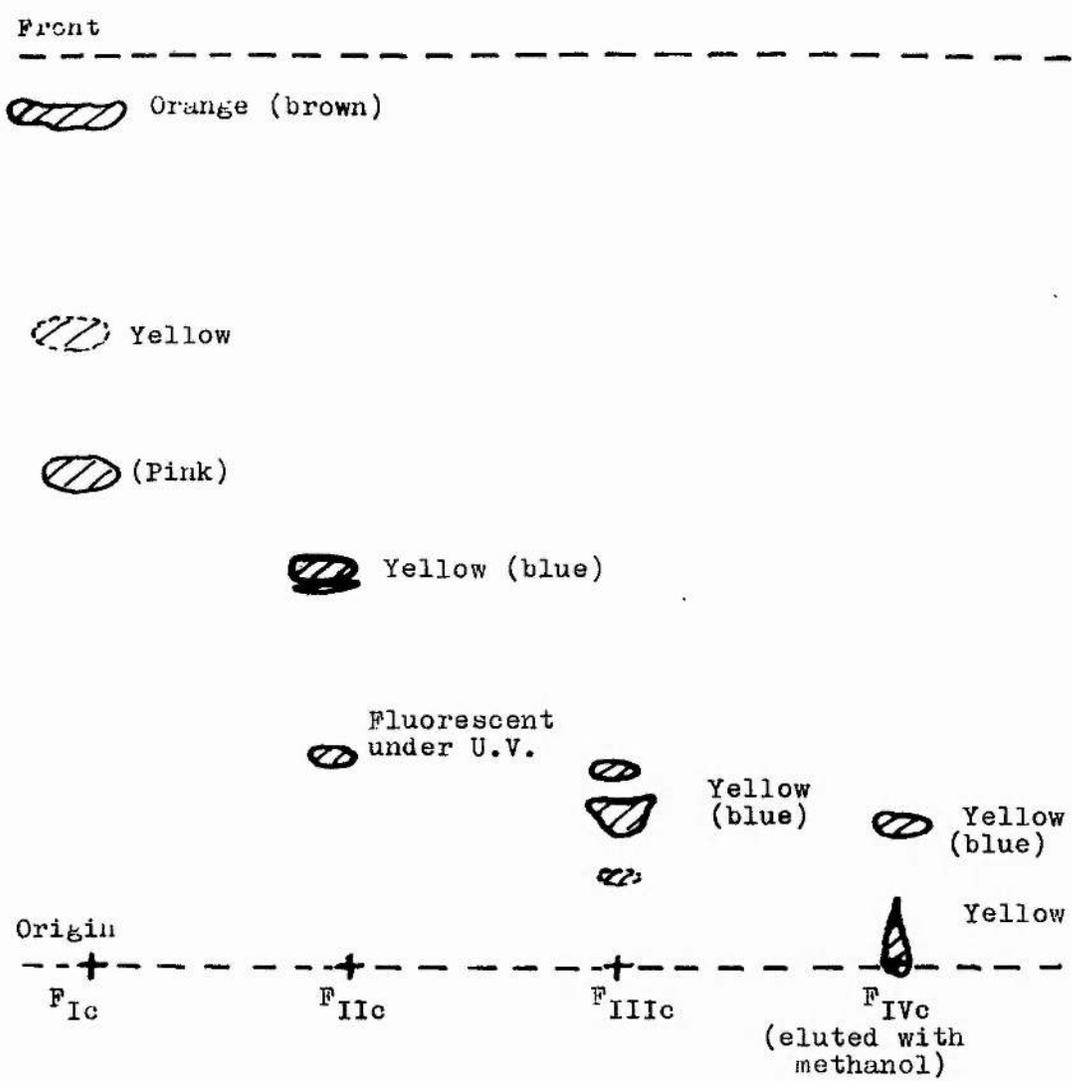


Fig. 16. T.L.C. on Silica Gel G (Merck) using benzene/methanol/acetic acid; 87/11/2; (v/v/v) as solvent, of the fractions obtained from column chromatography on CaCO₃ of the methanolic extract of *S. flava*. (The descriptions³ in the brackets refer to the colours obtained on staining with a saturated solution of SbCl₃ in chloroform.

1

that they were all composed of the same carotenoids.
[N.B. It was obvious from these results that some
further purification was necessary after separation
of the material on such a column.]

Thin-layer chromatography (T.L.C.)

The results of the trials with a variety of adsorbents activated for different lengths of time and at different temperatures, are tabulated below (Table 3), indicating the R_f values obtained for the standard dyes Sudan Yellow and p-aminoazobenzene, with carbon tetrachloride as solvent.

TABLE 3

The R_f values obtained for the dyes Sudan Yellow and p-aminoazobenzene (p.a.b.) on a variety of adsorbents activated to different extents, employing carbon tetrachloride as solvent.

Adsorbent	Time of Activation (hours)	Temperature of activation			
		100°C		160°C	
		R_f values of Sudan Yellow	R_f values of p.a.b.	R_f values of Sudan Yellow	R_f values of p.a.b.
MgO	$\frac{1}{2}$	0.26	0.09	0.42	0.19
	1	0.34	0.17	0.28	0.10
	3	0.27	0.10	0.36	0.10
Al ₂ O ₃	$\frac{1}{2}$	0.47	0.05	0.25	0.05
	1	0.31	0.06	0.27	0.05
	3	0.37	0.07	0.22	0.05
SiO ₂	$\frac{1}{2}$	0.14	0.03	0.17	0.03
	1	0.13	0.04	-	-
	3	0.12	0.02	0.13	0.02

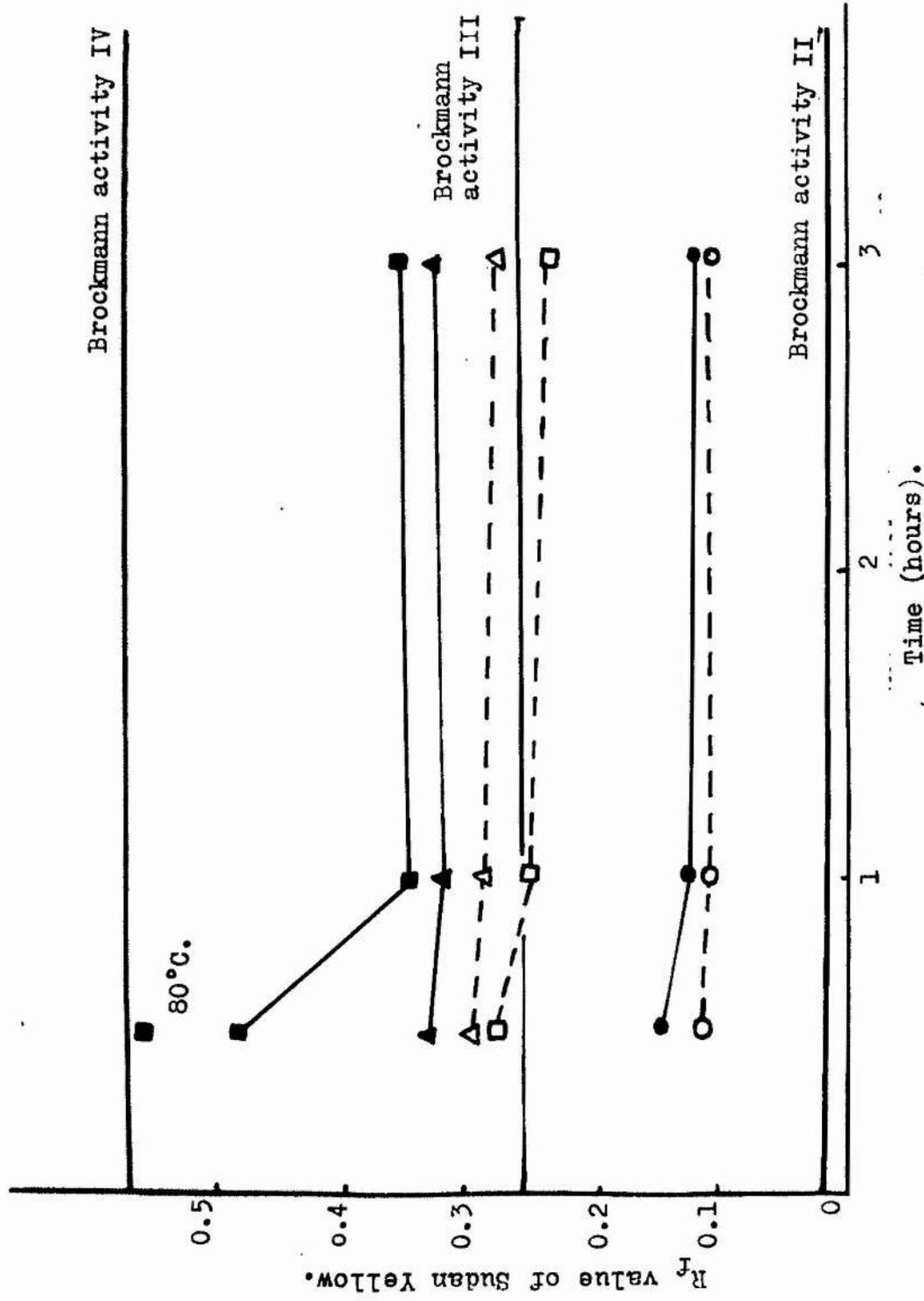


Fig. 17. Graph indicating the degrees of activation of various adsorbents on the Brockmann scale.
 Silica Gel G; 100°C. ● — ● ; 160°C. ○ — ○
 Alumina; " ■ — ■ ; " □ — □
 Magnesium oxide; " ▲ — ▲ ; " △ — △

By using these dyes, and that particular solvent, it was possible to compare the R_f values obtained, with those quoted for the dyes on the Brockman-Schodder scale of alumina activity (Brockman and Schodder, 1941), used by Randerath (1966). In this way it was possible to plot a graph of the activity of each adsorbent with the time and temperature of activation, on the Brockman-Schodder scale of activity, using as a reference, the R_f values of Sudan Yellow (Fig. 17).

On a qualitative basis, taking into account the resolution obtained and the sharpness of the bands, as well as the amount of material which could be loaded onto the plate without loss of resolution, the materials tested were as follows, in decreasing order of excellence:

- Silica Gel G (Merck)
- Alumina
- Magnesium oxide
- Calcium carbonate.

For these reasons, and for the ease and consistency of activation, it was decided to use Silica Gel G (Merck) for most of the further T.L.C. work.

The use of mixed adsorbents

The results of chromatograms with mixed adsorbents are given in Table 4.

Table 4

The efficiency of mixed adsorbents in the chromatography of standard dyes

Adsorbent	Time of development. (mins.)	R _f value (Sudan Yellow)	R _f value (p-aminazobenzene)	Resolution
1. Silica Gel G.	13	0.21	0.05	Good
2. Silica Gel G + "Hyflo":1/1(w/w)	10	0.35	0.15	Fair
3. Silica Gel G + "Hyflo":1/2(w/w)	6	0.37	0.15	Bad
4. Silica Gel G + kieselguhr:1/1(w/w)	19	0.35	0.15	Bad

On the basis of these results, a mixture of Silica Gel (Merck)/"Hyflo", 1/1 (w/w), was tried as an adsorbent for column chromatography, using tomato carotenoids. This work will be discussed in the section on tomato carotenoids.

Front	No.	R _f value
	1.	0.96 ± 0.01
	2.	0.60 ± 0.01
	3. 4.	0.42 ± 0.01 0.41 ± 0.01
	5.	0.26 ± 0.01
	6.	0.19 ± 0.01
	7.	0.06 ± 0.01
Origin		

Fig. 18. A typical T.L. chromatogram of the carotenoids of *S. flava* run on a 250 μ layer of Silica Gel G with benzene/methanol/acetic acid; 87/11/2; (v/v/v), under reproducible conditions.

Reproducible R_f values in T.L.C.

A typical chromatogram run under the conditions for reproducibility is shown in Fig. 18. At least ten such chromatograms were run, and the R_f values of the components of the mixture averaged.

These averages are as follows:- (This solvent yielded a better degree of resolution than did the solvent used in the preparative T.L.C., separating the cis-isomers of the larger fractions. In order to relate the picture obtained by reproducible T.L.C. to the main fractions dealt with in greater detail at a later stage, these latter are included in the table).

<u>Spot number</u>	<u>R_f value</u>	<u>Main Fraction No.</u>
1	0.96	Fraction 1
2	0.60	" 2
3	0.42)	" 3
4	0.41)	
5	0.26)	" 4
6	0.19)	
7	0.06)	

All these R_f values have a degree of error of ± 0.01 . It was later proved that some of the spots resolved represented the cis- isomers of all trans fractions.

Other thin-layer techniques

1. Reverse phase chromatography

The technique was carried out as described in the section on methodology. Three non-polar carotenoids, present in very small quantities, were retained almost at the edge of the section impregnated with olive oil. The main bulk of the carotenoids, however, consisting of much more polar carotenoids, were not resolved at all. No worthwhile results were obtained by the technique except that it was shown that more than one non-polar, and presumably carotene, fraction was present in the bacterial extract. In straightforward adsorption chromatography using the polar solvent benzene/methanol/acetic acid; 87/11/2; (v/v) (Rothblat et al., 1964), the non-polar materials could not be resolved.

2. "Wedge-strip" method

The main use to which this technique was put, viz. the comparison of the polar materials in saponified and unsaponified bacterial extracts, has already been described in the section on saponification (see page 184).

Front



Origin



Fig. 19. T.L.C. on Silica Gel G of the carotenoids of *S. flava*.
Solvent: ether/light petroleum; 70/30; (v/v).



Fig. 20. T.L.C. on Silica Gel G of the carotenoids of *S. flava*.
Solvent: light petroleum/acetone; 95/5; (v/v).

Solvents

The results obtained by the use of the solvent benzene/methanol/acetic acid; 87/11/2; (v/v), have already been reported in the previous section.

(a) The results of a chromatogram of the carotenoids of S. flava run on Silica Gel G with ether/light petroleum; 70/30 (v/v), are shown in Fig. 19. The visible spots of carotenoid are identified by the fraction numbers given to them as a result of the final separation of the pigments by preparative T.L.C., which will be described later. The solvent was insufficiently polar to resolve the bulk of the bacterial carotenoids.

(b) The other non-polar solvent which was tested was light petroleum/acetone; 95/5; (v/v). It can be seen from a comparison of the R_f values obtained with the two solvents that the latter is slightly less polar than the former (Fig. 20). As was later discovered by the chromatography of tomato carotenoids, both solvents were ideal for the resolution on mono-hydroxylated carotenoids.

Front

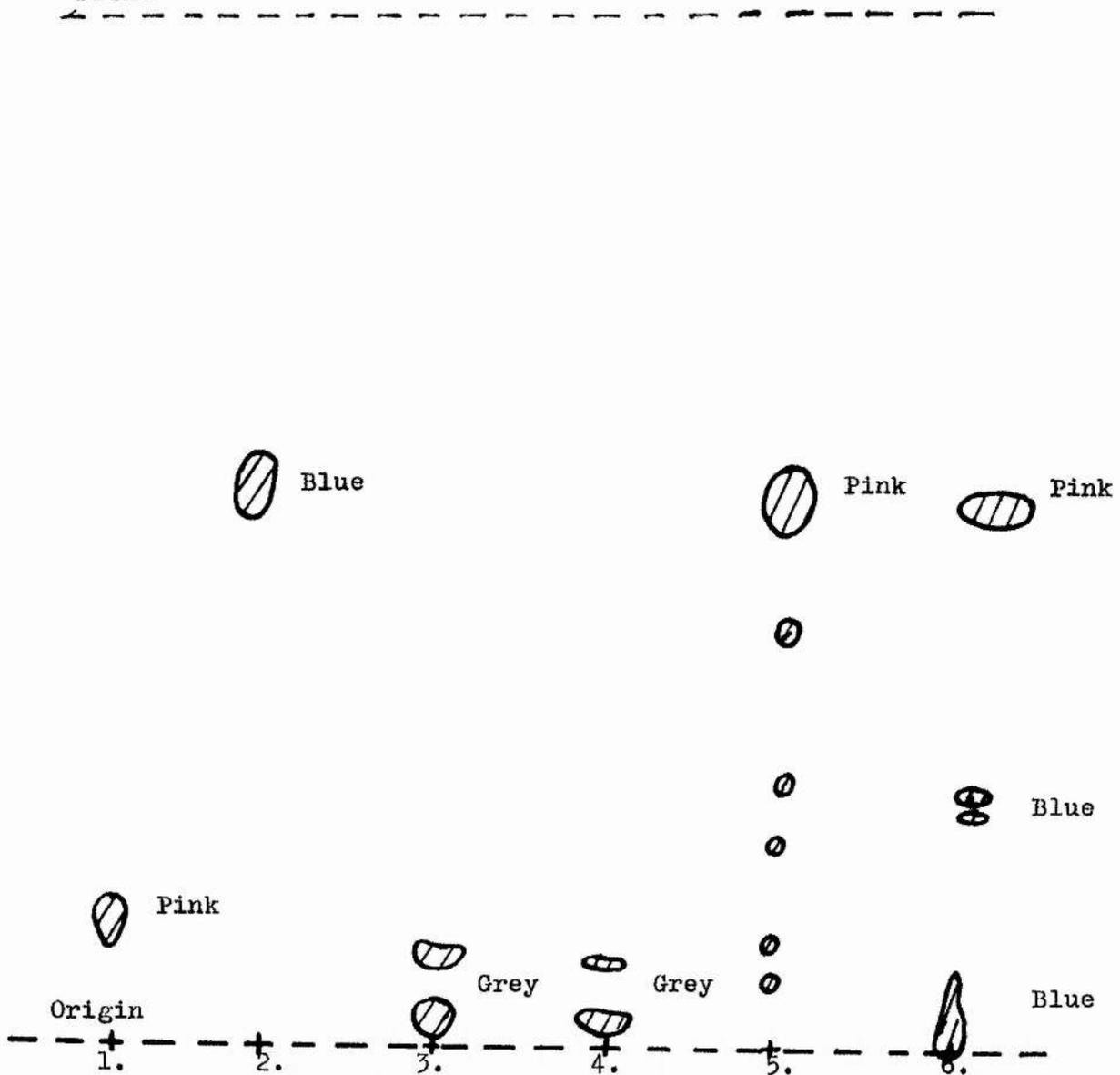


Fig. 21. T.L.C. on Silica Gel G of *S. flava* extract run against standard steroids. Solvent: ether/ light petroleum; 70/30;(v/v). Stain: $SbCl_3$ in chloroform. 1. Deoxycorticosterone. 2. Testosterone propionate. 3. Corticosterone. 4. Hydrocortisone. 5. Cholesterol. 6. Extract of *S. flava*.

1. *S. flava*
2. *S. flava*
+ cholesterol.
3. *S. flava*
+ lecithin.
4. Lecithin.
5. Cholesterol.

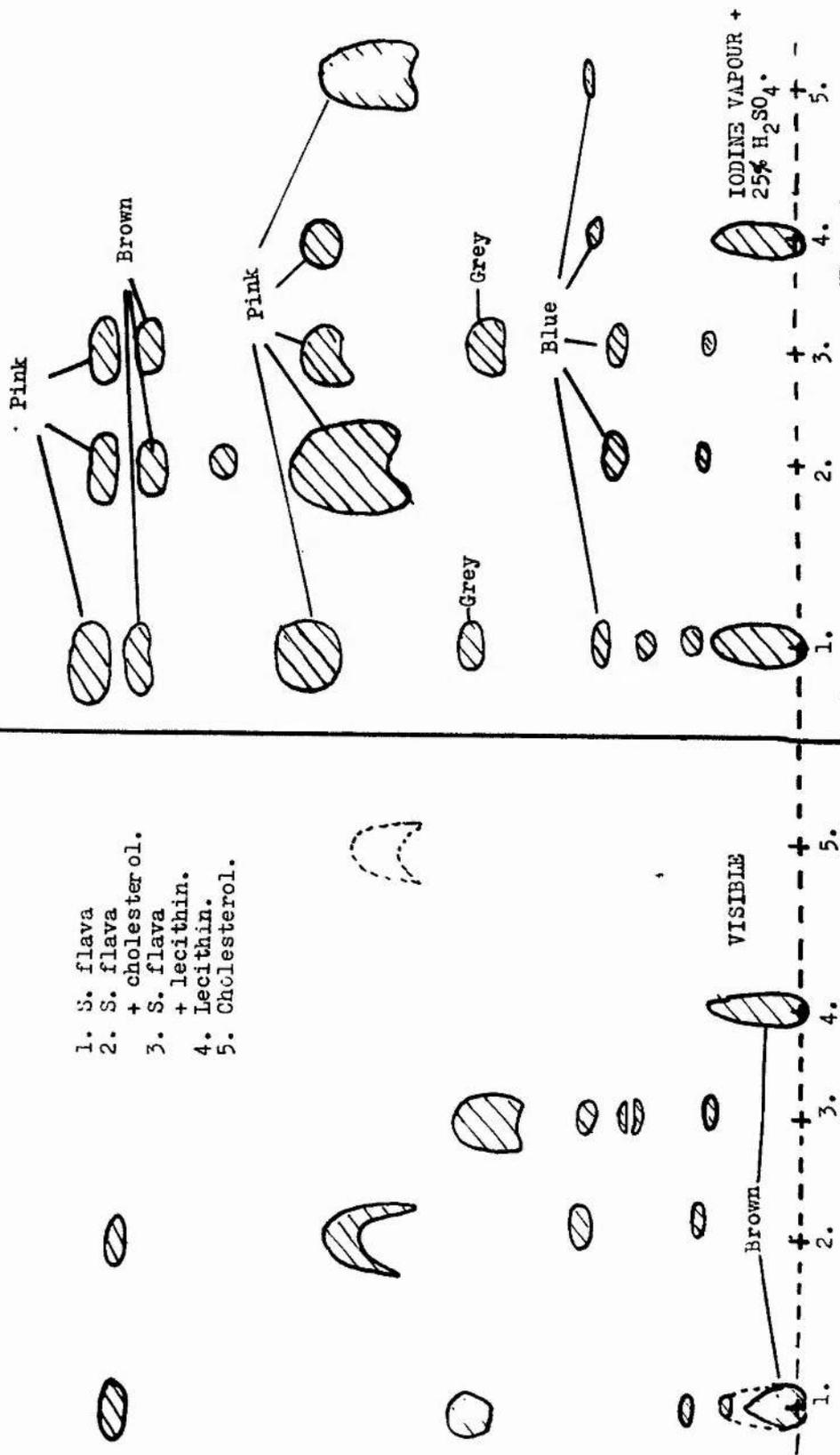


Fig. 22. T.L.C. on Silica Gel G, illustrating the effects of large amounts of lipid contaminants on the resolution of the carotenoids of *S. flava*.
Solvent: Benzene/methanol/acetic acid; 87/11/2; (v/v/v).

The use of lipid standards other than carotenoids,
in the T.L.C. of *S. flava* extracts.

(a) Co-chromatography with steroids

The results of the chromatographic comparison of the saponified extracts of *S. flava* with a series of steroid standards is shown in Fig. 21. It is obvious that the pink spot appearing in the saponified extract of *S. flava* after staining with $SbCl_5$ in chloroform had an identical colour and R_f value to that of cholesterol (R_f value 0.53). Although no further investigation was made of this fraction, it would appear to be cholesterol.

(b) Effects of lipid contaminants on T.L.C. of carotenoids

The purpose of this experiment was to test the hypothesis resulting from the chromatographic studies on the unsaponified extracts of *S. flava*, that large amounts of contaminating colourless lipids distorted the resolution of the carotenoids by occluding them. The chromatogram (Fig. 22) shows quite clearly that the presence of a large amount of cholesterol does in fact greatly distort the chromatographic separation, raising

the R_f value of the main mobile carotenoid fraction from 0.35 to 0.51. After staining with iodine vapour and 25% aqueous H_2SO_4 the cholesterol was stained a brilliant pink colour. This pink fraction was also present in the S. flava extract, a further indication that the bacterium contained cholesterol.

It was difficult to assess whether or not the polar saponifiable lipid, lecithin, did in fact occlude the pigments of S. flava, but there was some indication that the carotenoids were in fact carried with the front of the polar lipid. The lecithin used was extremely impure, and evidently contained cholesterol.

The dangers of interpretation of chromatograms obtained by the use of the S-chamber are well illustrated by Fig. 23 which shows that under these conditions the cholesterol has actually split the main mobile carotenoid fraction, (R_f 0.19), to produce apparently two free fractions, R_f values 0.22 and 0.19.

Preparative T.L.C.

(a) Correct thickness of layer

Thicknesses above 500 μ decreased the resolution to such an extent that contamination of the carotenoid fractions with colourless lipids became inevitable. Layers thicker than 500 μ were also costly in materials. The optimum thickness was therefore 500 μ .

(b) Choice of solvent

It was mentioned in the experimental section that the solvent used for much of the analytical chromatography viz. benzene/methanol/acetic acid; 87/11/2 (v/v), (Rothblat et al., 1964), had disadvantages when it came to be applied to preparative work. The main failing was that it contained an appreciable amount of acetic acid (2%). As mentioned in the introduction, the presence of any acidity at all could affect the carotenoids, producing isomers, and possibly dehydrating allylic alcohols. Apart from these considerations, it proved almost impossible to rid the eluted fractions of acetic acid. It was found that the acetic acid absorbed strongly in the ultra-violet region of the spectrum.

For these reasons it was decided to develop a new

solvent with approximately the same polarity, but without acetic acid as a constituent. The solvents tried were combinations of chloroform and methanol. The R_f values of the standard dyes Sudan Yellow and p-aminoazobenzene on Silica Gel G obtained with these solvents are listed in Table 5, along with those obtained with benzene/methanol/acetic acid; 87/11/2; (v/v).

Table 5

R_f values of the standard dyes Sudan Yellow and p-aminoazobenzene on Silica Gel G using as solvent combinations of chloroform and methanol.

Solvent	<u>R_f values</u>	
	Sudan Yellow	p-aminoazobenzene
1. Chloroform	0.57	0.38
2. Chloroform/methanol: 9/1: (v/v)	0.92	0.55
3. Chloroform/methanol: 5/1: (v/v)	0.98	0.80
4. Chloroform/methanol: 4/1: (v/v)	0.98	0.82
5. Chloroform/methanol: 1/1: (v/v)	0.98	0.86
6. Chloroform/methanol: 1/4: (v/v)	1.00	1.00
7. Benzene/methanol/acetic acid: 87/11/2	0.95	0.57

As can be seen from the results, the solvent with polarity most akin to that of benzene/methanol/acetic acid: 87/11/2 (v/v), was chloroform/methanol: 90/10: (v/v).

Using this solvent, the carotenoids of S. flava were resolved into four main fractions, which were called, in order of increasing polarity, Fractions 1, 2, 3, 4. Where necessary, the results of other chromatographic separations are related to this system of classification.

The approximate R_f values for these fractions are given below (Table 6). They were, of course, subject to a certain fluctuation, as the preparative T.L.C. was not done under carefully controlled conditions. There was no danger of confusion between the fractions, however, due to the wide differences in polarity between them.

Table 6

R_f values of the main fractions obtained by preparative T.L.C. of the total carotenoids of S. flava on Silica Gel G with, as solvent, chloroform/methanol: 90/10:(v/v).

Fraction	R_f value (Silica Gel G)
1	0.95 \pm 0.03
2	0.55 \pm 0.03
3	0.40 \pm 0.03
4	0.10 \pm 0.03

These were the R_f values obtained in chromatography tanks unlined with chromatography paper. It was found that if the tanks were lined, the R_f values were depressed to such an extent that there was insufficient resolution between Fractions 3 and 4 to be certain that they would be fully separated.

The experiments to find the maximum amount of carotenoid which could be safely resolved on a single plate were carried out using the carotenoids extracted from tomatoes, and the details will be given in the appropriate section of the results. The maximum loading was found to be 200 μ gm. per plate with a layer 500 μ thick. This meant that a total weight of 1 mgm. of carotenoid could be chromatographed on five plates run simultaneously in a single tank.

Paper chromatography

The results of the chromatography of the various fractions and their derivatives on either silica or kieselguhr-filled papers will be presented along with the results of all the other tests carried out on the fractions, where they will be more in context.

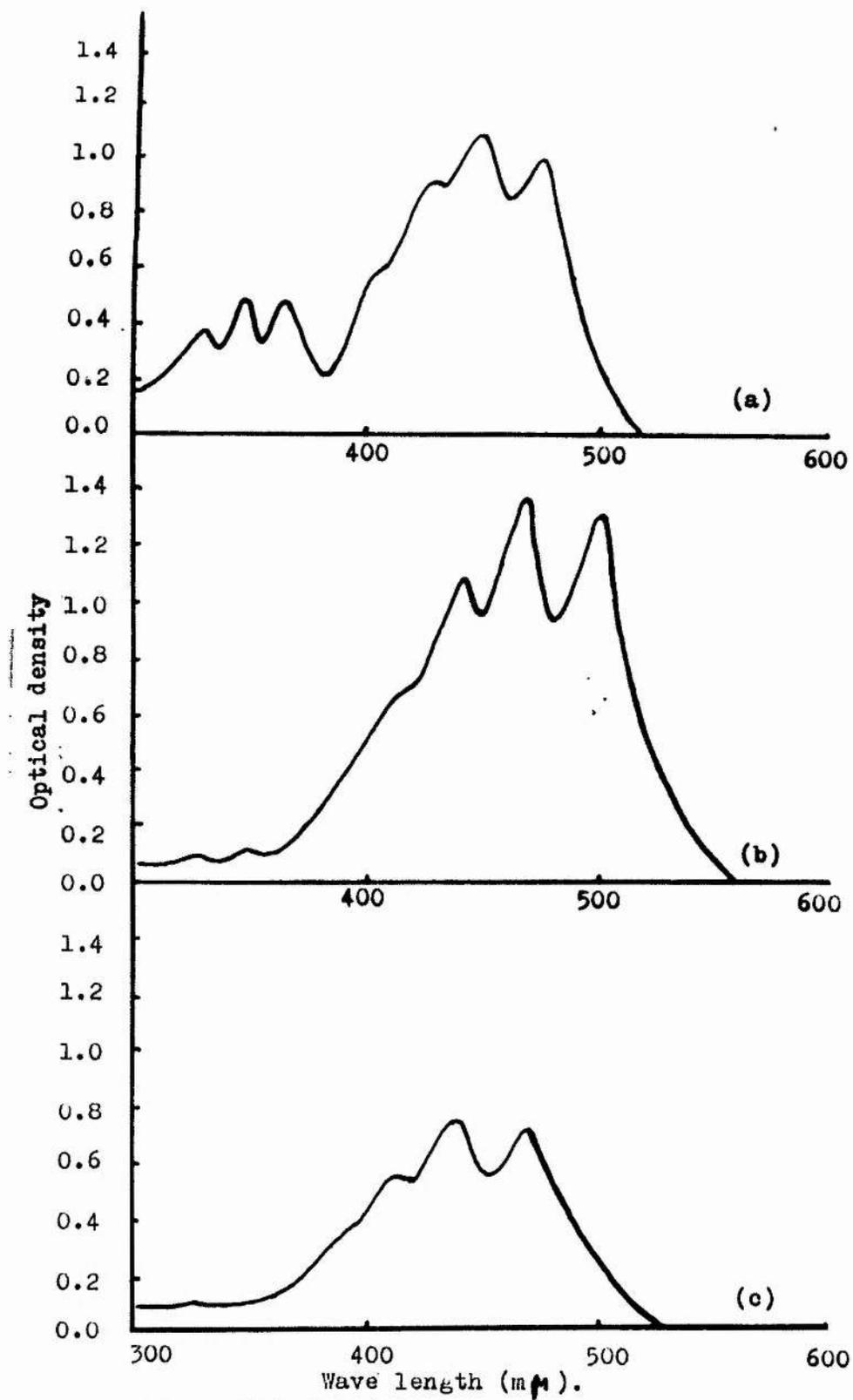


Fig. 24(a),(b),(c). Spectra of tomato carotenoid fractions resolved by column chromatography on alumina.

The use of carotenoids extracted from tomatoes for experimental purposes, and for the preparation of standards.

Column chromatography of the total extract from tomatoes with alumina as the adsorbent, produced six fractions, some of which were carefully examined spectrophotometrically and by T.L.C. on Silica Gel G and alumina.

Fraction 1, as can be seen from examination of the spectrum (Fig. 24a), consists of two materials with absorption peaks at 330, 347, 366 m μ , and at 425, 449, 475 m μ . From their polarities, and spectrum, these were identified as phytofluene (absorption peaks in light petroleum, 331, 348, 367 m μ (Davies, 1961)) and β -carotene (absorption peaks in light petroleum, 425, 451, 482 m μ (Goodwin, 1955)). The slightly lower absorption maxima could be accounted for by the occurrence of some stereomutation. The β -carotene was further identified by co-chromatography with an authentic commercial crystalline sample.

Fraction 2 had traces of β -carotene, but consisted mainly of a single large fraction with an absorption spectrum having peaks in light petroleum at 442, 468, 500 m μ . There was also a small peak at 360 m μ , indicating the presence of cis isomers (Fig. 24b). This material was identified as lycopene (absorption maxima in light petroleum, 446, 472, 505 m μ (Isler and Schudel, 1963)).

Fraction 3, because of its polarity and spectrum (peaks of maximum absorption at 442, 468, 500 m μ) was identified as rhodopin (1-hydroxy-1,2-dihydrolycopene).

Fractions 4 and 5 consisted of a large number of mono-hydroxylated derivatives of the carotenes, each occurring in very small concentration.

Fraction 6, as far as could be ascertained, consisted of a single polar carotenoid. Its spectrum in methanol (Fig. 24c) with peaks at 414, 439, 468 m μ was identical to that of the more polar carotenoids from S. flava. This fraction was co-chromatographed with the carotenoids of S. flava, S. lutea, and M. lysodeiktus, and will be reported on in the appropriate section.

Column chromatography (2)

The mixture of Silica Gel G and "Hyflo": 1/1 (w/w), was found to be too slow and too adsorbent, and was abandoned.

The reasons for choosing preparative T.L.C. for the isolation of pure carotenoids can be summed up as:

- (a) Lack of resolution in column chromatography.
- (b) The % recovery of materials from both methods was approximately equal.
- (c) Column chromatography demands the use of quite large amounts of materials.

Crystallisation

Lycopene was crystallised and chromatography of a solution of the washed crystals on Silica Gel G in light petroleum showed only one visible fraction, but the spectrum of the solution indicated that the crystalline material was not, in fact, all-trans. The absorption peaks in the visible region at 441, 468, 500 m μ were lower than those reported for all-trans-lycopene (446, 472, 505 m μ (Isler and Schudel, 1963)), which suggested the presence of cis isomers, a fact which was confirmed by the presence in the ultra-violet region of two small peaks at 344 and 361 m μ . Thus it appeared that crystallisation of a carotenoid from the solution of a mixture did not result in the isolation of a pure all-trans compound.

The use of Rhodamine 6G and Silica Gel G F₂₅₄ to locate colourless lipids.

When 75 μ gm. of tomato extract were run on a Silica Gel G layer impregnated with Rhodamine 6G, the only colourless lipid which could be identified was phytofluene. As the presence of Rhodamine

21

constituted a difficulty in eluting the materials,
the technique was not pursued further.

When chromatograms developed on Silica Gel G F₂₅₄
with added fluorescent indicator were viewed under
ultra-violet light at 254 m μ , no more colourless lipids
could be located than on the adsorbent without the
added indicator. This too was not pursued further.

The preparation of carotene standards from tomatoes

In the experiments to find the maximum loading of carotenoid which could be resolved on a single Silica Gel G (Merck) layer 500 μ in thickness, test chromatograms were run with a loading of up to 2 mg. of carotenoid. It was found that 200 μ gm. of tomato carotenoid was the maximum amount of material which could be resolved without danger of overlapping of the constituents.

Both Silica Gel G (Merck) and alumina (Merck) were employed in the preparation of standard carotenes. Although the silica was a more effective adsorbent from the point of view of activity and load, there existed tables which listed the elution order of carotenoids from alumina (Davies, 1965) which greatly facilitated the identification of the carotenes. The fractions of light petroleum boiling between 40-60°C. and 60-80°C. were both tried as solvents for resolving the tomato carotenes. The R_f values and R_x values with reference to Sudan Yellow, for the identifiable carotenes, on both fully activated adsorbents, and in both solvents, are given in Table 7. Authentic α -carotene and neurosporene were run as standards.

TABLE 7

The R_f and R_x values of identifiable carotenes from tomato chromatographed on Silica Gel G and alumina, using as solvents, light petroleum b.p. 40-60°C. and light petroleum b.p. 60-80°C.

Adsorbent	Identity of carotene	Light pet. b.p. 40-60°C.		Light pet. b.p. 60-80°C.	
		R_f	R_x (Sudan Yellow)	R_f	R_x (Sudan Yellow)
Silica Gel G.	(Squalene?)	0.41	--	--	--
	α -carotene	0.22	11.1	0.34	10.8
	phytoene	0.21	11.0	0.34	10.8
	β -carotene	0.20	10.5	0.30	10.0
	phytofluene	0.16	8.3	0.25	8.0
	γ -carotene	0.11	5.0	0.19	6.0
	lycopene	0.06	3.3	0.09	3.1
	polar fractions	0	0	0	0
Alumina (Brockman grade III)	phytoene	0.56	4.5	0.60	4.7
	phytofluene	0.50	4.0	0.58	4.2
	β + α -carotene	0.49	3.9	0.54	4.1
	lycopene	0.18	1.4	0.22	1.4
	neurosporene	0.13	1.0	0.17	1.1
	polar fractions	0	0	0	0

Two points of interest resulting from these chromatograms were:-

1. The order of elution of phytofluene and β -carotene is different on Silica Gel G and alumina.
2. A colourless material runs on Silica Gel G with the same R_f value as squalene (Davies, 1965).

By running preparative chromatograms on alumina and Silica Gel G (Merck), the following identifiable carotenoid were isolated:

Phytoene
Phytofluene
All trans β -carotene
[β -carotene
Lycopene
Neurosporene

The yield of carotene from a typical preparative T.L.C. is shown below:-

Table 8

The recovery of carotenes from a typical preparative
T.L.C. of tomato carotenoids.

Adsorbent: Silica Gel G (Merck)
Layer thickness: 500 μ
Solvent: Light petroleum 60-80°C. (Triple developed)
Loading: 90 μ gm.
Recovery: 75%

<u>Carotenes and precursors</u>	<u>% of total</u>
Phytoene	23
Phytofluene	10
ζ -carotene	4
Neo- β -carotene	8
β -carotene	10
Neo-lycopene	9
Lycopene	30
Remainder	6

The controlled deactivation of alumina.

In an attempt to achieve a degree of activity suitable for the resolution of the non-polar carotenes, ϵ -, α -, β -carotene, an alumina plate was deactivated as described, and β -carotene from tomatoes chromatographed with authentic commercial α -carotene (Sigma Ltd.) in light petroleum as solvent. The variation of the R_f values of the β -carotene with different times of exposure to the air is shown below (Table 9):-

Table 9

The variation in R_f value of β -carotene on alumina with decreasing activity of that adsorbent.

Time of exposure to the air (mins.)	R_f value of β -carotene
40	0.70
30	0.66
20	0.63
10	0.50
0	0.07

No resolution of α - and β -carotene was obtained at any degree of activation. The object of the exercise, named

2.

to locate and elute any ϵ -carotene which might have been present in the tomato extract, was thus not accomplished. According to the figures of Davies (1965), ϵ -carotene runs ahead of α - and β -carotene on alumina. The reasons for attempting to isolate ϵ -carotene will be discussed in the final section of the thesis.

One thing the experiment did illustrate, however, was the rapidity with which the alumina became deactivated on exposure to the air.

Analysis of the carotenoids

Fraction 1. This, the least polar of the main fractions, constituted 5% of the total carotenoid isolated. The partition ratio of the entire fraction between light petroleum and 95% aqueous methanol was 100/0.

Co-chromatography of the fraction on silica-filled chromatography paper (Whatman S.G.81) with the carotenes and precursors isolated from tomatoes, viz. phytoene, phytofluene, α -carotene, β -carotene, lycopene, ζ -caroten neurosporene, indicated that none of the components resolved from the fraction on the same silica-impregnated paper were identical with any of the identified carotenes from tomatoes or commercial samples.

The results of the preparative isolation of the components of Fraction 1 on Whatman S.G.81 chromatography paper are given below in Table 10. The R_f of phytofluene is included for the sake of comparison.

TABLE 10

Possible carotenes and precursors from Fraction 1, resolved by preparative chromatography on Whatman S.G.81 chromatography paper, using as solvent light petroleum.

Band	Behaviour under U.V. light	R _F	Colour	max. in methanol (m μ)
F. 1(a)	Fluorescent	0.48	Colourless	-
F. 1(b)	Fluorescent	0.40	Colourless	-
F. 1(c)	Absorbent	0.28	Yellow	392, 424, 448
F. 1(d)	Fluorescent	0.17	Colourless	-
F. 1(e)	Absorbent	0.12	Yellow	408, 431, 450
F. 1(f)	Absorbent	0.11	Yellow	406, 428, 450
F. 1(g)*	Absorbent	0.07	Yellow	395, 415, 438
F. 1(h)*	Absorbent	0.06	Yellow	394, 415, 438
F. 1(i)**	Absorbent	0.03	Yellow	414, 438, 469

Notes:- * The R_F value of phytofluene on Whatman S.G.81 with light petroleum as solvent was 0.56.

** The λ_{max} of F. 1(i) are consistent with the presence of a chromophore containing 9 conjugated double bonds. This is probably the parent hydrocarbon of the polar carotenoids in this bacterium.

Fraction 2. This fraction constituted 8% of the total carotenoid. The results of the tests carried out on this fraction are shown in Table 11.

TABLE 11

The results of chemical tests carried out on Fraction 2 in an attempt to gain information as to its nature.

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction - 2 (original plus ester). Final number of spots detected - 1 (ester)	Single hydroxyl group present.
Tertiary hydroxyl group.	Negative	No tertiary hydroxyl group present.
Reduction with LiAlH_4	Negative	No other reducible group present.
Oxidation with nickel peroxide.	One α - β unsaturated aldehyde formed.	One allylic hydroxyl group separated from polyene chain.
Iodine isomerisation	One isomer induced (Neo U) (20% of original converted to isomer)	"
Partition Ratio. Light petroleum/95% aqueous methanol.	73/27	Polar compound, value consistent with mono-hydroxyl compound.
I.R. Spectroscopy.	No <u>cis</u> peaks Peak at 1050 cm^{-1}	All- <u>trans</u> form Primary hydroxyl group.
Mass Spectrometry.	Parent ion at m/e 686; mass measurement gave the empirical formula as $\text{C}_{50}\text{H}_{70}\text{O}$.	C_{50} carotenoid fraction.

The R_f values of the fraction, its derivatives, and isomers, obtained on kieselguhr filled paper are given in Table 12.

TABLE 12

R_f values and spectra of Fraction 2, its derivatives, and isomers obtained by chromatography on kieselguhr-filled chromatography paper, using, as solvent, light petroleum/acetone: 98/2: (v/v).

Sample	R_f value	$\lambda_{max.}$ in methanol(m μ)
All <u>trans</u> fraction 2	0.74	-, 415, 439, 469.
Neo U isomer	0.61	331, 413, 437, 466.
Monoacetate	0.95	-, 415, 439, 469.

Fraction 3. This fraction constituted 26% of the total carotenoid. The results of the tests carried out on this fraction are shown in Table 13.

TABLE 13

The results of the chemical tests carried out on Fraction 3 in an attempt to gain information as to its structure.

Test	Result	Comment
Acetylation	Max. number of spots detected during reaction - 3 (original, mono- and diester) Final number of spots detected - 1 (diester)	Two hydroxyl groups present.
Tertiary hydroxyl group.	Negative	No tertiary hydroxyl group present.
Reduction with LiAlH_4	Negative	No other reducible group present
Oxidation with nickel peroxide	One mono- and one di α - β unsaturated aldehyde formed.	Two allylic hydroxyl groups separated from polyene chain.
Iodine isomerisation	One isomer induced (Neo U) (33% original converted to the isomer)	-
Partition ratio light petroleum/45% methanol.	29/71	Polar compound, value consistent with the presence of two hydroxyl groups.
I.R. Spectroscopy.	No <u>cis</u> peaks Peak at 1050 cm^{-1}	All <u>trans</u> form Primary hydroxyl group.
Mass Spectrometry.	Parent ion at m/e 702 Mass measurement gave the empirical formula as $\text{C}_{50}\text{H}_{70}\text{O}_2$.	C_{50} carotenoid fraction.

The R_f values for this fraction, its derivatives and isomers on kieselguhr-filled paper are shown in Table 14.

TABLE 14

A comparison of the R_f values obtained by chromatography on kieselguhr-filled paper with Fraction 3 from *S. flava* and P 439 from *Flavobacterium dehydrogenans*, and their respective derivatives.

Trans-carotenoid	R_f values on kieselguhr-filled paper		
	Solvents		
	acetone/ light pet. 2/98(v/v)	acetone/ light pet. 5/95(v/v)	acetone/ light pet. 10/90(v/v)
Fraction 3	Free carotenoid		0.62
	mono-acetate		0.66
	di-acetate	0.65	
	mono-aldehyde		0.50
	di-aldehyde		0.80
Dehydrogenans P 439	Free carotenoid		0.62
	mono-acetate		0.66
	di-acetate	0.65	0.98
	mono-aldehyde		0.50
	di-aldehyde		0.80

TABLE 15

Absorption maxima in visible region of Fraction 3 from
S. flava and Dehydrogenans P 439.

Carotenoid	Stereoisomer	<u>Absorption maxima (mμ) in</u> <u>light petroleum methanol</u>	
Fraction 3 <u>S. flava</u>	Trans	415, 439, 469	
	Neo U	412, 436, 466	
	Iodine cata- lysed equili- brium mixture	414, 437, 468	
Dehydrogenans P 439	Trans	(394), 416, 439.5, 470	416, 439, 469
	Neo U	415, 436, 465	414, 436, 465
	Iodine catalys- ed equilibrium (394), 416, 438, 468 mixture	415, 437, 467	

The fraction and its derivatives were compared spectrally and chromatographically with the bacterial carotenoid pigment "P 439" and its corresponding derivatives. This material was isolated from Flavobacterium dehydrogenans by Dr. O.B. Weeks and Dr. S.L. Jensen (Jensen and Weeks, 1966) as was mentioned in the Experimental Section. The results of the comparative tests are shown in Tables 14 and 15. From mass spectral analysis, these two workers concluded that the carotenoid "P 439" contained 50 carbon atoms in its skeleton. In this respect, as in all the other comparative tests tried the two bacterial carotenoids, "P 439" from Flavobacterium dehydrogenans, and Fraction 3 from S. flava, appeared to be identical. A paper recording this fact has been recently published. (Jensen, Weeks, Strang, and Thirkell, 1967). More will be said about this fraction in the discussion.

Fraction 4. This fraction constituted about 61% of the total carotenoid pigments. It was further resolved into four fractions on kieselguhr-filled chromatography paper, using light petroleum/acetone: 60/40 (v/v). The four sub-fractions were identified from their absorption spectra as follows: (Table 16).

TABLE 16

The further fractionation of Fraction 4 on kieselguhr-filled chromatography paper, including the R_f value of the acetate of Fraction 4c.

Fraction	Spectrum in methanol (μ)	R_f value on kieselguhr- filled paper light pet./ acetone(60/40)	% of total	Identification
4a	331, 414, 437, 468	0.99	6.5	Neo B isomer
4b	331, 414, 438, 468	0.83	10.5	Neo A isomer
4c	416, 439.5, 469.5	0.77	53.0	All trans
4d	331, 414, 435, 467	0.69	30.0	Neo U isomer
Acetate of 4c	416, 439.5, 469.5	0.67*		

* The solvent used in the case of the acetate of Fraction 4c was light petroleum/acetone: 90/10: (v/v).

Fraction 4c, the all-trans isomer, and that present in the greatest concentration, was tested in more detail and the results shown in Table 17.

TABLE 17

The results of the chemical tests carried out on Fraction 4c in an attempt to gain information as to its structure.

Test	Result	Comment
Acetylation	Initial number of spots detected - 1. Max. number of spots detected during reaction - 7. Final number of spots detected - 1	At least three primary or secondary hydroxyl groups present.
Tertiary hydroxyl group	Negative	No tertiary hydroxyl group present.
Reduction with LiAlH_4	Not carried out on this fraction.	-
Iodine isomerisation	Three isomers induced:- One Neo B isomer. One Neo A isomer One Neo U isomer	Equiv. to fraction 4(Equiv. to fraction 4(Equiv. to fraction 4(
Partition ratio	100/0	Very polar compound
Methylation of carboxyl group	Results uncertain	Carboxyl group present
I.R. Spectroscopy.	No <u>cis</u> peak No absolute conclusion could be reached from the traces as to the nature of the hydroxyl groups or as to the presence or absence of a carboxyl group.	All <u>trans</u> compound

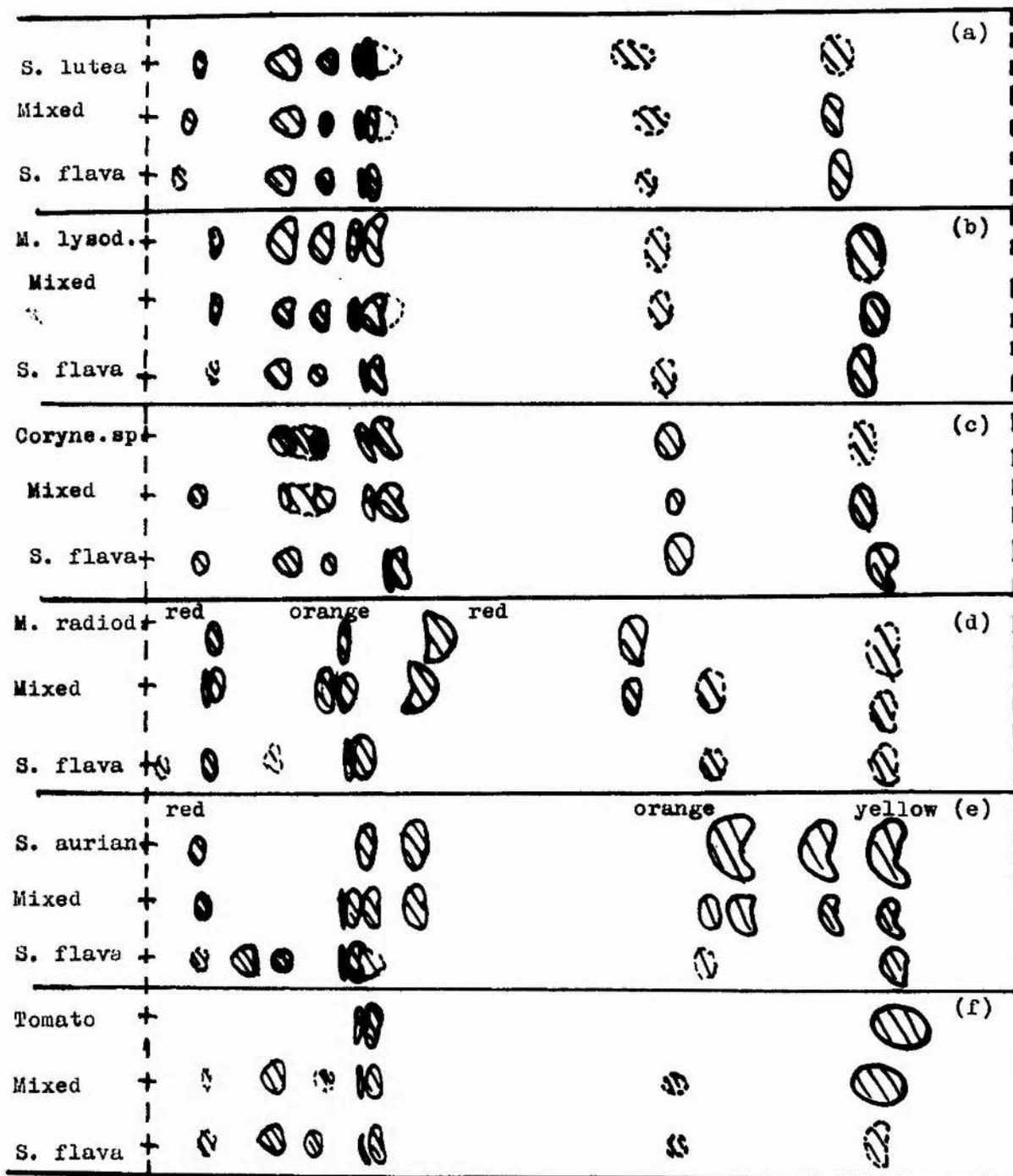


Fig. 25(a)-(f). Co-chromatography on Silica Gel G of the carotenoids from various sources with those of *S. flava*. Solvent: benzene/methanol/acetic acid; 87/11/2; (v/v/v). *M. lysod.*- *M. lysodeiktus*; *Coryne. sp.*- *Coryneform* species; *M. radiod.*-*M. radiodurans*; *S. aurian.*-*S. aurantiaca*.

2

Comparison of the carotenoid pigments from *S. flava*
with those from other pigmented micro-organisms.

The chromatograms comparing the bacterial carotenoids are illustrated in Fig. 25. The bacterial carotenoids which are of the greatest interest are those from *S. lutea*, *M. lysodeiktus*, and the species of Coryneform bacterium.

The carotenoids from *S. lutea* were extracted and co-chromatographed with those of *S. flava* repeatedly under various conditions, in light petroleum/acetone: 95/5: (v/v) as well as with benzene/methanol/acetic acid: 87/11/2: (v/v). On all occasions, the visible pigments of the two bacteria co-chromatographed exactly. The spectrum of the total extract of *S. lutea* was identical with that of the extract of *S. flava*, with peaks at 414, 439, and 468 m μ , and sufficient carotenoid material was extracted from *S. lutea* to allow preparative chromatograms to be run on Silica Gel G (Merck) in chloroform/methanol: 90/10: (v/v), the fractions eluted from which were found to have the same R_f values and spectrum in methanol as those of *S. flava*, viz:

Fraction 1	425, 447, 472 m μ
Fractions 2, 3, 4,	414, 439, 468 m μ

2

Unfortunately, the quantities isolated did not permit any further characterisation.

The carotenoids of M. lysodeiktus were investigated because of the publication of a study by Rothblat et al. (1964) on the pigments of this bacterium, which indicated a close similarity between the carotenoids present in M. lysodeiktus and those of S. flava.

The total extract of M. lysodeiktus displayed the same absorption spectrum in the visible region as S. flava, and seven fractions were resolvable by chromatography on Silica Gel G (Merck) with benzene/methanol/acetic acid: 87/11/2: (v/v), as was the case with S. flava. Co-chromatography of the carotenoids from the two bacteria on Silica Gel G in this solvent failed to separate the carotenoids from the two bacteria.

The reasons for examining the carotenoid pigments of the species of Coryneform bacterium have been mentioned in the introduction. The investigation of these pigments by Hodgekiss et al. (1954), indicated the presence of sarcinaxanthin and other carotenoids. Once more co-chromatography failed to separate the carotenoids from the S. flava and the Coryneform bacterium.

Carotenoids from tomatoes were chromatographed along with many of the bacterial extracts. As was mentioned in the section on tomato carotenoids, column chromatography on alumina allowed the isolation of a polar carotenoid which had the same absorption spectrum in the visible region as that of the more polar carotenoid from S. flava. This tomato carotenoid co-chromatographed exactly with Fraction 3 from S. flava (reproducible R_f value - 0.42). The cis isomer (reproducible R_f value 0.41) was also evident in the tomato extract, giving further evidence of identical nature of the two carotenoids. (It is uncertain whether the cis isomers discovered in this work are naturally occurring or induced by the extraction and chromatographic procedures).

The quantitative comparison of the pigment production of S. lutea and S. flava under identical conditions of culture is given below, Table 18. Under these conditions the carotenoid production of the two bacteria was identical within the limits of experimental error.

TABLE 18

A comparison of the pigment production of *S. lutea*
and *S. flava* grown under identical conditions.

Bacterium	Sample	Total pigment extract- ed. (mgm.)	Wt. of dry bact- erial rem- nants. (mgm.)	mgm. pigment/ mgm. bact- remnants. (mgm.)	Average
<u><i>S. lutea</i></u>	1	13	39.6	0.33	
	2	18	57.7	0.32	0.31
	3	26	84.8	0.31	
<u><i>S. flava</i></u>	1	10	27.8	0.36	
	2	8	25.6	0.31	0.32
	3	9	29.8	0.30	

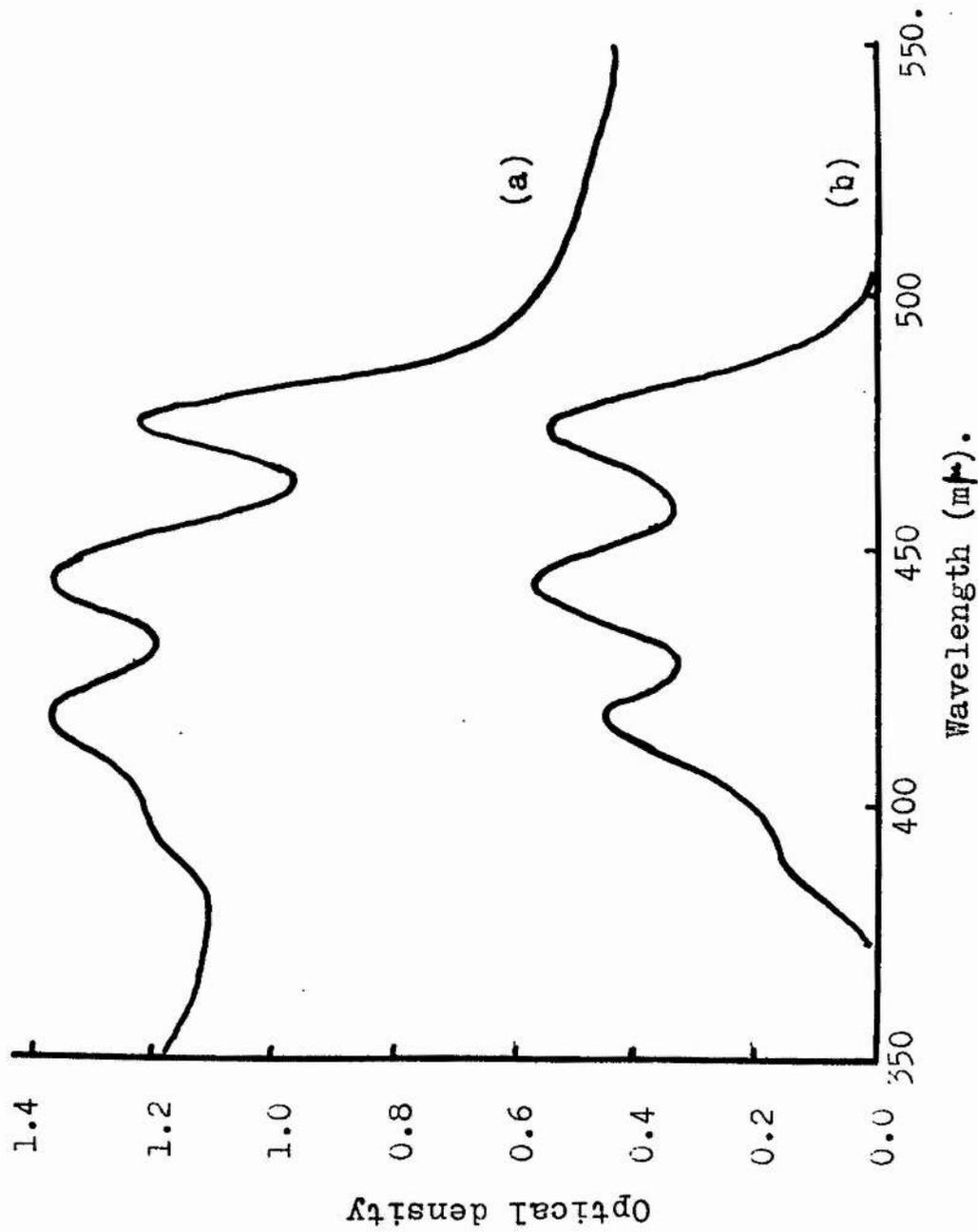


Fig. 26. (a) Spectrum in the visible region of the lysate of *S. flava*. (b) The carotenoid absorption curve corrected for random light scattering.

Experiments with the protoplast membrane of *S. flava*.

Lysis of the bacteria

It was found that lysis of *S. flava* under the stated conditions, followed by observing the fall in the optical density of the solution, was completed within one hour. No increase of the concentration of lysozyme caused any further fall in the optical density.

Spectral analysis of the initial lysate and the protoplast membranes after differential centrifugation.

The spectrum, in the visible region, of the translucent yellow suspension resulting from the lysis of *S. flava* is shown in Fig. 26. In this region of the spectrum, the only peaks are those of the carotenoid at 417, 444, 475 m μ . These peaks were at slightly longer wavelengths than those of the extracted carotenoid in methanolic solution. After correction for light scattering due to the particulate nature of the suspension, the actual form of the spectrum was identical to that of the free pigment (Fig. 26). In the ultra-violet region (Fig. 27), two peaks were present, at 235, and 257 m μ . As the blank against which the spectrum of the lysate was measured contained lysozyme, neither of these two peaks were due to the presence of this protein in solution.

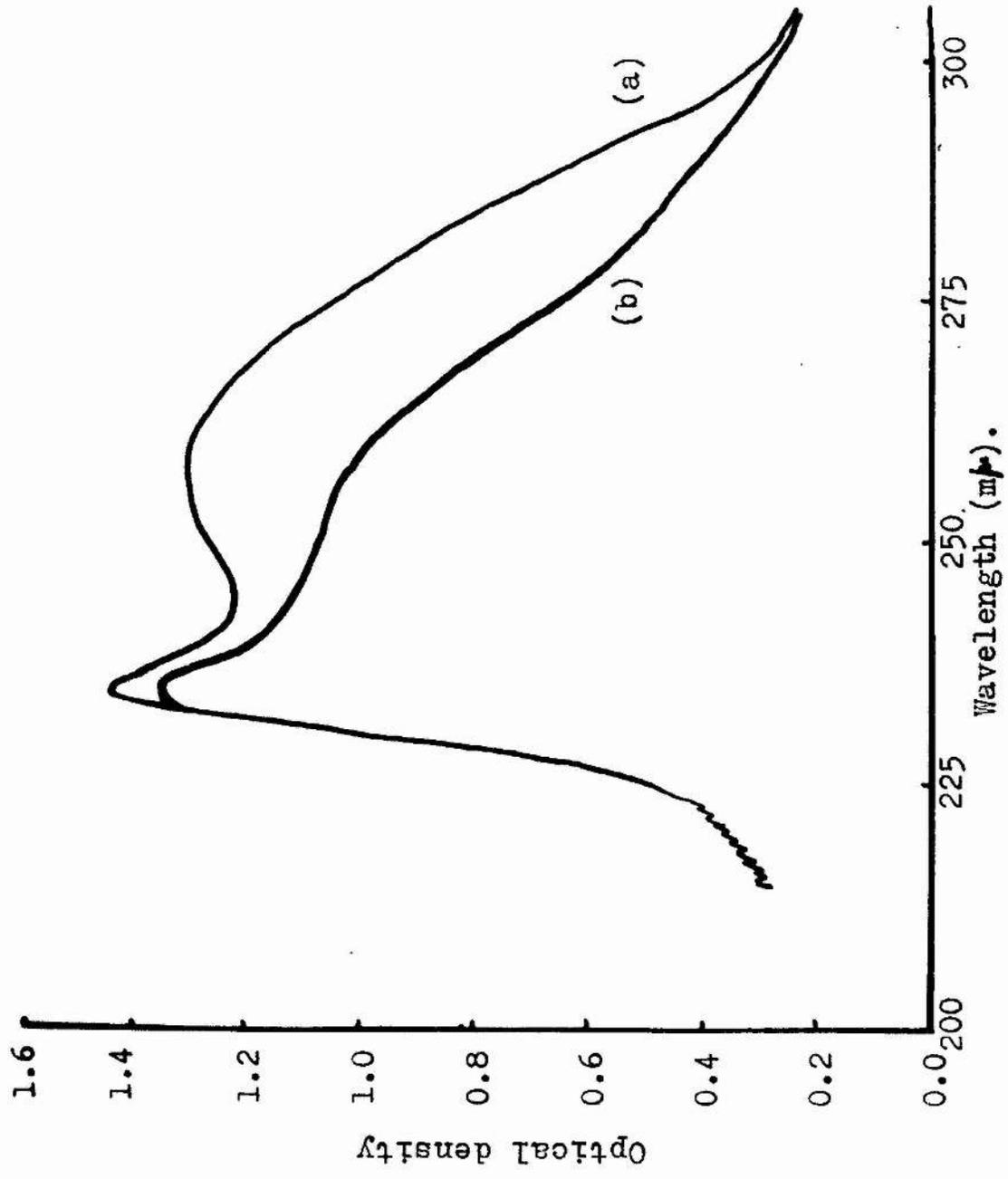


Fig. 27. Spectrum in the ultraviolet region of the lysate of *S. flava*, (a) before, and (b) after, differential centrifugation.

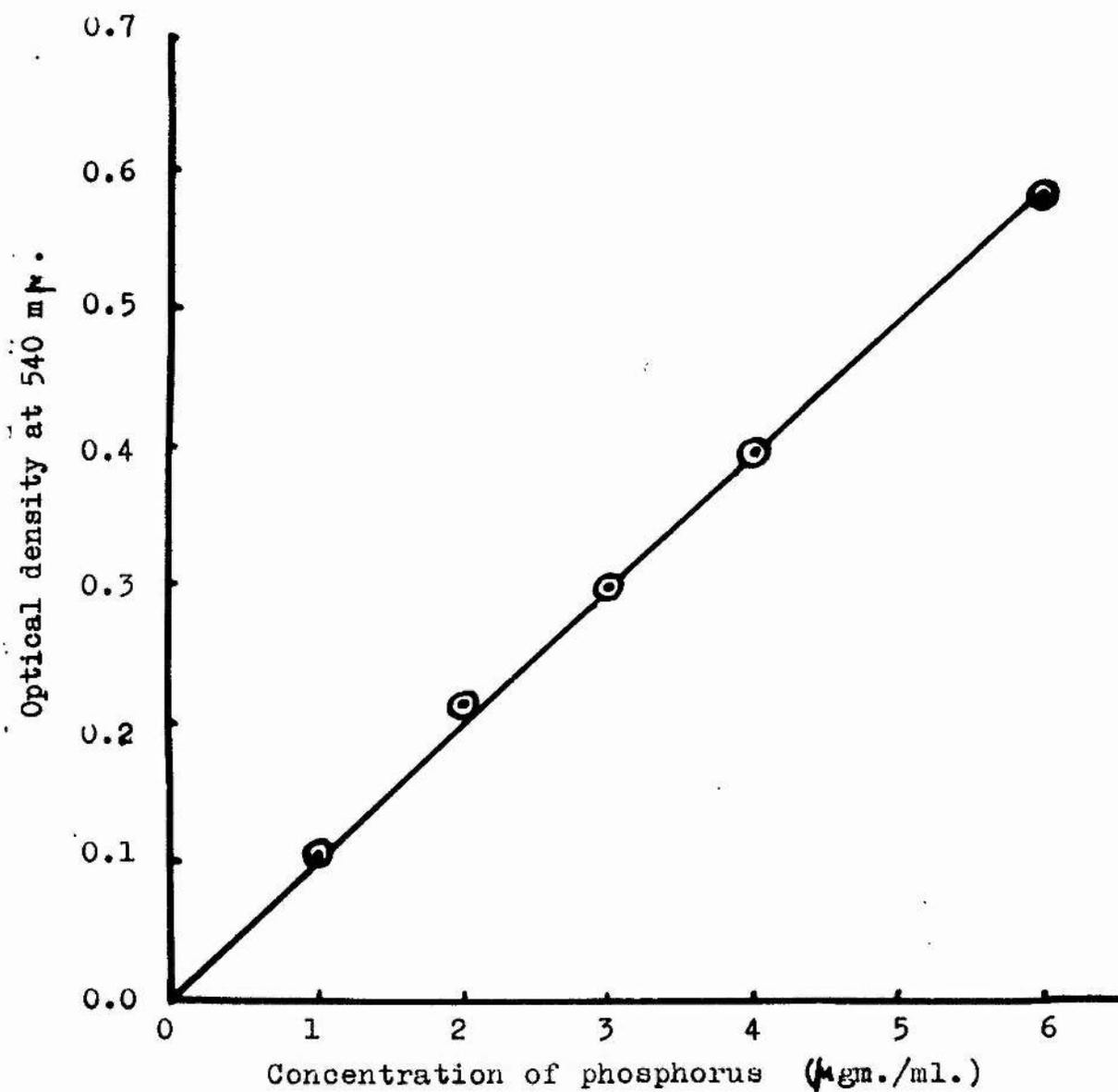


Fig. 28. Standard curve of phosphorus concentration against colour developed in the amidol/ammonium molybdate reaction.

23

The spectrum of the membranes after purification by differential centrifugation showed the same five main peaks, at 235, 257, 417, 444, 475 $m\mu$. The ratio of the peak at 257 $m\mu$ to that at 235 $m\mu$, which was 0.94 in the original lysate, had now fallen to 0.5, indicating that the membranes had been freed of much of the material absorbing at 257 $m\mu$, possibly ribonuclear material (Fig. 27)

Analysis of the protoplast membranes

The standard curve for the estimation of phosphorus is shown in Fig. 28.

(a) The results of the analyses of the entire lyophilised bacteria are presented in Table 19.

TABLE 19

Results of analysis of entire lyophilized S. flava cells and protoplast membranes
in terms of % by weight.

Fraction	entire bacteria	Membranes	Comment
Weight	100.00	34.5 ± 2.0	Average of 4 preparations
Phosphorus	0.41	0.08	8.5% total bacterial P in membrane
Nitrogen	9.77	8.92	32% total bacterial N in membrane
Carotenoid	0.0150	0.0434	100% of total bacterial carotenoid in membrane

(b) The comparison of the % of weight of the carotenoid with reference to the entire lyophilised cell estimated first by extraction from whole bacteria with methanol, and then by finding the amount present in the purified protoplast membrane is given in Table 20.

It can be seen from the close agreement of the figures, that the entire carotenoid content of S. flava is located in the protoplast membranes.

(c) Four estimations were made of the % of the total bacterial dry weight represented by the protoplast membranes prepared in the manner described. The average result was $34.5\% \pm 2\%$. From this average value the % of the total bacterial nitrogen and phosphorus represented by the nitrogen and phosphorus of the protoplast membranes were calculated, and are presented in the relevant table (Table 19).

TABLE 20

Comparison of the % of carotenoid present in entire bacteria, and in the protoplast membranes from known weights of entire bacteria.

Wt. of entire lyophilised bacteria (mg.)	Carotenoid extracted with methanol. (mg.)	% carotenoid of total bacterial wt.	Average %
194	0.023	0.012	
412	0.048	0.016	0.016
148	0.027	0.017	
286	0.050	0.017	
Wt. of entire lyophilised bacteria from which the membranes were extracted.	Total carotenoid present in protoplasm membranes.	"	"
257	0.035	0.014	0.014
286	0.040	0.014	0.014

TABLE 21

Results of Tests on Fraction S₂

Test	Result	Comment
1) Ammonium sulphate precipitation	Yellow fraction precipitated at between 60-70% saturation. Supernatant after centrifugation was colourless.	? Indicative of fairly low m.wt. compound.
2) Addition of 10% T.C.A.	Pale yellow flocculation. Supernatant colourless after centrifugation.	
3) Extraction with ether (previously saturated with distilled water)	No colour extracted.	Checked spectrophotometrically
4) S ₂ at pHs 3-11 extracted with ether.	No colour extracted. Yellow precipitate below pH 3 (Isoelectric point in this region?)	Suggests that bonding of carotenoid to protein is not primarily due to charged groups.
5) S ₂ made 4M with respect to urea and extracted with ether.	No colour extracted.	Suggests that (a) carotenoid binding does not involve hydrogen bonds (b) disruption of the secondary and/or tertiary structure of the protein component does not seem to facilitate release of pigment.
6) S ₂ + thioglycollic acid extracted with ether.	No colour extracted.	
7) S ₂ in 4M urea + thioglycollic acid extracted with ether.	No colour extracted.	

8) (a) Addition of acetone.

Pale yellow flocculation.

(b) aq. acetone suspension extracted with ether.

39% original pigment extracted with ether.
61% remains in aq. acetone layer.

1. T.L.C. showed pigment extracted to be almost entirely fraction 3 and no polar fraction 4; 61% of pigment remaining corresponds to % of pigment in entire bacteria extracted as fraction 4.

2. Binding of carotenoid to protein (or carbohydrate) not electrostatic (Ref. Cheesman, 1967).

9) Boil solution for 10 minutes.

No precipitation.

No change in absorption spectrum of material.

Suggests that carotenoid stabilizes protein and protein stabilizes carotenoid. Carotenoids are usually degraded when raised to 100°C. in air.

10) Extraction with ether after boiling for 10 minutes.

No colour extracted.

11) Expose to sunlight for 2 hrs. in presence of catalytic amounts of I₂. Follow absorption maxima.

Peaks in the visible region at 417, 445, and 475 mμ do not change.

When the carotenoid is bound, the induction of isomers is not easy, again suggesting stabilization of the carotenoid when complexed.

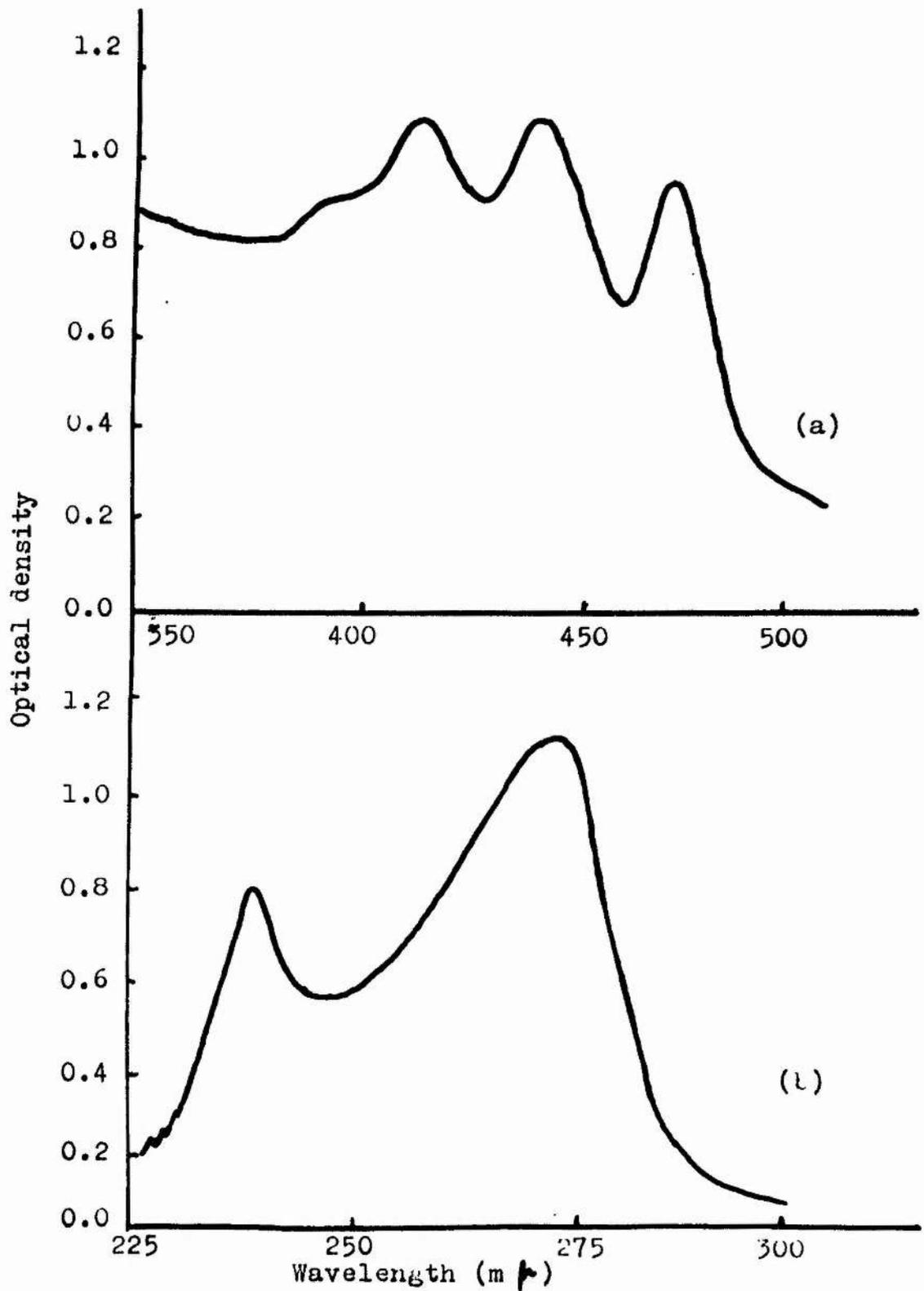


Fig. 29. Spectra in (a) the visible region, (b) the ultraviolet region, of the water-soluble fraction S2 from the protoplast membrane of *S. flava*.

Preparation and investigation of a water-soluble fraction of the protoplast membranes.

As a result of tests comparing the effectiveness of the different synthetic detergents, "Crill S 6", a non-ionic detergent (Croda Ltd.), was found to give the best results and was used in the subsequent preparations. The spectrum of the water-soluble fraction produced from the protoplast membranes by the action of "Crill S 6" is shown in Fig. 29. It is almost identical with that of a suspension of the entire protoplast membranes. The peaks in the visible region of the spectrum due to the carotenoid are at 417, 445, and 475 m μ .

Effects of light and air on the carotenoid in solubilized protoplast membranes.

There was no detectable change in the optical density at 600 m μ or at 445 m μ in either suspension throughout the 3 hours during which one suspension was exposed to the sunlight. The solution of α -carotene, however, had lost 12% of its original optical density.

When the optical densities were measured after one

2

had been exposed to the light from a fluorescent bulb overnight, it was found that it had fallen in both cases, but that the ratio of the absorption at 445 m μ to that at 600 m μ was unchanged.

When the concentration of carotenoid in the two suspensions was measured at the end of three hours on the second day, it was found to have fallen in both instances from 0.48 μ gm./ml. to 0.35 μ gm./ml., but there was no differential loss from the suspension which had been exposed to the light.

It would appear from these results that the carotenoids were well protected against light-induced degradation by their "in vivo" environment.

After exposure of the suspension of S₂ to sunlight in the presence of catalytic amounts of iodine for two hours, the absorption peaks in the visible region due to the carotenoid were still at 417, 445, and 475 m μ . In other words, there was no evidence of the formation of any isomers. From this, it appeared that the carotenoid were immutable while still bound in membrane fragments.

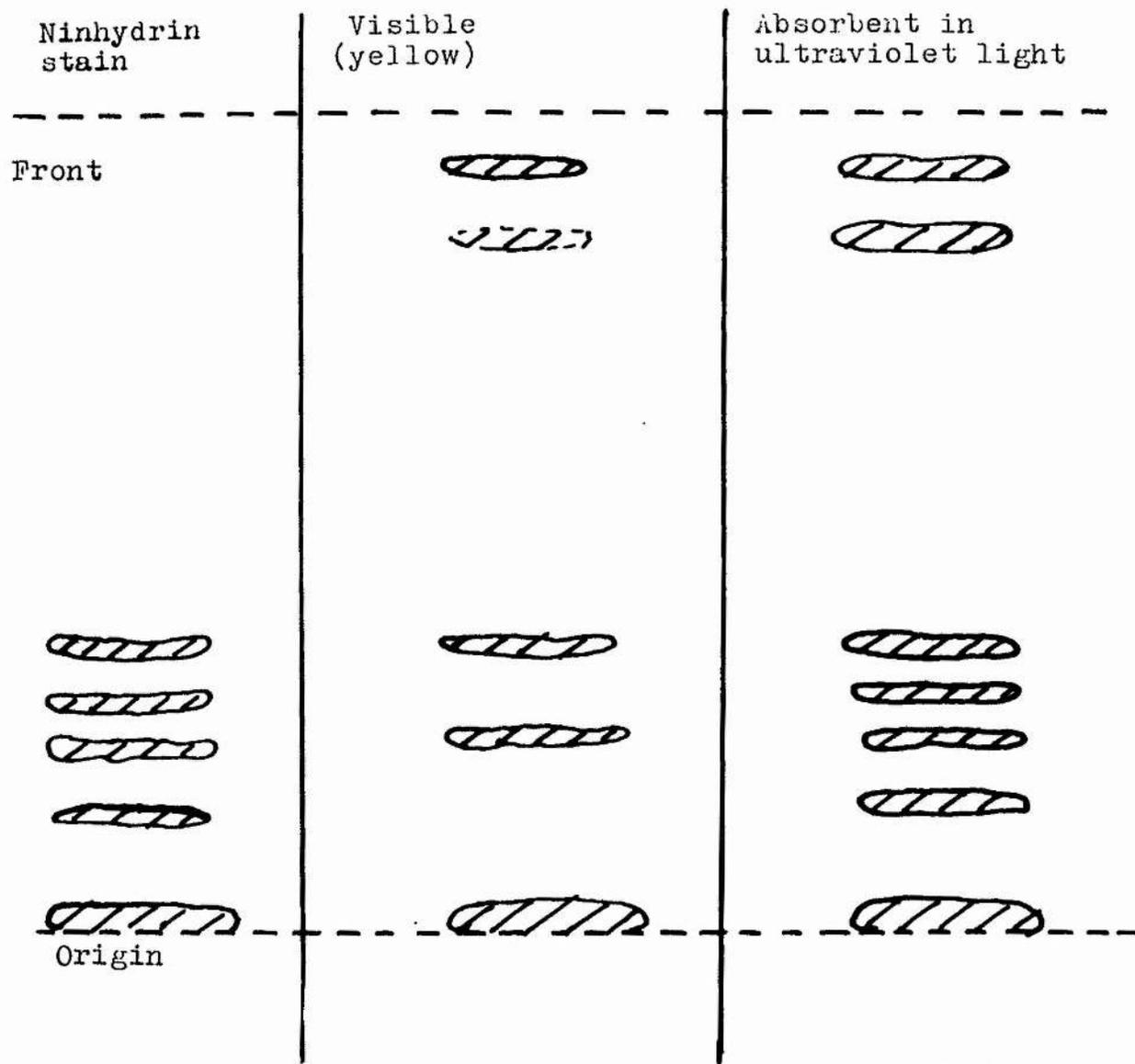


Fig. 30. T.L.C. on Silica Gel G of the water-soluble membrane fraction S2. Solvent: n-butanol/acetic acid/water; 70/12/25; (v/v/v).

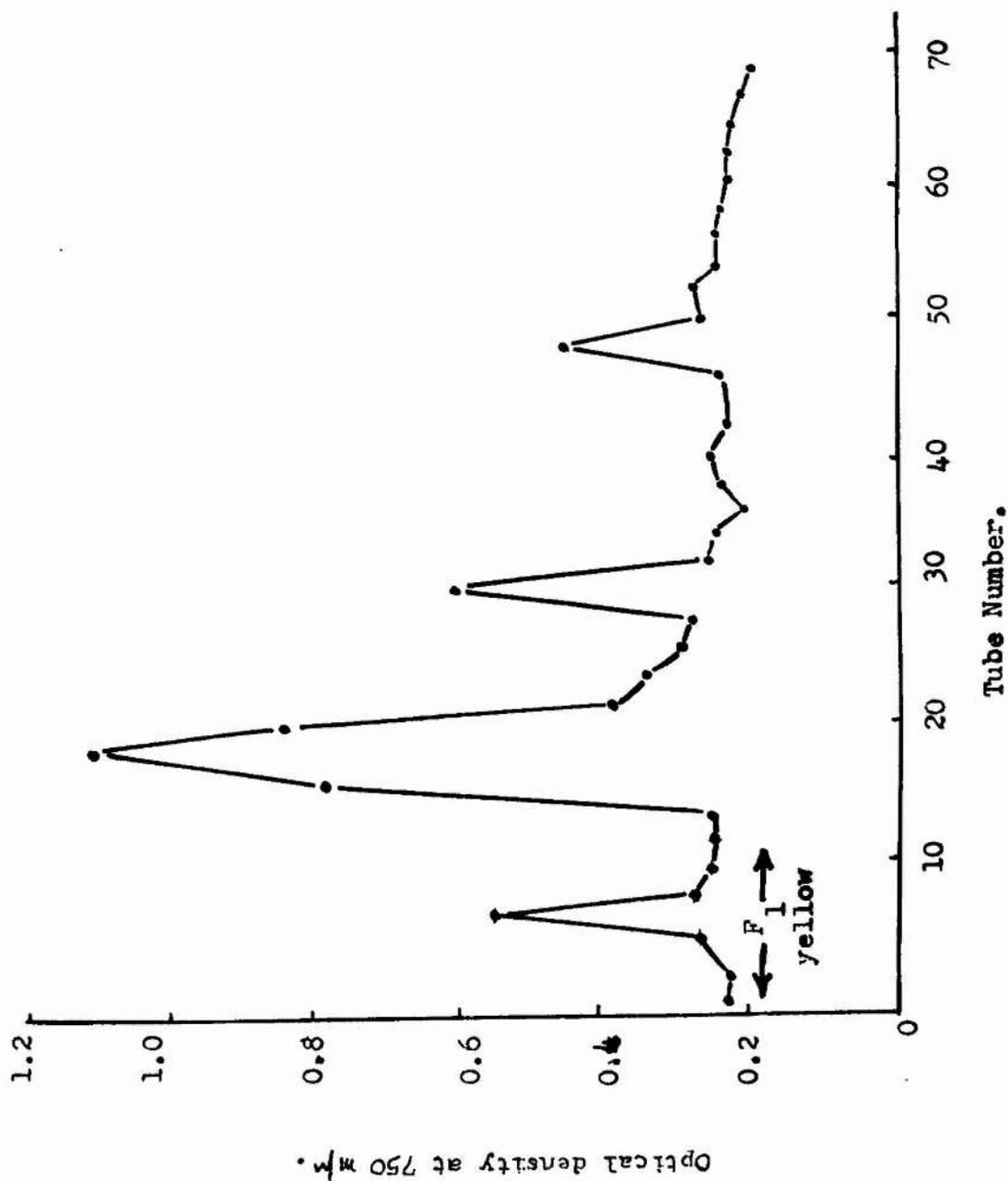


Fig. 31. Separation of water soluble membrane fragments (S2) on Sephadex G100, estimated by Folin-Lowry.

Thin-layer chromatography

The most useful solvent was found to be n-butanol/ acetic acid/water: 70/12/25 (v/v). This separated 5 visible yellow fractions. On staining with ninhydrin, only fractions 3, 5, 7 appeared to be associated with peptide material. Two other spots stained with ninhydrin but these were not associated with carotenoid. The R_f values are indicated in Fig. 30.

Gel filtration

1. Filtration of S_2 through a column of G 100 "Sephadex" gel yielded a coloured fraction, F_1 , and a number of other proteins which could be estimated only after staining with the Folin-Lowry reagent. The results are shown in Fig. 31. The coloured fraction, F_1 , represented about 10% of the protein estimated.
2. Filtration of S_2 on G 200 "Sephadex" gel yielded poorer results, indicating that a preliminary separation on G 100 gel was necessary.
3. F_1 filtered through G 200 gel was resolved into six fractions, the first of which was yellow, and represented about 20% of the total original fraction, F_1 , i.e. 2% of the protein estimated.

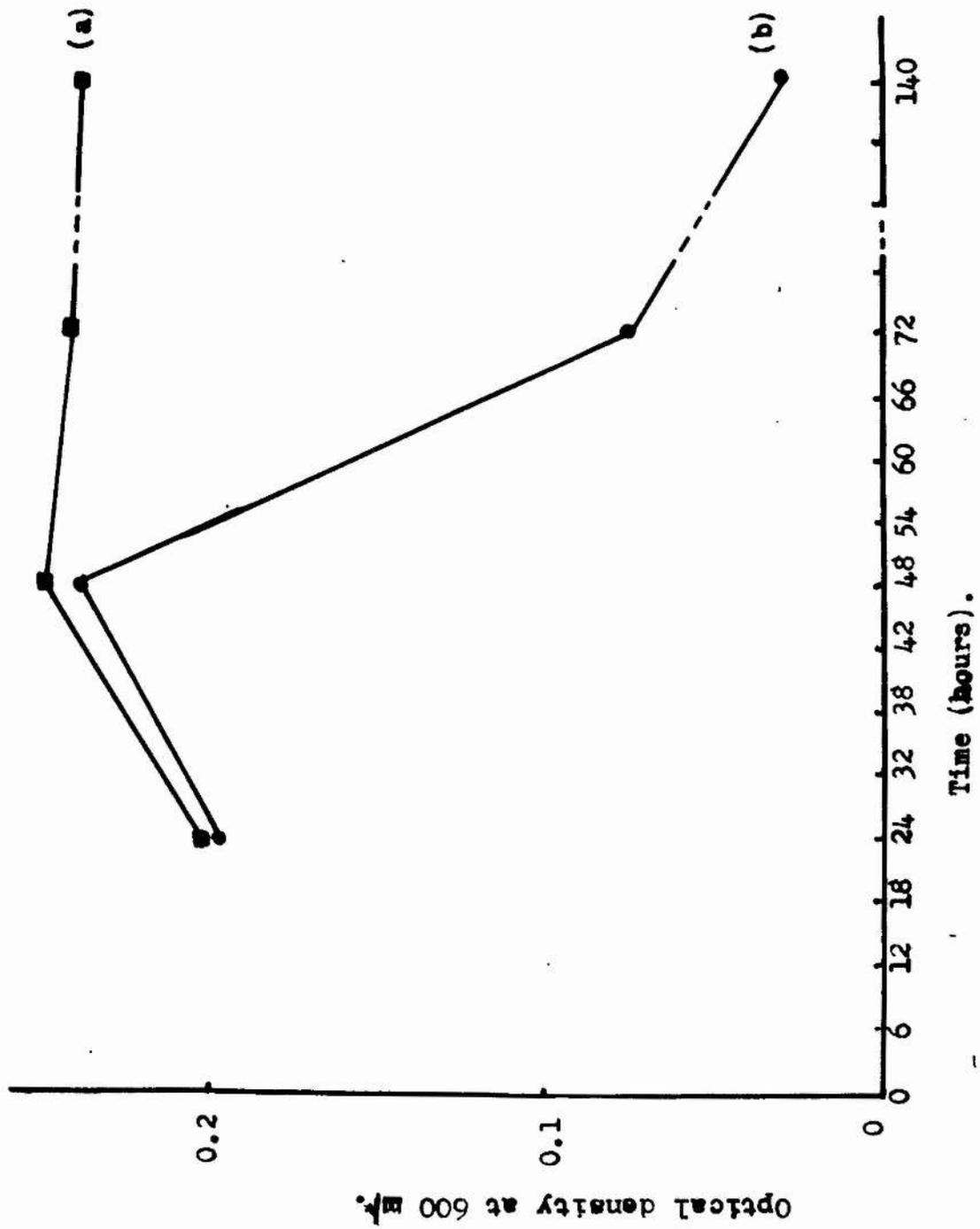


Fig. 32. The growth and autolysis of broth cultures of *S. flava* grown (a) in the light, (b) in the dark.

Electrophoresis in free buffer film

Of the different buffers which were tried, the best separation was achieved with phosphate buffer, pH 7.0. The protein in each collected fraction was estimated by the Folin-Lowry method. Only one yellow fraction was separated, but this was impure. The degree of purification achieved by this method was obviously not as good as that which could be achieved on Sephadex G 200. The most probable reason for this is that the conditions as used were not ideal for the system being separated.

Effects of light on a culture of *S. flava*.

The graph of the estimated bacterial numbers is shown in Fig. 31. It can be seen that the numbers of bacteria in the light- and dark-grown cultures were identical up to 48 hours after inoculation. At this point a divergence can be observed. In the case of the dark-grown cultures, death and autolysis of the bacteria evidently outruns the rate of multiplication of the cells and the estimable number of bacteria rapidly falls, until at the end of five days, the broth is almost clear. In the case of the bacteria grown in the light, the optical density of the culture remains almost at the level of the

48-hour maximum throughout the term of the experiment.

The parallel estimate of the carotenoid content indicated that at the end of 48 hours, there was 20% more carotenoid present in the light-grown bacteria than in the dark-grown.

DISCUSSION

D I S C U S S I O N

The main findings on the carotenoids of the bacteria investigated can be summarised as follows:-

1. S. flava contains a number of carotenoids which appear to lie in an orderly biosynthetic sequence, the xanthophylls all possessing the same chromophore.
 2. The xanthophylls of the series contain 50 carbon atoms in their skeleton, in contrast to all the previously reported carotenoids, which contain a maximum of 40 carbon atoms.
 3. The same series of carotenoids is present in other pigmented bacteria, not all of which are classified as Sarcina, or are even cocci, but which are all Gram +ve.
-

It is generally agreed that the insertion of the oxygen functions comes as a final stage in the biosynthetic sequence, after the formation of the carotenes (Goodwin, 1963). It would be more logical to deal first with the carotenes, and then proceed to the xanthophylls, but it is probably more convenient to start off by examining what

has been found out about the most fully characterised carotenoid isolated, the xanthophyll Fraction 3.

As mentioned in the section on results, this fraction was compared with the carotenoid isolated by Jensen and Weeks (1966), from Flavobacterium dehydrogenans, and called by them, P439. In all respects tested, the two carotenoids proved identical, and the results obtained by these authors in their attempts to elucidate the structure of P439 may be used in a consideration of the structure of Fraction 3 from S. flava.

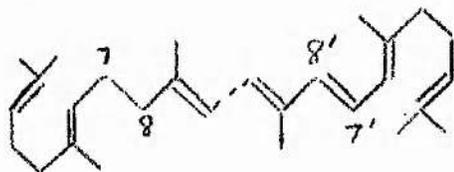
From its behaviour in adsorption chromatography the carotenoid was evidently polar, suggesting the presence of oxygen functions. Its partition ratio between light petroleum and 95% aqueous methanol was comparable to those quoted by Petracek and Zechmeister (1956), for dihydroxylated xanthophylls (c.f. 4,4'-dihydroxy- β -carotene; 22/78). Acetylation of the compound took place easily, and indicated (a) that the polar groups were primary or secondary hydroxyl groups, and (b) that there were two such groups present in the molecule.

The low polarity of the diester formed showed that there were no further oxygen functions present, and this

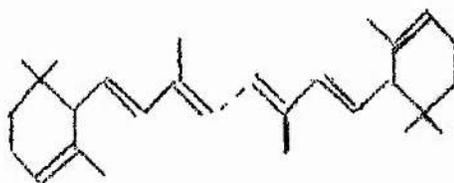
was confirmed by reduction of the ester with LiAlH_4 which resulted only in the formation of the original dihydroxyl compound.

The electronic spectrum of the xanthophyll suggests a chromophore of 9 conjugated double bonds. From analogy with other carotenoids whose structures have been determined, two chromophoric systems seem possible.

(a) A neurosporene type of chromophore with the 7,8-dihydro system.



(b) A chromophore which does not extend into the terminal ionone rings, as in the case of ϵ -carotene.



Previously authors investigating similar carotenoids in such bacteria as *M. lysodeiktu* (Rothblat et al., 1964) and a species of *Coryneform bacterium* (Hodgkiss et al., 1954), favoured the theory that the carotenoids contained

the neurosporene type of chromophore. (The relationship of these bacterial carotenoids to those of S. flava will be discussed at a later stage).

Evidence obtained from the oxidation of the hydroxyl groups, however, points to the latter chromophore in the case of the Fraction 3 from S. flava. Under the influence of nickel peroxide, one mono- and one di- α, β -unsaturated aldehyde were formed, the electronic spectrum of the carotenoid remaining unchanged. This reaction indicated that:-

- (a) the hydroxyl groups are allylic.
- (b) they are detached from the chromophore.

Jensen and Weeks (1966) carried out the same reaction on P439, and were able to transform the aldehyde groups formed into acids via their oximes and nitriles, proving that the hydroxy groups are primary in character.

From N.M.R. studies, these two authors considered that P439 contained two α -ionone rings, a suggestion which agrees well with the presence of two primary allylic hydroxyl groups detached from the chromophore. On this basis, it was considered that, if the precursor of these C_{50} xanthophylls was a C_{40} carotene, the other 10 carbon atoms being added at a later stage, the most likely

candidate for such a precursor was ϵ -carotene. This carotene was reported present in some strains of tomatoes by Trombly and Porter (1953). Consequently, an attempt was made to isolate this carotene from a number of types of tomatoes, without success, although a number of other identifiable carotenes, useful as standards, were obtained. Without a standard of ϵ -carotene, however, it was impossible to say, from the small quantities of carotene obtained from S. flava, whether or not this carotene was present in the bacterium. From the fact that neither of the normal carotene precursors, phytoene and phytofluene, could be located in the bacterial extracts, and that none of the coloured sub-fractions of Fraction 1 could be found to co-chromatograph with any of the standard carotenes, it would appear that a new biosynthetic sequence is involved.

A non-polar fraction with the same electronic spectrum as the xanthophylls was isolated in very small quantities from Fraction 1. This could be the parent carotene of the series, and from its relatively high polarity, might also be a C_{50} carotenoid.

Although the detailed structure of Fraction 3 remains unknown, on the grounds of mass spectrometric

analysis, it would seem that the extra 10 carbon atoms are not included in the skeleton in the form of side chains (Chapman, (1967), personal communication).

The fraction from S. flava called Fraction 2 is a mono-hydroxylated C₅₀ xanthophyll. Logically, it must be the immediate precursor of Fraction 3.

Fraction 4 was the most interesting of the carotenoid fractions isolated from S. flava, and unfortunately it was the least well characterised. The fact that it constituted 61% of the total carotenoid content bore testimony to its importance.

The main fraction could be further resolved into 4 sub-fractions, which were evidently isomers of each other. The sub-fraction present in the greatest quantity and having a definitely all-trans form, was further investigated.

The polarity of the material was extremely high, with a partition ratio between light petroleum and 95% aqueous methanol lower than any recorded by Petracek and Zechmeister (1956); (c.f. capsorubin; two keto and two hydroxy groups; ratio: 1/99; crocetin; two carboxyl groups; ratio: 4/96). This indicated the possible presence of carboxyl groups, but analysis by neither

2

chemical nor spectrophotometric methods could detect this group. Esterification clearly showed that some polar grouping other than hydroxyl must be present. Acetylation took place easily, with the formation of a large number of intermediates, but the final product was still relatively polar.

There is evidence that the polarity of the fraction is due to the conjugation of a xanthophyll and a sugar moiety. The main evidence for this comes from Weeks (personal communication, 1967) who found that corynexanthin, identified with Fraction 4, is such a complex. (The relationship between the two xanthophylls, corynexanthin and Fraction 4, and the possible role of such a xanthophyll-sugar complex in the bacterial structure, will be discussed later in this section.). If Fraction 4 does consist of a complex between a xanthophyll and a carbohydrate it would explain the large number of intermediates formed during acetylation, and also the polarity of the final product.

The comparisons of carotenoids produced by different bacteria in this thesis are based mainly on spectrophotometric analysis and co-chromatography. The exception is that of S. lutea, the carotenoids of

which were eluted and examined individually. The evidence suggests that many of the bacteria examined produced the same carotenoids.

The comparison of the pigments of S. flava with those of S. lutea was made partly because the carotenoids of the latter organism had received more attention than those of S. flava, and partly because of a report (Sobin and Stahly, 1942) that the two bacteria produced at least one carotenoid in common. Their findings were as follows:-

- (a) that S. lutea contained two carotenoid alcohols;
- (b) that S. flava contained one carotenoid alcohol which was identical with one of those produced by S. lutea
- (c) that no carotenes could be located;
- (d) that no carotenoid esters were present in either of the two bacteria.

Earlier work with the carotenoids of S. lutea was that of Chargaff and Dieryck (1933), who reported the presence of two carotenoids in the bacterium, one of which they considered to be a hydrocarbon and called "sarcinene". Nakamura (1936) believed that S. lutea contained an esterified xanthophyll. Takeda and Ohta (1941) gave the name "sarcinaxanthin" to the hydroxylated xanthophyll

which they crystallised from an extract of S. lutea. They considered that it might be a monohydroxy compound.

The author's findings were that the two bacteria, S. flava and S. lutea produced the same carotenoids, and that under the same conditions of growth, the carotenoid production of both was identical. Despite this, the colonies of the two species have a slightly different colour when cultured on nutrient agar. This may be due to some difference in the actual colonies of the bacteria.

Thus, according to these findings, more carotenoids are present in S. lutea than has been previously reported but it is possible to account for this fact by

- (a) the increased efficiency of modern methods of chromatography;
- (b) the small proportion of some of the fractions, particularly the least polar;
- (c) the possibility that some of the previous workers were using mutants which were unable to produce some of the more polar carotenoids.

Two trivial names have been given to the carotenoids of S. lutea. A word should be said about the validity of these two names in the light of recent developments.

2

The first of these is "sarcinene", given by Chargaff and Dieryck in 1933 to that fraction of the bacterial carotenoid which was epiphasic when partitioned between petrol ether and 90% aqueous methanol, and which had the same electronic spectrum as the more polar carotenoid material. They considered that the material must be a pure hydrocarbon. Partition experiments on bacterial extracts reported here show that the epiphase in such a system contains considerable amounts of the more polar carotenoids. Thus, while it is likely that a parent carotene with nine conjugated double bonds exists as a precursor of the more polar carotenoids, it is unlikely that this was the material partially isolated by Chargaff and Dieryck.

The other trivial name associated with the carotenoids of S. lutea is "sarcinaxanthin". This name was first given by Takeda and Ohta (1941) to an (apparently) single carotenoid which they crystallised. There was no attempt to characterise the carotenoid, apart from giving its melting point and electronic spectrum, and suggesting that it might contain one hydroxyl group. The name was next used by Hodgkiss et al. to describe a specific xanthophyll from S. lutea

which has been found to be identical to Fraction 3 of S. flava. This carotenoid is, of course, dihydroxylated. Finally, in the paper by Jensen et al. (1967), recording the identical nature of P439 and Fraction 3, the latter xanthophyll is called "sarcinaxanthin". Thus, rather by default than intention, a rather unspecific name has come to refer to a quite specific carotenoid, which occurs in bacteria other than Sarcina.

The carotenoids of M. lysodeiktus were compared with those of S. flava after the report of Rothblat et al. (1964), had indicated that there might be some similarities between the two. These workers reached the following conclusions:

- (a) that seven fractions could be detected in the carotenoids of M. lysodeiktus, all possessing the same electronic spectrum, with peaks at 414, 437, 467 m μ in methanol;
- (b) no carotenes were detectable;
- (c) that all the fractions were all-trans isomers;
- (d) that none of the fractions were acidic in character;

Co-chromatography of the carotenoids of M. lysodeiktus and S. flava indicated that the two bacteria produced identical carotenoids. The findings were in quite close

agreement with those of Rothblat et al. (1964), but differed in some respects. Seven carotenoid fractions were detected in both bacteria, but some of these were found to be *cis*-isomers, and one of the seven was the non-polar carotene fraction which had an electronic spectrum with peaks at 425, 447, 472 m μ ., differing from that of the xanthophylls.

The other bacterium whose carotenoids were reported to be similar to those of S. flava was the species of Corynebacterium (A 1032). The pigments were investigated by Hodgkiss, Liston, Goodwin, and Jamikorn (1954). They reported that:

- (a) there were present three xanthophylls with the same chromophore, yielding peaks at 416, 439, 469 m μ in ethanol. These they identified with (in order of increasing polarity) neoxanthin, sarcinaxanthin, and a highly polar carotenoid which they called corynexanthin (This last was also present in S. lutea from which they isolated the sarcinaxanthin);
- (b) no carotenes were detectable;
- (c) no keto, carboxyl, or epoxy groups could be detected in the carotenoids;

(d) that the xanthophylls might be derivatives of neurosporene.

The investigations recorded here agree quite well with these findings except in one major respect. The least polar xanthophyll was reported by Hodgkiss et al. to be neoxanthin. This fraction co-chromatographed exactly with the mono-hydroxy C₅₀ carotenoid from S. flava. At the time when the study on the carotenoids of the Coryneform bacterium was carried out (1954), the structure of neoxanthin was unknown, but it was later found to be 3,3',5,(or 6)-trihydroxy-6,(or 5)-hydro, 5',6' epoxy-β-carotene. The reasons against the identification of neoxanthin with the least polar carotenoid from the Coryneform species are as follows:

- (a) the high polarity of neoxanthin;
- (b) no co-chromatographic evidence is offered by Hodgkiss et al.;
- (c) the absence of epoxy groups in the Coryneform carotenoids;
- (d) while epoxy groups are common among the carotenoids found in photosynthetic tissues, they are as yet completely unknown in non-photosynthetic bacteria (Jensen, 1965).

2

On the basis of these facts, it is reasonable to assume that the original identification was wrong, and that the Coryneform species A 1032 contains the same set of carotenoids as does S. flava.

The most polar of the xanthophyll fractions isolated by Hodgkiss et al., and named by them "corynexanthin", appears to be identical to Fraction 4 from S. flava, and to be also present in S. lutea and M. lysodeiktus. On the basis of precedence, the trivial name of "corynexanthin" could be given to the xanthophyll fraction in question from these bacteria.

The other two bacteria whose carotenoids were compared with those of S. flava were S. aurantiaca and M. radiodurans. M. radiodurans can be considered to be of the Sarcina type as it shows the characteristic tetra grouping of the cells. The electronic spectra of the total carotenoid extracts from both bacteria are quite different from that of S. flava. A solution of the unresolved carotenoids from S. aurantiaca shows peaks of absorption at 391, 411, 435 m μ in methanol (Holford, 1966), and a similar solution of the pigments of M. radiodurans has a single peak at 475 m μ , suggesting the presence of conjugated keto group(s) (Hart, 1966).

Co-chromatography of these carotenoids with those of S. flava confirms that the bacteria have little in common as regards their carotenoid production.

Two points arise from these results:

- (a) bacteria which are classified together, e.g. Sarcina, do not necessarily produce the same carotenoids, underlining the weakness of pigmentation as a criterion of classification;
- (b) bacteria with quite different biochemical characteristics in other respects, may produce identical carotenoids. Thus evidence has been obtained that bacteria from such groups as Sarcina, Micrococcus, Corynebacterium, and Flavobacterium produce certain C₅₀ carotenoids in common.

Two further observations, one incidental, the other possibly less so, were made as a result of the co-chromatography experiments.

There occurs in tomatoes a carotenoid with exactly the same electronic spectrum and chromatographic characteristics as Fraction 3 (sarcinaxanthin) from S. flava. This finding was purely fortuitous, and as it did not bear directly upon the subject of the

thesis, it was not pursued. It would be impossible to state categorically, on the basis of electronic spectroscopy and chromatography alone, that sarcinaxanthin and the carotenoid from tomatoes were one and the same. Nevertheless, the fact that it gives rise to a single cis-isomer, with the same R_f value as the cis-isomer of sarcinaxanthin, adds weight to the supposition that the two are identical. If this were the case, and a C_{50} carotenoid were present in tomatoes, it would indicate that either two biosynthetic pathways existed, one synthesising C_{40} carotenoids, the other C_{50} compounds or that the C_{50} carotenoid did in fact have C_{40} carotenoids as precursors.

The other point is that all the bacteria whose carotenoids were co-chromatographed with those of S. flava, possessed at least one highly polar fraction with equivalent chromatographic properties to the largest component of Fraction 4 from S. flava. The polarity of these carotenoid fractions makes it seem unlikely that they are free xanthophylls. If they too are complexed to unknown carbohydrates, as appears to be the case in S. flava, then the widespread occurrence

of such carotenoid complexes in carotenogenic bacteria favours the suggestion that such complexes play some role in maintaining the structure of the membranes.

This brings us to the final section of the work reported here, the protoplast membranes of S. flava, and the carotenoids within these membranes.

The membranes of S. flava isolated by the author constituted on average 34% of the total dry weight of the bacteria. This is a much higher proportion than reported by Gilby et al. (1958), for M. lysodeiktus (9%), and by Weibull (1953) for B. megaretium (10%). It is uncertain whether this discrepancy was due to species differences or to different techniques of preparation of the membranes. The composition of the membranes was in close agreement with those of M. lysodeiktus analysed by Gilby et al. (1958). Although time did not permit the analysis of the S. flava membranes with reference to carbohydrate content, the reported proportion of 20% by weight for M. lysodeiktus indicates that a considerable amount of carbohydrate is present, some of which could be complexed to carotenoids.

Careful quantitative estimations proved conclusively that the entire bacterial carotenoid content was located

in the protoplast membranes. It was impossible to go deeply into the binding of the carotenoids in the membranes, but it was established that the binding was a firm one. Attempts to release the carotenoids by the disruption of the protein of the membranes, by boiling and by the chemical breaking of specific bonds, failed. The membrane-bound carotenoids were also resistant to the action of light and iodine. Fraction 4, for which there exists indirect evidence that the carotenoid is complexed to a carbohydrate, is not released when the soluble membrane fraction is precipitated with acetone, giving further support to the theory that the carotenoids may play some part in maintaining the structure of the membrane. It is significant that ether extraction of the aqueous acetone solution after precipitation extracts approximately only 39% of the total carotenoid content of the solubilised membrane material, leaving 61% of the carotenoid still bound in the precipitated material. This 61% is the exact proportion of the total carotenoid made up by Fraction 4. Chromatographic analysis of the saponified ether extract revealed that Fraction 4 was entirely absent, and the main detectable carotenoid was Fraction 3.

(Fractions 1 and 2 were present in such small concentrations as to be undetectable in the quantities employed).

From the results presented here, there is no evidence for the existence of a specific homogeneous protein/carotenoid particle in S. flava, such as was reported by Saperstein and Starr (1955) from C. poinsettia, although solubilisation of the protoplast membranes did release particulate material sedimenting at high gravitational forces, which contained both protein and carotenoid in intimate association. There were, however, other lipids present in the particles, and presumably also carbohydrate.

Further work would be required to elucidate the structural function of carotenoids within the protoplast membrane, if indeed they do have such a function. If this could be proved, however, it would shed new light on the functions of the carotenoids in non-photosynthetic bacteria. It has been proved from both photosynthetic bacteria (Griffiths et al., 1955) and carotenogenic non-photosynthetic organisms (Kunisawa and Stanier, 1958), that carotenoids protect the cells against photodynamic destruction. It is thought that the seat of the photodynamic action is the protoplast membrane. It is here

that the potential photosensitisers such as cytochromes and flavins are located (Mathews and Sistrom, 1960). The suggestions as to the mechanism of the protective action of the carotenoids include one that the carotenoids absorb harmful radiations preferentially but this explanation is not adequate to account for the protective influence of the carotenoids in the presence of photosensitisers such as chlorophyll or toluidine blue which do not absorb at the same wavelengths as the carotenoids. Mathews and Sistrom (1960) considered that the basis of the photodynamic destruction of normally carotenogenic bacteria in which the formation of carotene had been chemically inhibited, was the disruption of the membrane, and they found some evidence for this in the inactivation of some membrane-located enzymes. The evidence suggesting that some part of the carotenoid content may perform some structural function in the membranes has already been considered, but one final experiment augmented the argument. It was found that the effect of light on a growing culture of S. flava was to increase the amount of carotenoid present, and to prevent the eventual autolysis of the bacteria. This experiment in itself is too fragile

a piece of evidence to suggest that the greater content of carotenoid and the apparently increased strength of the cellular membrane are linked; but it might provide a useful basis for further investigation.

To sum up, the conclusions, both proven and proposed reached on the biosynthetic sequence of the carotenoids of *S. flava*, and their incorporation into the protoplast membranes, can be represented diagrammatically:-

	Carotenes	-	may be C ₄₀ with the subsequent addition of 10 carbon atoms, or may be C ₅₀ . The evidence favours the latter conclusion.
Methanol extractable material	Mono-hydroxylated C ₅₀ xanthophyll		- Fraction 2
	Di-hydroxylated C ₅₀ xanthophyll		- Fraction 3 (sarcinaxanthin)
	Possible complex with carbohydrate		- Fraction 4
<hr/>			
Protoplast membrane	Water soluble complex containing protein and lipids other than carotenoids.		
	Integral part of the protoplast membrane structure.		

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S U M M A R Y

1. Studies on the carotenoid pigments of S. flava indicated the presence of four main fractions, the amount of each being in direct proportion to its polarity. Little could be discovered about the least polar fraction, containing carotenes, and probably the colourless precursors, except that none of the normal carotene precursors appeared to be present. The three xanthophylls were found to be C₅₀ carotenoids with a chromophore of nine conjugated double bonds. All contained primary hydroxyl groups.
2. Comparison by spectroscopy and chromatography of the carotenoids of S. flava with those of some other bacteria, indicated that the occurrence of these C₅₀ carotenoids might be quite widespread. S. lutea was found not only to produce the same carotenoids, but also to have the same level of pigmentation as S. flava, when the bacteria were grown under the same conditions.
3. Investigations into the protoplast membrane of S. flava proved conclusively that all the carotenoid was contained within the membrane. A water-soluble

fraction was obtained from the membrane, and studies showed that it was a lipo-protein complex, in which the bulk of the carotenoid was tightly bound.