SC

STUDIES ON LIPID METABOLISM IN THE EXTREMELY HALOPHILIC BACTERIUM HALOBACTERIUM CUTIRUBRUM

by

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Candidate ~~Supervisor~~ Supervisor Supervisor

TO MY PARENTS

who provided the genes

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ABSTRACT

Cells of the obligate halophile Halobacterium cutirubrum were grown to the stationary phase in the presence of ^{32}P-orthophosphate, ^{35}S-sulfate, ^{14}C-labelled acetate, malonate, mevalonate and glycerol and in the presence of 1,(3)-^{3}H- and 1, 3-^{14}C -glycerol mixture and 2-^{3}H- and 1, 3-^{14}C-glycerol mixture as precursors. ^{32}P was incorporated into the phosphatide components in proportion to their relative concentrations, thus: phosphatidyl glycerol phosphate (diether analogue) > unidentified minor phosphatide > phosphatidyl glycerol (diether analogue). ^{32}S-sulfate was incorporated mostly into the glycolipid sulfate ester component, but small amounts of ^{35}S also appeared in a more polar sulfolipid and in a minor unidentified sulfolipid.

^{14}C-Mevalonate was the most efficient precursor for the phytanyl chains, followed in decreasing order by acetate and glycerol. Only traces of ^{14}C from the malonate precursor were incorporated, and these appeared entirely in the phytanyl groups. A considerable proportion of ^{14}C from the glycerol precursor was found in the glycerol and sugar moieties of the lipids. Little or no labelling of the water-soluble moieties of the lipids was observed with the other carbon precursors.

Only traces of ^{14}C-labelled fatty acids were detected with any of the ^{14}C precursors; with acetate in particular, only 0.3% of the ^{14}C in the total lipids was found in fatty acids. The synthesis of fatty acids was confirmed by the use of cell-free enzyme system and ^{14}C-malonyl-CoA as a precursor. Although 0.5 - 0.6% of the ^{14}C in the precursor was incorporated in fatty acids, it is believed that the fatty acid synthetase system is depressed by the high salt concentrations. It is concluded that the predominant biosynthetic route for the lipid chains is the mevalonate pathway for synthesis of isoprenoid groups, and that the malonyl-CoA pathway for synthesis of fatty acids is operating at a very low level of activity.

Studies on the metabolism of ^{14}C and ^{32}P-glycerol phosphate by whole cells and cell-free enzyme preparations showed that H. cutirubrum does not utilize α-GP as such, but it contains an active glycerol phosphatase which catalyzes the hydrolysis of α-GP into glycerol and P_i. The latter two components then pass into the cells where glycerol is phosphorylated by a glycerol kinase specifically to form sn-glycerol-3-phosphate.

sn-Glycerol-3-phosphate was formed not only by glycerol phosphorylation, but also by reduction of dihydroxyacetone phosphate by L, α-glycerol phosphate dehydrogenase.

Using tritiated glycerol, the incorporation of ^{3}H into lipids of H. cutirubrum cells varied with the position of the ^{3}H label in the precursors supplied. Total lipids and lipid moieties from cells grown in 2-^{3}H-glycerol lost almost completely the ^{3}H activity. In contrast, when cells were grown in 1(3)-^{3}H-glycerol, the phytanyl groups and the sugar residues of the lipids retained about 50% of the ^{3}H activity while the glycerol moiety of the diphytanyl glycerol ether retained about 100% of the ^{3}H activity. It is concluded that glycerol is metabolized via three separate pathways: (1) by the reverse of the Embden-Meyerhof cycle, to sugars; (2) by glycolysis, to acetate and then to isopentenyl pyrophosphate through the mevalonate pathway; (3) by a new pathway, in which glycerol is converted to diphytanyl glycerol ether by an as yet unknown series of reactions, probably involving dihydroxyacetone.

Direct studies on the biosynthesis of phosphatides of H. cutirubrum by short-term labelling of total lipids with $^{32}P_i$ was undertaken. During the first minute of labelling, an unidentified phospholipid was initiated which bears a precursor-product relationship between it and PGP-diether analogue. It is concluded that the unidentified phospholipid may be the immediate phosphorus-containing precursor of the major phosphatide PGP-diether analogue.

RESUME

Les cellules de l'Halobacterium cutirubrum nécessairement halophile ont été cultivées jusqu'à atteindre une phase stationnaire en présence de ^{32}P-orthophosphate, ^{35}S-sulfate, d'acétate, de malonate, de mevalonate et de glycérol marqués au ^{14}C et en présence d'un mélange de ^{3}H-1(3)- et ^{14}C-1(3)-glycérol et d'un mélange de ^{3}H-2- et ^{14}C-1(3)-glycerol comme précurseurs. Le ^{32}P a été incorporé dans les constituants phosphatides en proportion avec leurs concentrations relatives, ainsi: phosphatidyl glycérolphosphate (analogue diéther) un phosphatide mineur non identifié > phosphatidyl glycérol (analogue diéther). Le ^{32}S-sulfate a été incorporé pour la plus grande partie dans le constituant glycolipide sulfate, mais de petites quantités de ^{35}S sont également apparues dans un sulfolipide plus polaire et dans un sulfolipide mineur non identifié.

Le ^{14}C-mévalonate s'est révélé etre le précurseur le plus efficace pour les chaînes phytanyles, suivi par ordre décroissant par l'acétate et le glycérol. Seulement des traces de ^{14}C à partir du précurseur malonate ont été incorporées et celles-ci sont apparues entièrement dans les groupes phytanyles. Une proportion considérable de ^{14}C a partir du glycérol précurseur a été trouvée dans les fragments glycérol et sucres des lipides. Peu ou pas du tout de marquage des fragments hydrosolubles des lipides a été observé avec les autres précurseurs carbonés.

Ce sont seulement des traces d'acides gras marqués au ^{14}C qui ont été détectées, quels qu'aient été les précurseurs; en particulier avec l'acétate, seulement 0.3% du ^{14}C dans les lipides totaux a été trouvé dans les acides gras. La synthèse des acides gras a été confirmée par l'utilisation d'un système d'enzyme en dehors des cellules et de ^{14}C-malonyl-CoA comme précurseur. Bien que 0.5 - 0.6% du ^{14}C dans le précurseur ait été incorporé dans les acides gras, nous pensons que le systeme synthetase des acides gras est inhibé par de hautes concentrations en sels. On en conclut que le processus bio-synthétique prédominant pour les chaînes lipides est la voie mévalonate pour la synthèse des groupes isoprénoides et que la voie malonyl-CoA pour la synthèse des acides gras se produit à un très faible niveau d'activité.

Des études sur le métabolisme du ^{14}C et ^{32}P-glycérol-phosphate par les cellules intactes et par les préparations d'enzyme faites en dehors des cellules ont montré que H. cutirubrum n'utilise par l'α-GP en tant que tel mais contient une glycérolphosphatase active qui catalyse l'hydrolyse de l'α-GP en glycérol et en phosphate minéral. Ces deux derives constituants passent alors dans les cellules où le glycérol est phosphorylé par une glycérol kinase spécifiquement pour former du sn-glycérolphosphate-3.

Le sn-glycérolphosphate-3- a été formé non seulement par phosphorylation du glycérol mais aussi par réduction du phosphate de dihydroacétone par le L-α-glycerolphosphate déshydrogénase.

En utilisant du glycérol tritié, l'incorporation de ^{3}H dans les lipides des cellules de H. cutirubrum variaient avec la position du marquage en ^{3}H dans les précurseurs utilisés. Les fragments de lipides totaux et les lipides eux-mêmes obtenus à partir de cellules cultivées dans le ^{3}H-2 glycerol ont perdu presque complétement l'activité en ^{3}H. En revanche, quand les cellules ont été cultivées dans le ^{3}H-1(3)-glycerol, les groupes phytanyles et les résidus sucres des lipides ont conservé environ 50% de l'activité en ^{3}H alors que le fragment glycérol du diphytanyl glycérol éther a retenu 100% environ de l'activité en ^{3}H. On en conclut que le glycérol est métabolisé via trois processus différents: (1) par l'inverse d'un cycle d'Mebden-Meyerhof, en sucre; (2) par le cycle de glycolyse en acétate puis en pyrophosphate d'isopentényle en suivant la voie mévalonate; (3) par une nouvelle voie, dans laquelle le glycérol est transformé en diphytanyl glycerol éther par une série encore inconnue de réactions, probablement en faisant intervenir la dihydroxyacétone.

Des études directes sur la biosynthèse des phosphatides de H. cutirubrum par des marquages rapides des lipides totaux avec du ^{32}P-phosphate minéral ont été entreprises. Pendant les premières minutes de marquage, un phospholipide non identifié s'est formé ce qui est en faveur d'un rapport-précurseur-produit entre celui-ci et l'analogue PGP-diéther. On en conclut que le phospholipide non identifié pourrait être le précurseur immédiat contenant du phosphore de l'analogue phosphatide PGP-diéther majeur.

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(A) INTRODUCTION

I. Historical Background

Since the dawn of history, man has recognized the necessity of salt for life. Since one of the main sources of salt is sea water, it is not surprising that the technology for preparing sea salt has a long history.

The art of extracting salt from sea water was first described in one of the oldest Chinese treatizes, the "Peng Tzao-Kau-Mu", translated as the "Man-Plant-Classification" and which was dated about 2700 B.C. (Baas-Becking, 1931). The Chinese observed that when the sea water was left for a long time, its color became pinkish-red. The reddening of the concentrated sea water and boiled brines, and the production of deep red-colored salt niterbeds has also long been noticed and described by the ancient Egyptians. However, the relation between the concentrated salt solution, the red coloration and the growth of some microorganisms was not recognized until 1771 by Monro and then later by Joly (1840), Grube (1840) and Marren and Marren (1841). The occurrence of microorganisms in highly concentrated solutions was unexpected, since salt has long been used (for instance by the Chinese) as a preservative against microbial deterioration of food and other protein products. Nevertheless, it was not until the work of Klebahn (1919) and Harrison and Kennedy (1922) that it was realized that among the red-colored microorganisms

(algae and fungi) living in this very salty environment is a highly specialized kind of bacteria. These bacteria live and proliferate in concentrated brines and require almost saturated sodium chloride solutions for optimum maintenance and growth. Accordingly, they are classified as "extreme or obligate halophiles" to distinguish them from moderately halophilic organisms which require much lower salt concentrations.

In the past fifty years, bacteriologists have given fairly detailed descriptions of the taxonomy of the halophilic organisms and have also studied their salt requirements [Baas-Becking (1928); Benecke (1933); Hof (1935); Smith and ZoBell (1937 and 1946); ZoBell et al (1937) and Volcani (1940)]. However, studies on the biochemical basis for the absolute salt requirements of these halophiles were largely neglected until the early 1950's when Gibbons' group (1952) in Canada first described the biochemical peculiarities of the extreme halophiles. Since then, a great deal of new and interesting information has been reported on the unique biochemical and biophysical characteristics of extreme halophiles [see reviews by Flannery (1956), Ingram (1957) and Larsen (1962 and 1967)]. These unusual characteristics will now be discussed in some detail.

II. Literature Review on Extremely Halophilic Bacteria

1. Culture Conditions

Organisms which grow best in the presence of 20% sodium chloride or more have been assigned to one of two types of extremely halophilic bacteria. The halococci were assigned to the family Micrococcaceae and were collected in part under the genus Micrococcus and in part under the genus Sarcina. The rod-formed or irregularly-shaped halobacteria have been collected under the genus Halobacterium and assigned to the family Pseudomonadaceae (Bergey's Manual, 7th ed., 1957).

Cells of the red-pigmented, rod-formed, gram negative halophile, Halobacterium cutirubrum are non-spore formers and lophotrichlously flagellated when motile. They grow optimally in media containing 25% (w/v) sodium chloride and lyse when exposed to lower salt concentrations. A minimum of 12 - 20% NaCl is required for growth to occur, a figure differing considerably from strain to strain (Kurochkin, 1962). Proteins (such as peptone, trypton or yeast autolysate) and amino acids seem to be the most readily utilized carbon sources.

In attempts to design a chemically defined medium, Dundas et al (1963) described one which contained, besides inorganic salts and cytidylic acid, ten amino acids. The amino acids valine, methionine, leucine and isoleucine were essential for growth, while the others stimulated growth. However, they observed that in the chemically defined medium cell multiplication was not rapid and the lag phase was longer than in a complex medium containing a protein digest.

Onishi et al (1965) designed a synthetic medium in which H. cutirubrum and other halophiles grew just as well as on a complex medium. This medium contains fifteen amino acids, two nucleotides (adenylic and uridylic acids) and glycerol, besides the inorganic salts. The amino acids arginine, leucine, lysine and valine proved essential; each of the others was stimulatory when in a mixture. Although the addition of ammonium chloride at an optimum concentration of 0.2 - 0.5% led to a considerable decrease in the duration of the lag period for growth of H. cutirubrum (Onishi et al, 1965), later experiments by Onishi and Gibbons (1965) showed that the stimulatory effect is due to the utilization of amino acids and the requirement for ammonium ions is not specific.

Onishi et al (1965) examined the growth of H. cutirubrum and Sarcina littoralis on a chemically defined medium supplemented with, among other growth factors, different water soluble lipid precursors (such as α-glycerophosphate, glycerol, dihydroxyacetone phosphate, etc.). They showed that adding as little as 0.01% glycerol or 0.1% dihydroxyacetone led to as good growth as in the complex medium of Sehgal and Gibbons (1960).

Gochnauer and Kushner (1969) showed that the growth of H. cutirubrum, H. halobium and H. salinarium was stimulated by the addition of galactose, glucose, Na-lactate or glutamate to the synthetic medium of Onishi et al (1965). This stimulation was most marked when the medium was supplemented with extra K^+. Although these carbohydrates stimulate growth, the medium does not become acidic; this may explain why standard bacteriological tests indicate that many extreme halophiles do not "utilize" carbohydrates.

Concerning the inorganic constituents of the culture media, it should be emphasized that the extreme halophiles have a specific requirement for sodium chloride for growth. Numerous experiments have been reported in which no growth was obtained when sodium chloride was replaced with other salts or compounds in the culture medium; for a review see Larsen (1962). Sodium ions can, however, be partially replaced by potassium ions (Brown and Gibbons, 1955; Christian, 1956) and to a very small extent by magnesium ions (Weber, 1949).

Brown and Gibbons (1955) reported that the extreme halophiles require unusually high concentrations of magnesium ions (0.1 - 0.5 M) in the culture medium for good growth. Growing in a medium low in Mg^{++}, the normally rod-shaped halobacteria take on a coccoid form which is maintained even after transfer back to a medium containing a high concentration of Mg^{++}. The same authors reported that the requirement of the extreme halophiles for K^{+} is only slightly higher than that reported for other non-halophiles (about 2 mM). This is particularly interesting in view of the fact that the intracellular K^{+} concentration of extreme halophiles is extremely high (about 4 M). This implies that these cells can concentrate K^{+} very effectively.

In 1960, Sehgal and Gibbons reported good growth of various halophiles with 10 p.p.m. Fe^{++} and 0.05 p.p.m. Mn^{++}. Optimum concentrations of other inorganic components which are normally present in the complex culture media have not been reported.

The extreme halophiles multiply relatively slowly even under the most favorable conditions designed. The shortest generation time obtained for the halobacteria is not much less than 7 hours, and for the halococci about 15 hours (Larsen, 1967). Holmes et al (1965) have reported that a strain of H. salinarium yielded 8 - 10 g (wet weight) of cells per liter in a yeast extract-trypticase culture medium, provided they used about a 25% inoculum. H. cutirubrum was found to yield 10 - 12 g (wet weight) of cells per liter in a yeast extract-casaminoacids culture medium using about 4% inoculum containing about 120 mg cells (dry weight).

2. Intracellular Salt Content and Composition

The fact that the extreme halophiles require a very high concentration of NaCl in their growth medium raised questions concerning the intracellular salt content of these organisms.

Experiments based on chloride determination in packed cells (Gibbons and Baxter, 1953; Holmes, 1964) or on the basis of cryoscopic measurements on heated cell pastes (Christian, 1956; Christian and Ingram, 1959) have provided convincing evidence that the total intracellular salt concentration of the extreme halophiles is indeed very high and reaches values comparable to that in the growth medium.

Although K^+ is a minor component of the medium in which the halophilic cells are normally cultured (0.3 M), it is the major component of the internal salt (4.0 M) (Brown and Gibbons, 1955; Christian, 1956 and Christian and Waltho, 1962). Internal Na^+

concentration (1.0 M) was found to be much less than the external concentration (4.0 M) while the Cl^- concentration appeared to be somewhat lower internally than extracellularly (Christian and Waltho, 1962).

The very high K^+ content of the extreme halophiles might bear a relationship to the finding by Christian and Waltho (1961). Testing a number of different non-halophilic bacteria, they established a relation between the K^+ content of the cells and their tolerance towards sodium chloride. The higher the salt tolerance the higher was the internal K^+ content when the cells were grown in ordinary media. A high internal K^+ content thus seems to be a general characteristic of bacteria possessing the ability to live in NaCl brines, regardless of whether the organisms normally live in the brine or just possess a potential ability to do so.

3. Effect of Salt on the Uptake of Metabolites

Stevenson (1966) studied the mechanism of metabolite uptake by the extremely halophilic bacterium H. salinarium. He found that glutamate uptake is specifically dependent upon a high concentration of NaCl. Replacing the Na^+ by equimolar concentrations of K^+, or the Cl^- by equimolar concentrations of acetate, gave a negligible uptake of the amino acid by the cells.

Some salts have been found to fill the same functions when they replace NaCl. Thus, KCl activates all the individual enzymes [Baxter and Gibbons, 1954, 1957); KCl and Na acetate interact weakly with proteins, protect the halobacteria from structural

transformations and lysis when replacing NaCl in iso-osmolar concentrations. KCl or any other salt cannot, however, replace NaCl for growth of these organisms, which suggests some specific function for NaCl in the cells. NaCl being the only salt that stimulates the mechanism for the uptake of glutamate by H. salinarium (Stevenson, 1966) points to a specific function of NaCl in the halophiles possibly connected with the process of oxidative phosphorylation, or some other transport (uptake) processes.

4. Nucleic Acids - Content and Composition

The data available on the nucleic acid content of the extreme halophiles are quite limited. Smithies et al (1955) analyzed a strain of H. salinarium and found, on a salt-free dry weight basis, 15% total nucleic acids of which 4.3% was DNA. These values are comparable to those commonly found for other bacteria.

In experiments on the nucleic acid content of halophilic bacteria, Smithies and Gibbons (1955) noticed that the cells of many halophilic bacteria were sticky when handled. They suggested that, due to extreme fragility of the cells, there might be a leak of DNA, during mechanical stress or under unfavorable nutritional conditions; and that DNA adhered to the cells, causing its stickiness.

Ormerod (cited in Larsen, 1967) chemically determined the base composition of bulk RNA and DNA of H. salinarium. He found that the base composition of RNA is as commonly found in bacteria:

G, 33%; A, 24%; C, 22%; U, 20%; PU/PY, 1.4%; $\frac{A+T}{G+C}$, 0.16%.

The composition of DNA is as commonly found in members of the genus Pseudomonadaceae (Belozersky and Spirin, 1960) to which the halobacteria bear much resemblance in many other respects:

G, 31%; A, 17%; C, 34%; T, 17%; PU/PY, 0.94%; $\frac{A+T}{G+C}$, 0.53%.

The same results were found by Baxter (1961, 1964) and Joseph et al (1963).

Baxter (1961, 1964) investigated further the nucleic acids of H. salinarium strain I. He chromatographed cell-free extracts on two different cellulose ion exchange materials (DEAE- and ECTEOLA-cellulose) using an eluent with increasing NaCl concentration. The elution patterns obtained for DNA and TNA were identical to those for a non-halophilic pseudomonad. Melting curves for DNA preparations gave T_m values which, according to the relationship between G + C concent and T_m found by Marmur and Doty (1959), fitted well with the data obtained by Ormerod (cited in Larsen, 1967). Baxter concluded that, on the basis of the criteria tested, there is no reason to believe that the nucleic acids of the halobacteria differ in their fine structure from those of non-halophilic organisms.

Joshi et al (1963) investigated the DNA of H. salinarium strain I. Centrifugation in a CsCl density gradient gave one major and one minor band at positions corresponding to G+C contents of 67% and 58%, respectively. The major, higher density band did not represent a denatured form of the lower density band. The minor, lower density band was estimated to contain about 20% of the total DNA. Thus, the two components pooled should display a G+C content of about 65% if the position of each band truly reflected their G+C content (Sueoka et al, 1959 and Rolfe and Meselson, 1959). A value of about 65% G+C has actually been found for the bulk DNA by Ormerod (cited in Larsen, 1967). Further, the melting curves of the bulk DNA determined by Joshi et al (1963), suggested the presence of two components. A strain of H. cutirubrum gave similar results, but the satellite DNA represented only about 10% of the total.

DNA satellite bands are rare in bacteria and may be due to foreign episomal elements (Sueoka, 1964). Joshi et al (1963) emphasize that this possibility cannot be ruled out for the halobacteria tested by them. The amount of satellite (10 - 20% of the total DNA) was, however, surprisingly large. At present, the significance of the findings of Joshi et al await further elucidation.

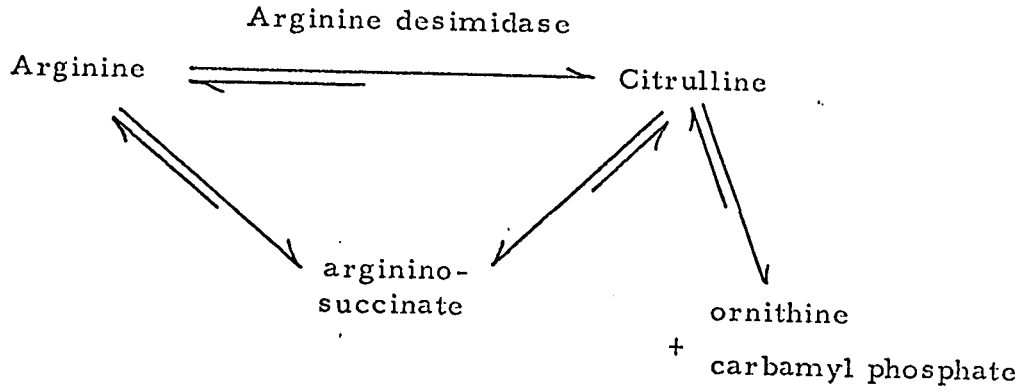
Bayley and Kushner (1964) reported that about 50% of the total RNA of H. cutirubrum was ribosomal, about 30% soluble and about 15% present in a fraction that contained mainly cell-envelope fragments. However, detailed work on the RNA's of the extreme halophiles has not yet been carried out.

5. Amino Acid Metabolism

Very little work has been done on the general metabolic patterns in the extreme halophiles. The high salt requirements of the halophiles for growth and survival suggests that basic biochemical differences might exist between their metabolic pathways and those of non-halophilic organisms. However, on the basis of present knowledge of the nutritional characteristics and the chemical composition of the halophilic cells, their general metabolic patterns would appear to be essentially the same as those in non-halophiles.

The extreme halophiles occur and grow best on protein digest. They seem to metabolize amino acids readily, as shown from experiments on chemically defined medium, whereas carbohydrates are generally not attacked. Gibbons (1957) reported proteolytic activities in several strains of halophilic bacteria, suggesting that metabolizing amino acids is a common property of the halophiles.

Dundas (1965) and Dundas and Halvorson (1966) reported the only detailed studies on the amino acid metabolism in extreme halophiles. Using a strain of H. salinarium they showed the following reactions take place:



Arginine was shown to be essential for growth, but Dundas and Halvorson (1966) found that citrulline could replace arginine and they concluded that citrulline seemed to be converted to arginine via the arginino-succinate route.

These metabolic conversions are quite similar to those found in most non-halophilic bacteria capable of degrading arginine, but the unusual character seems to lie in the halophilic nature of the enzymes.

6. Salt Effects on Ribosomes, and Protein Synthesis

The ribosomes of the extreme halophile, Halobacterium cutirubrum have been shown to be similar to the ribosomes of E. coli with respect to the size of the particles and the gross chemical composition, i.e. 70S particles consist of 60% RNA and 40% protein (Bayley and Kushner, 1964). However, H. cutirubrum ribosomes, in contrast to other ribosomes, are only stable in solutions containing

about 4 M KCl and 0.1 - 0.4 M Mg^{++}. At lower K^+ and Mg^{++} concentrations, H. cutirubrum ribosomes dissociate into 52 S and 31S subunits and these may be further degraded by further lowering of K^+ and Mg^{++} concentrations to give 42 S, 33 S and 22 S particles and acidic, soluble proteins (Bayley, 1966a). The latter three types of particles contain 80% RNA which represents the bulk of the RNA in the native 70 S ribosomes. The dissolved protein constitutes about 75% of the ribosomes and displays acidic properties as shown by electrophoresis.

The requirement for KCl to maintain the 70 S particles is quite specific. When KCl was replaced by iso-osmolar NaCl, dissociation to 52 S particles and also complex aggregation phenomena occurred. Replacing KCl by other chlorides of the alkaline series resulted in an extensive degradation of the 70 S particles. A requirement for Mg^{++} to maintain integrity is a well-known property of ribosomes from other organisms. However, ribosomes from H. cutirubrum require 10 - 100 times the Mg^{++} concentration required by E. coli for stability (Bayley, 1966a).

Amino acid analysis and gel electrophoresis showed the acidic nature of the protein released by ribosomes dissociation in low salt concentration. The protein remaining in the particle fraction is relatively basic. Analysis of K^+ and Mg^{++} showed that potassium ions were easily removed from the particles by washings with dilute salt solutions; a certain amount of Mg^{++} was more firmly bound to the particles (Bayley, 1966a). Bayley (1966b) also suggested that a function of the Mg^{++} is to stabilize the configuration

of the ribosomal RNA rather than to take part in the binding of the acidic protein to RNA. This suggestion is in accordance with that for the function of Mg^{++} in ribosomes of other organisms. Since the ribosomes of the halophilic bacteria are distinguished by having a high content of acidic protein, Bayley (1966b) pointed out that the extremely high intracellular concentration of K^{+} may neutralize the negative charges on the acidic protein and RNA, thereby enabling the latter to aggregate and form stable and functionally active ribosome structure.

In spite of the unusual ionic environment in which the cell-free system of extremely halophilic bacteria operated, Bayley and Griffiths (1968a and b) were able to demonstrate that the incorporation of amino acids by a ribosomal preparation from H. cutirubrum cells represented true peptide synthesis and not non-specific absorption. The same authors showed that ribosomes were essential for the incorporation of labelled amino acids at the C-terminal ends of the growing polypeptide chains in the protein synthesizing system of H. cutirubrum. Also, activation and transfer of amino acids depended on distinct synthetase and transferase enzymes and on t-RNA. Griffiths and Bayley (1969) provided, perhaps, the strongest evidence showing the polypeptide synthesis was taking place under the direction of m-RNA where they found that after preincubating the H. cutirubrum cell-free system, to destroy endogenous messenger, the protein synthesizing system responded to added synthetic polynucleotides.

Addition of NaCl to the peptide synthesizing system improved the amino acids incorporation and extended the optimum range of Mg^{++} concentrations to lower values. Bayley and Griffiths (1968b) showed that in studies with synthetic messengers, Na^{+} also affected the fidelity of translation. Whether these effects were due entirely to the more effective lowering of water activity by Na^{+} compared to other ions such as NH_4^{+} or whether they demonstrated further specificity of Na^{+} in the system was not yet clear.

Apart from the requirement for high concentrations of K and Na salts, the peptide synthesizing system was found to be remarkable in two other respects. One is the close similarity in all essential features to protein synthesizing systems from non-halophilic organisms. The other is that the system still depends to some degree on comparatively low concentrations of other cations. Thus the amino acid incorporation is enhanced by low concentrations of NH_4^{+} and depends specifically on a relatively very low concentration of Mg^{++} (0.02 - 0.04 M). This Mg^{++} concentration is not greatly different from that required by non-halophilic systems (Bayley and Griffiths, 1968 a and b).

7. Effect of Salt on Enzyme Activity

Baxter and Gibbons (1954, 1956, 1957) were the first to investigate the response of a number of enzymes towards sodium chloride and other salts at different concentrations. In all cases, they found good or maximum activity at salt concentrations presumably corresponding to those existing within the cell under normal culture conditions. At low salt concentrations, most of the enzymes showed little or no activity. They concluded that most of the enzymes of the extreme halophiles are adapted to function at very high NaCl concentrations and could properly be designated halophilic. Larsen (1962) and Holmes et al (1965) investigated about twenty different enzymes and found that generally all enzymes in extreme halophiles were extremely halotolerant, and most of them were also strikingly halophilic.

In attempts to test the effect of salts other than NaCl on the activity of halophilic enzymes, it appeared that several alkaline cations and the anions Br^- and NO_3^- could replace Na^+ and Cl^- respectively. Potassium chloride was especially potent in this respect, and some enzymes required almost exclusively high concentration of K^+ for maximum activity (Holmes and Halvorson, 1963 and 1965a). The strongly stimulatory effect of both K^+ and Cl^- upon the enzymes of the extreme halophiles brings to mind the finding that these two ions are the major intracellular ionic constituents in these bacteria besides Na^+. It seems reasonable to assume that Na^+, K^+ and Cl^- play an important role in the cells by keeping the enzymes in an activated form (Baxter and Gibbons, 1954, 1957; Holmes and Halvorson, 1963 and 1965b, and Hochstein and Dalton, 1967 and 1968a).

The function of the sodium and potassium chlorides was not limited to an activation of the enzymes. These salts also acted as stabilizers of the enzymes. Baxter and Gibbons (1954, 1957) and Holmes and Halvorson (1963, 1965a and b) have shown that after removal of salts from enzyme preparations, from H. salinarium, by dialysis against water, the enzyme activity could not be restored by addition of solid NaCl. If, however, the salt-free preparation was dialyzed against 25% NaCl, a number of enzymes such as alkaline phosphatase regained full activity even if they had been kept in a salt-free environment for a relatively long period of time. Malate or alanine dehydrogenase, for example, could restore about 60% and 70% of the original activity respectively. On the other hand, ethanol dehydrogenase and aspartate aminotransferase did not regain activity upon dialysis of salt-free preparations against 25% NaCl; these enzymes became completely and irreversibly denatured in the absence of salt. The phenomenon that enzyme activity could be restored from salt-free preparations by dialysis against 25% NaCl had provided a very important means of effecting the purification of enzymes from extreme halophiles (Holmes and Halvorson, 1965 a).

Holmes and Halvorson (1965 b) confirmed the halophilic character of the enzyme malate dehydrogenase purified from H. salinarium. Their detailed studies of the enzyme activity at salt concentrations lowered by dialysis against water, suggested that most of the enzyme molecules were equally sensitive to salt deprivation. The inactivation by removal of salt was markedly inhibited by the presence of NADH + H. The restoration of the enzyme activity by

dialysis of the salt-free preparation against 25% NaCl was slow. Activity was regained more slowly than the rate at which salt entered the dialysis bag, suggesting that the reactivation is a complex process, probably comprising conformational changes in the protein molecules. Kinetic studies suggested either that reactivation did not follow a first-order reaction, or that more than one species of malate dehydrogenase, each with its characteristic reactivation rate, was present.

Baxter (1959) conducted studies on the halophilic activity of the enzyme lactate dehydrogenase. He proposed that a "function of the salt is to decrease the electrostatic repulsion between ionized groups within the enzyme molecule. Only at high salt concentrations will the molecule attain the conformational structure in which it is active as a catalyst". By lowering the salt concentrations, the molecule will expand to an inactive conformation.

The studies of Holmes and Halvorson (1965 b) suggest a similar explanation for a function of salt with malate dehydrogenase. They also reported a relation between salt concentration and pH value for optimal activity, suggesting that ionized groups within the enzyme molecule might play a role both in the activity of the enzyme and in the reactivation from the salt-free state. Also, Holmes and Halvorson (1965 b) observed that the sedimentation rate, determined by centrifugation in sucrose gradients, decreased upon lowering the salt concentration. This was difficult to reconcile with a disaggregation of the molecule since virtually all of the enzyme activity could be regained by increasing the salt concentration. However, it seemed more probable that lowering of the sedimentation rate reflected an increase in the partial specific volume of the molecule.

In contrast to the findings by Baxter and Gibbons (1954, 1956), Baxter (1956) and Hochstein and Dalton (1967) that enzymes prepared from extremely halophilic bacteria required high concentrations of monovalent cations to neutralize intramolecular electrostatic charges for the maintenance of enzymatically active conformations, Hochstein and Dalton (1968 b) reported that the concentration of NaCl required for maximum activity of NADH dehydrogenase, prepared from the extreme halophile AR-I, depended on the assay buffer (see below). They speculated that enzymes obtained from halophilic bacteria might not require concentrations of cations much different from enzymes obtained from non-halophilic cells. Rather, they might be stabilized and activated at low concentrations of specific cations. In the absence of these cations, they were maintained in a functional and stable state by extraordinarily high concentrations of monovalent cations.

The same authors showed that in the presence of Tris, maximum oxidation occurred at approximately 2.5 M NaCl. With imidazole, maximum oxidation was observed at 1 M NaCl. Two additional buffer effects were present: significantly greater activity was observed when the assay was carried out in imidazole rather than Tris buffer; and the rate of NADH oxidation as a function of NaCl concentration increased in a hyperbolic manner when the assay was carried out in imidazole, while in Tris, the response appeared to be sigmoidal. Hochstein and Dalton (1968 b) also showed that the ability of $MgCl_2$ to satisfy the cation requirement was affected by the

buffer. In the presence of imidazole, maximum oxidation took place at 5 mM $MgCl_2$. In the presence of Tris, no activity was observed at $MgCl_2$ concentrations less than 10 mM, and the level of activity was extremely low.

Hochstein and Dalton (1968 b) also showed that 0.25 - 1.0 M lysine activated the NADH dehydrogenase with the activity at the latter concentration equivalent to the rate observed with 1.0 M NaCl. Polylysine of an average molecular weight of 3000 satisfied the cation requirement at 100 μM; a preparation of average molecular weight 40,000 was effective at 10 μM. In terms of ionic strength, polylysine 3000 (I = 0.011) was about 15 times more effective than polylysine 40,000 (I = 0.17) at these concentrations and the former was essentially as effective as 5 mM $MgCl_2$ (I = 0.015).

Moreover, studies by the same authors on the ability of spermine to stabilize the enzyme revealed that it was effective in maintaining NADH dehydrogenase activity. When incubated in 0.1 M Tris buffer (pH 7.4) containing 0.1 M NaCl, the enzyme activity decayed exponentially with a half-life of 18 min. In the presence of 3.3 mM spermine, the half-life increased to 150 min., while in 33 mM spermine, half of the initial activity was lost after 220 min. When the experiments were carried out at a more alkaline pH, the rate of decay was significantly less. At pH 8.5, the half-life in 0.1 M NaCl increased to 30 min., while in the presence of 3.3 mM spermine the half-life was 200 min. After 120 min., the dehydrogenase had lost little activity when incubated in the presence of 33 mM spermine, and after 25 h, retained approximately 50% of the initial activity.

Similar results have also been obtained for glycerol dehydrogenase enzyme prepared from H. cutirubrum in which it was found that this enzyme functioned in the absence of added cations other than low concentrations of spermine (Hochstein and Dalton, 1968 b).

8. Cell Envelope of Extremely Halophilic Bacteria

(a) Salt requirements and morphology

The extremely halophilic bacteria require high concentrations of salt for growth and preservation of their structure. Under the phase contrast light microscope the intact organisms show no unusual features. The extremely halophilic bacteria such as Halobacterium cutirubrum, H. salinarium and H. halobium require high concentrations of salt for growth and preservation of their structure. When the salt concentration is lowered, growth ceases and the long slender rods assume first irregular and finally spherical shapes. Ultimately they lyse. In older cultures of H. halobium, the cells develop central light areas, apparently gas vacuoles, which occupy a considerable part of the total cell volume. The chemical nature of the gas is not known. Under the electron microscope, shadowed preparations reveal a surface structure consisting of a regular hexagonal arrangement of spherical particles about 130 Å in diameter (Larsen, 1962). When the salt concentration is lowered, growth of the cells ceases and the long slender rods gradually lose their regular surface pattern and finally assume spherical shapes (Abram and Gibbons, 1960 and Boring et al, 1963). Below 1.0 M NaCl

concentration, the gas vacuoles open to form sheet-like structures and the cell membranes begin to disintegrate rapidly so that less and less material remains sedimentable at 1.2×10^6 g x min. (Stoeckenius and Rowen, 1967). The concentration of NaCl required for growth and maintenance of shape of extreme halophiles ranges between 3 and 5 M. Concentrations of 0.1 - 0.5 M Mg^{++} and $1.3 - 2.5 \times 10^{-3}$ M K^+ are also required for optimal growth. No salt has been found that could replace NaCl to any significant extent. Lysis, however, can be prevented in the absence of NaCl by other salts, such as NH_4Cl, KCl and LiCl, in high concentrations, and also by relatively low concentrations of $MgCl_2$ and $CaCl_2$ (Larsen, 1962).

The studies reported above on the behavior of whole cells support the contention of Abram and Gibbons (1961) that the cell envelope of the halobacteria is very sensitive, and that a function of NaCl is to maintain chemical bonds in the envelope structure. Evidence has been presented that the disintegration of the cell envelopes in hypotonic solutions is not enzymically mediated (Brown, 1963; Mohr and Larsen, 1963; Kushner, 1964; Onishi and Kushner, 1966) and that it is pH-dependent in a way similar to that of the lysis of the whole cells (Brown, 1963; Kushner et al, 1964). The disintegration process in the complete absence of salt is also strongly temperature-dependent (Brown, 1963; Kushner et al, 1964), and seems to involve at least two separate processes, one being more rapid than the other, Kushner et al, 1964).

The loss of structural rigidity and lysis of intact cells and cell envelopes upon salt depletion is poorly understood at present. It is generally assumed that a high charge density exists in the surface membrane which, upon removal of the counter ions, disrupts the structure (Abram and Gibbons, 1960, 1961; Brown, 1963, 1964 a and b; Kushner et al, 1964; Kushner and Onishi, 1966; Mohr and Larsen, 1963; Onishi and Kushner, 1966; Stoeckenius and Rowen, 1967). An unusually high content of dicarboxylic amino acids in the protein of cell envelope fractions has been demonstrated (Brown, 1963; Kushner et al, 1964; Kushner and Onishi, 1966 and 1968). While these findings are consistent with the assumed mechanism of lysis, they do not explain the specific NaCl requirement for growth and maintenance of the rod shape of the cells.

(b) Structure of envelopes

The term "cell envelope" is used in accordance with Salton's (1964) recommendation to designate the structure (or structures) containing the cytoplasm and comprising both the structure conferring shape and rigidity upon the cell (wall) and the cytoplasmic membrane. Brown (1963, 1964 a and b) and Brown and Shorey (1963), on the basis of electromicrographs, concluded that halobacteria do not possess a cell wall but are bounded only by a cell membrane. Their preparation of isolated cell envelopes seemed to consist of vesicles with only a unit membrane as the limiting structure. However, recently Stoeckenius and Rowen (1967), using

electronmicroscopy, have shown the presence of a cell wall and of an intracytoplasmic membrane in the halophilic cells when they are fixed with formaldehyde in the presence of 1% $CaCl_2$. Intracytoplasmic membranes are few in number before the cultures reach the end of the log phase, are difficult to detect in sections of whole bacteria, and are not very conspicuous in shadowed preparations. However, they are found in higher concentrations in lysates of whole cells than in lysates of envelope fractions (Stoeckenius and Rowen, 1967).

While these intracytoplasmic membranes look like typical unit membranes in cross-section (Stoeckenius and Rowen, 1967), their surface pattern, and their plate-like structure distinguish them from all other membranes so far described in bacteria or other cells, with the possible exception of certain blue-green algae (Bowen and Jensen, 1965 and Jost, 1965). Their function is still not completely known, although they seem to be related to the formation of gas vacuoles(Stoeckenius and Rowen, 1967). The fraction of the lysate in which they are concentrated contains membrane lipids. However, its lipid content cannot be used as an evidence for the membrane nature of these structures since this fraction was not homogeneous. Thus the term intracytoplasmic membranes does not necessarily mean that these structures have a composition, fine structure and function comparable to those of other cellular membranes. In recent studies, Scherphof et al (private communication) found that their highly purified intracytoplasmic (vacuole) membrane preparation composed entirely of lipids and cannot, therefore, be considered as "classical membranes".

Cho et al (1967) found that the envelope of H. halobium was more complex than a single unit membrane; they suggested that the cell was surrounded by a cytoplasmic membrane and a wall. They found an unusual organelle inside the cytoplasm in the form of parallel striated strands. These may or may not be similar to the intracytoplasmic membranes described by Stoeckenius and Rowen (1967).

Larsen et al (1967) isolated gas vacuoles in cells of Halobacterium sp. strain 5. The vacuoles are bounded by a membrane which reveals itself in electron micrographs of thin sections as a 1-layered structure about 30 Å thick. Although evidence for the exact chemical nature of the vacuole gas content is indirect, it is believed that the gas is not carbon dioxide or oxygen, but might possibly be nitrogen (Larsen et al, 1967).

(c) Chemical composition

Smithies et al (1955) were the first to analyze the gross chemical composition of the cell envelope of a halobacterium and reported it to consist mostly of lipoprotein. In recent years, more detailed investigations have been made and have led to a number of interesting findings.

Proteins: - The total protein content of halophilic bacterial cell envelopes was estimated to be roughly between 45 and 75% (Kushner et al, 1964; Brown and Shorey, 1963) and quite acidic in nature (Brown, 1963; Kushner et al, 1964; Kushner and Onishi, 1966). In fact, they are more acidic than the acidic proteins normally occurring in the common gram-negative bacteria such as E. coli (Holmes et al, 1965).

Chemical analysis has shown that envelopes and whole cells of H. halobium, H. salinarium and H. cutirubrum lack muramic acid and α-ε-diaminopimelic acid, associated with the cell walls of most other bacteria (Brown and Shorey, 1963 and Kushner et al, 1964; Kushner and Onishi, 1966, 1968). Envelopes of these bacteria are mainly lipoproteins, whose total amino acid composition has been determined showing unusually high content of dicarboxylic amino acids (Brown and Shorey, 1963; Kushner et al, 1964; Kushner and Onishi, 1966). Kushner and Onishi (1968) examined the envelopes of H. cutirubrum for teichoic acid and for D-amino acids, especially for D-alanine and D-glutamic acid, found in the walls of many other bacteria. They concluded that these envelopes did not contain teichoic acid or detectable amounts of any known D-amino acids.

Interesting observations on the acidic nature of the envelope protein have been contributed by Brown (1965). He titrated H. halobium envelopes electrometrically with acid and base, and compared the results to similar titrations on the envelope material after dissolving the envelopes by removing NaCl from their environment. He found that all detectable carboxyl groups were titrated on envelopes suspended in NaCl solutions strong enough to keep the envelope intact, i.e. 4M NaCl. However, at this NaCl concentration, many basic groups, which would be titrated in envelope material dissolved in water, would escape titration. He concluded that the basic groups in the envelope material were "buried" in the intact envelope, possibly by being bound to acidic groups of the phospholipid

components. This finding supports the idea that at physiological pH-values, the intact envelope is strongly negatively charged at or near its surface by the dominance at this location of ionized carboxyl groups.

Carbohydrates: - In contrast to Salton's finding of high contents of sugars in envelope hydrolysates of gram-negative bacteria (1964), only small amounts of sugars were detected in halophilic cell-envelope hydrolysates by many investigators (Brown and Shorey, 1963; Kushner et al, 1964; Brown et al, 1965). Brown and Shorey (1963) and Kushner et al (1964) failed to detect muramic acid in the envelope hydrolysates of H. salinarium, H. halobium and H. cutirubrum which shows the lack in these organisms of mucopeptide (rigid layer) of the type found in other bacteria (Salton, 1964). However, regarding the effect of antibiotics on cell wall and cell membrane synthesis, it has been reported that proliferating cells of H. salinarium were as sensitive to penicillin as those of other gram-negative bacteria (Mohr and Larsen, 1963); and since it was established that penicillin inhibits mucopeptide synthesis in gram-negative bacteria as was firmly established for gram-positive bacteria (Plapp and Kandler, 1965), this was taken to be an indication of the presence of a mucopeptide structure in the H. salinarium envelope. However, since no muramic acid could be demonstrated in the halophiles(Brown and Shorey, 1963; Kushner et al, 1964), penicillin sensitivity of H. salinarium might therefore reflect an interference with the formation of some other envelope components (Larsen, 1967). This view has been supported by the finding that D-cycloserine, a compound known to interfere similarly with muco-

peptide synthesis in gram-negative and gram-positive bacteria (Plapp and Kandler, 1965), has no effect at all on the proliferating cells of H. salinarium (Larsen, 1967).

Lipids: - Cells of H. cutirubrum contain about 2.0 - 3.5% of total lipids (including pigments) on a salt-free, dry-weight basis (Sehgal et al, 1962). About 93% of the lipids are phosphatides, the remainder being carotenoid pigments and hydrocarbons (Tornabene et al, 1969). Kushner et al (1964) found that envelopes of H. cutirubrum had a total lipid content of 22%. Kates et al (1963, 1965 a and b) estimated the concentration of the main phospholipid component to form 13% of the salt-free dry-weight of the envelope. It was reported that the cell envelope of H. halobium contained 17 - 23% lipid (Brown, 1965); Sehgal et al (1962) and Cho and Salton (1966) found only traces of, if any, fatty acids in hydrolysates of this lipid. Kates et al (1966) indicated that the presence of only traces of fatty acid might be possibly derived from lipids in the culture medium.

The lipids of the extremely halophilic bacteria were strikingly different from those of non-halophiles in many respects. They have been found to consist virtually exclusively of derivatives of a long-chain diether of glycerol (Sehgal et al, 1962; Kates, et al, 1963; Faure et al, 1963, 1964; Kates et al, 1966). The structure of this diether (Scheme I) was established by chemical degradation (Kates et al, 1965; Joo et al, 1968) as 2, 3-di-O-(3, 7, 11, 15-tetramethylhexadecyl)-sn-glycerol(2, 3-di-O-phytanyl-sn-glycerol)*.,

*The stereochemical numbering system for glycerol derivatives proposed by the Commission on Biochemical Nomenclature, IUPAC-IUB (1968), is used throughout the present work.

i.e. the hydrocarbon groups contain only one alkyl group, namely the C_{20} branched chain phytanyl group linked to glycerol with ether bonds rather than ester linkages. The 2, 3-configuration of the glycerol diether is unusual, since the diglycerides derived from naturally occurring phosphophatides and glycolipids have the 1, 2-configuration.

Each of the phytanyl groups in the diether contains three asymmetric centers, and these have been shown by Kates et al (1967 a) to have the configuration 3-R, 7-R and 11-R. It is of interest that the configuration of the corresponding asymmetric centers in α-tocopherol is also all-R (Mayer et al, 1963).

The lipids of H. cutirubrum are in general highly acidic. They contain about 10% of acetone-soluble lipids (including orange-red carotenoid pigments, squalene, di- and tetrahydrosqualene and vitamin K (Tornabene et al, 1969), about 65% of phosphatides (Kates, 1967 a; Kates et al, 1965 b) and about 25% of a glycolipid sulfate (Kates et al, 1967 b). The major phosphatide, accounting for about 85% of the lipid-P has been shown by degradative studies (Kates et al, 1965) and chemical synthesis (Joo and Kates, 1968 and 1969) to have the structure 2, 3-di-O-(3'R, 7'R, 11'R, 15'-tetramethylhexadecyl)-sn-glycerol-1-phosphoryl-3''-sn-glycerol-1''-phosphate (phosphatidyl-glycerophosphate (PGP)-diether form). The glycerophosphate moiety has been established as the 3-isomer (Joo et al, 1968) (see Scheme I). A minor phosphatide component, accounting for about 10% of the total lipid-P has been shown by synthesis to have the structure

2,3-di-O-phytanyl-sn-glyceryl-1-phosphoryl-3'-sn-glycerol (PG-diether form) (Joo and Kates, 1969). It should be noted that the configuration of each of the glycerol moieties in these phosphatides is opposite to that of the corresponding glycerol in the diester form of phosphatidyl glycerol and phosphatidyl glycerophosphate found in plants and bacteria (E. coli). (See Scheme I).

The glycolipid sulfate also has an unusual structure. It has the same diphytanyl glycerol ether present in the phosphatides and contains glucose, mannose and galactose as component sugars in equimolar proportions (Kates et al, 1967b). The sulfate group has been shown to be attached to the C-3 position in galactose. Two structures (see Scheme 1) have been postulated differing only in the arrangement of the sugar groups.

The cell envelopes of the halophilic bacteria also contain carotenoids (for example α- and β-bacterioruberin) the amount varying with the culture conditions (about 0.1% of the total lipids) but increasing with the age of the culture (Baxter, 1960; Dundas and Larsen, 1963)*. The carotenoids seemed to protect the cell envelopes against photochemical damage. Although the mechanism of protection is not known yet, it seems reasonable to suggest that the carotenoid molecules act by quenching electronic excitation impressed by light upon the pigment systems of the envelope. This implies that the carotenoid molecules are spatially located very close to the photoactivated system (Dundas and Larsen, 1963).

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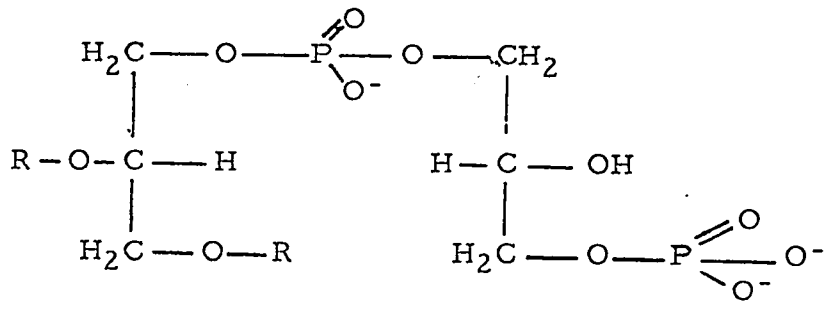
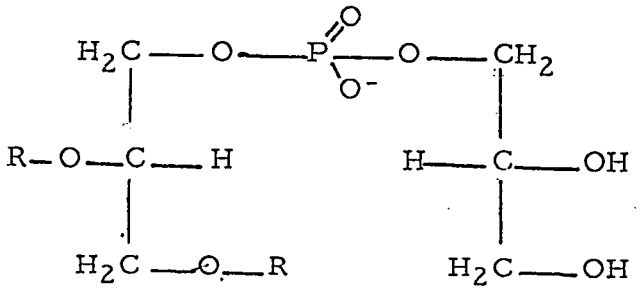
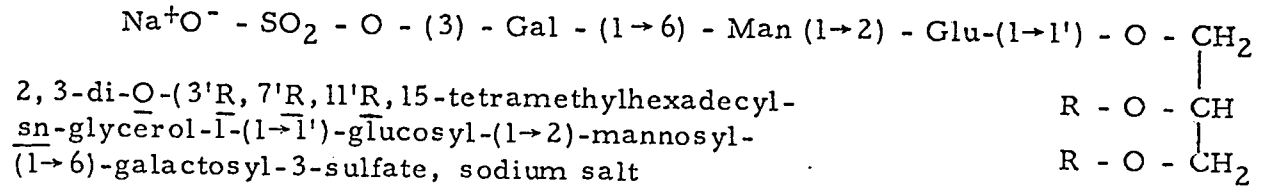
* Recently it was found that growing H. cutirubrum cells in chemically defined medium supplemented with increasing amounts of glycerol, decreased the pigmentation density (Gochenauer and Kushner, private communication).



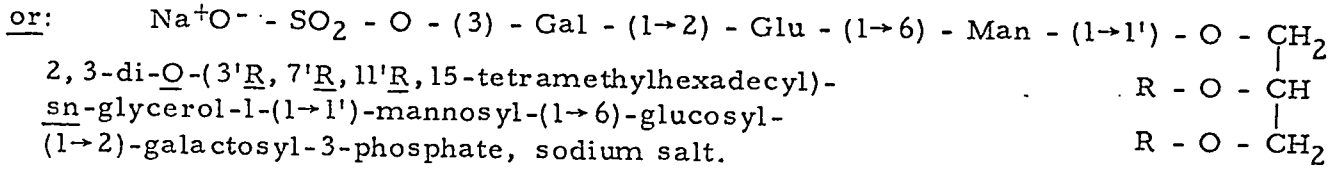
2, 3-di-O-(3'R, 7'R, 11'R, 15-tetramethylhexadecyl)-sn-glycerol-1-phosphoryl-3''-sn-glycerol-1''-phosphate. [Phosphatidyl glycerophosphate (PGP)-diether form].



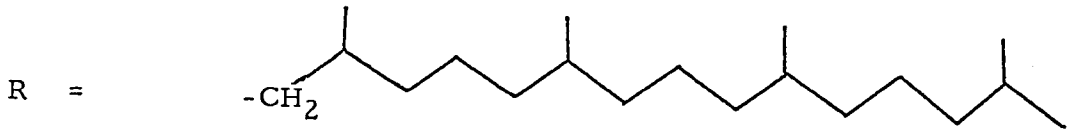
2, 3-di-O-(3'R, 7'R, 11'R, 15-tetramethylhexadecyl)-sn-glycerol-1-phosphoryl-3''-sn-glycerol [Phosphatidyl glycerol (PG)-diether form]



2, 3-di-O-(3'R, 7'R, 11'R, 15-tetramethylhexadecyl-sn-glycerol-1-(1→1')-glucosyl-(1→2)-mannosyl-(1→6)-galactosyl-3-sulfate, sodium salt



2, 3-di-O-(3'R, 7'R, 11'R, 15-tetramethylhexadecyl)-sn-glycerol-1-(1→1')-mannosyl-(1→6)-glucosyl-(1→2)-galactosyl-3-phosphate, sodium salt.



3'R, 7'R, 11'R, 15-tetramethylhexadecyl group

($C_{20}H_{41}$ phytanyl)

SCHEME I. Major phospholipids and glycolipid sulfate in H. cutirubrum

Moreover, Kates (1967a) suggested a role played by the glycerol diether-derived lipids in the survival of halophilic bacteria. The ether-linked alkyl groups are extremely stable over a wider pH-range than ester-linked fatty acids and hence they are not susceptible to direct hydrolysis. The fact that the glycerol diether moiety is derived from L-glycerol might be advantageous, since all the known animal, plant and bacterial phospholipases are specific for phosphatides having diglycerides derived from D- (or, l-) glycerol.

III. Statement of the Problem

The unusual nature of the lipids in the extremely halophilic bacteria prompted an investigation of the biosynthetic pathways involved in their formation. As a first approach, studies were made on incorporation of orthophosphate ^{32}P and sulphate ^{35}S into phospholipid and sulfolipid components of whole cells of the organism Halobacterium cutirubrum. The incorporation of ^{3}H-glycerol and of ^{14}C-labelled acetate, malonate, mevalonate and glycerol as precursors of long-chain hydrocarbon groups into the lipids of actively grown cells of H. cutirubrum, was also studied. Also, since there was no conclusive evidence for the formation of fatty acids by extreme halophiles, it was of great interest to determine whether or not extremely halophilic bacteria possess an active fatty acid synthetase system.

Moreover, the presence in H. cutirubrum of phosphatides in which the glycerol moieties have the opposite configuration to that in non-halophiles suggested that their biosynthetic pathways might differ from the established pathways in other organisms. In non-halophilic organisms, phospholipids are synthesized, according to Kennedy's pathway (1961) by O-alkylation of sn-glycerol-3-phosphate followed by dephosphorylation. The glycerol moieties in both the diglyceride and the glycerophosphate residues have the D-configuration. In the halophilic lipids (the diether analogue), the same glycerol moieties have the L- configuration (Scheme I). If the diether analogue is synthesized in an

analogous manner to diglycerides, its formation implies either that the precursor (GP) has the D-form, or that no conversion of configuration took place. Since L-α-glycerophosphate may arise by phosphorylation of glycerol with ATP catalyzed by the enzyme glycerokinase or by the glycolysis pathway through the reduction of dihydroxyacetone phosphate with the enzyme L-α-glycerophosphate dehydrogenase, it was therefore necessary to investigate the existence of these enzymes and to establish their stereospecificity and/or the configuration of the glycerophosphate synthesized.

Since work on the biosynthesis of glycerophosphatides in non-halophilic micro-organisms points to α-glycerophosphate and phosphatidic acid as key compounds, it was of interest to study the role played by both of these compounds and of other precursors (such as cytidine phosphate derivatives), in the biosynthesis of phospholipids of the extreme halophilic bacteria using both cell-free systems and whole cells of Halobacterium cutirubrum. Also, short-term labelling of total lipids was examined in attempts to identify intermediates which might be involved in the biosynthesis of these phospholipids.

(B) MATERIALS AND METHODS

I. Organism

The obligate aerobe, red-pigmented, extremely halophilic bacterium, Halobacterium cutirubrum was the test organism used throughout the present study. The organism was isolated, screened and purified originally by Dr. A. G. Lochhead at the Animal Research Institute, Research Branch, Canadian Department of Agriculture, Ottawa, Ontario. Stock cultures were maintained on agar slants by adding 2.0% agar to the growth medium.

II. Culture of Organism

Cells of the extremely halophilic bacterium, Halobacterium cutirubrum were grown at 37°C on a rotary shaker in the complex standard liquid medium described by Sehgal and Gibbons (1960). The medium was composed of (g/l): casamino acids (Difco), 7.5; yeast extract (Difco) 10.0; trisodium citrate, 3.0; potassium chloride, 2.0; magnesium sulfate heptahydrate, 20.0; sodium chloride, 250 and ferrous sulfate heptahydrate, 0.05. The ingredients were dissolved in 800 ml of distilled water, the pH was adjusted to 7.5 - 7.8 with 1N sodium hydroxide and the medium was autoclaved for 15 minutes at 120°C.

It was then filtered to remove any precipitate, the pH was adjusted to 6.5 - 7.0 with 1 N HCl and the medium made up to one liter. Some 20% of the magnesium and some phosphate are removed in the precipitate (Sehgal and Gibbons, 1960). However, sufficient of both ions remains in the medium for optimal growth. For routine cultures, the medium ingredients were dissolved in 1 liter distilled water without sterilization and the pH was adjusted to 6.5 - 7.0 with 1 N HCl.

Cells used for inoculation were grown in the standard complex liquid medium (Sehgal and Gibbons, 1960) for 48 hrs. at 37°C; they were harvested by centrifugation at 6000 x g for 10 min., washed twice by resuspension in 4 M NaCl, 0.03 M KCl and 0.1 M $MgSO_4$ (hereafter referred to as the 4M salt solution), centrifuged and finally suspended in the 4M salt solution to give an optical density of 0.22 at 660 mμ. Fifty ml of the medium in 250 ml Erlenmeyer flasks provided with a side-tube (12 cm. length, 1.5 cm. int. diameter) for turbidity measurements, were inoculated with 1 ml of this cell suspension. Incubation was carried out at 37°C on a rotary shaker at 150 r.p.m. Growth was estimated by turbidity measurements (optical density) at 660 mμ on a Coleman Junior spectrophotometer using the uninoculated medium in a matched Erlenmeyer flask as a blank. Cells were harvested in their stationary phase (130 hours; optical density in side tube, 0.611 ≏ 1.0 g dry weight of cells per liter of culture) by centrifugation at 8000 x g, washed twice in the 4M salt solution and suspended in the same salt solution to give an optical density of 0.22 at 660 mμ (60 mg dry cells/100 ml); this suspension was used for the subsequent experiments.

III. Preparation of Cell Fractions

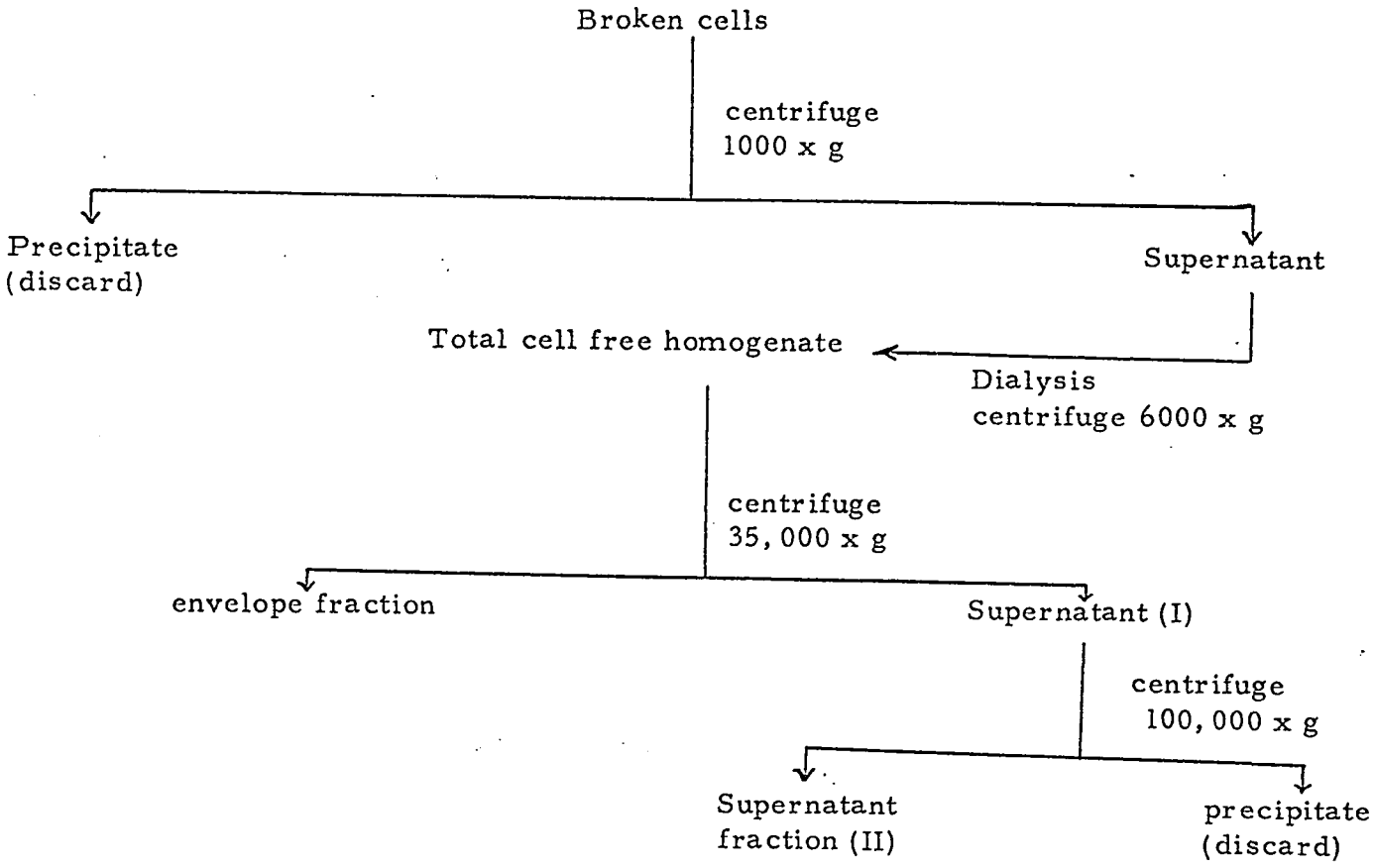
(a) For this purpose actively growing cells were required; H. cutirubrum cells were therefore harvested after 72 hours (log phase) and washed as described above, and suspended in the 4M salt solution to a cell density of about 60 mg dry weight per ml. To break the cells, 7.5 ml lots of the cell suspension were placed in 15 ml bottles and shaken with 1.5 g of number 12 Ballotini beads on a Mickle disintegrator at 120 cycles/min. for one hour at 0°C. This procedure caused about 20% breakage of cells as judged by inorganic phosphate release (Kushner et al, 1964). Intact cells were removed by centrifuging twice at 6000 x g for 10 min. The resulting cell-free suspension, designated as the total homogenate, was diluted to 15 ml with salt solution and dialyzed for 2 hrs. at 0°C against the 4M salt solution. To prepare cell envelopes, the dialyzed total homogenate was centrifuged at 35,000 x g for half an hour at 0°C and the sediment, which contained envelopes and cell fragments, was washed twice by resuspension in 15 ml of the 4M salt solution at 0°C and centrifuged briefly. The ribosomes, the polysomes and the water-soluble materials remained in the supernatant fraction (Kushner et al, 1964). The total homogenate, envelopes and supernatant fractions were used immediately after preparation.

(b) Modified procedure

In order to remove finely divided membrane particles, procedure (a) was modified as follows:

H. cutirubrum cell fractions were prepared by a modification of the procedure of Stoeckenius and Rowen (1967) as follows (see Scheme II). Cells from one liter of culture were suspended in 20 ml 4M salt solution and broken at 0° as described above. The broken cells were centrifuged at 1000 x g for 3 min. to remove unbroken cells and debris and the supernatant was dialyzed against 4M salt solution for 1 hour at 0°. The dialyzed solution was cleared by centrifugation at 6000 x g for 5 min. and diluted to 30 ml with 4M salt solution; this preparation is designated total cell-free homogenate. 20 ml of this total homogenate was spun at 35,000 x g for 20 min. to give "supernatant I" and the envelope fraction as sediment; the latter was washed once in 4Msalt solution, centrifuged and suspended finally in 20 ml salt solution. The "supernatant fraction I" was further centrifuged at 100,000 x g for 30 min. to remove any fragmented envelope particles; this final supernatant was used in the experiment.

Scheme II. - Summarizing the Procedure used for the Preparation of Different Cell Fractions of H. cutirubrum



IV. Lipid Extraction and Fractionation

Lipids were extracted by the method of of Bligh and Dyer (1959) as modified by Kates et al (1965, 1966). The washed cells were suspended in salt solution to a concentration of 20 - 60 mg of cells (dry weight) per ml. To one ml of the cell suspension was added 3.75 ml of methanol-chloroform (2:1, v/v); the mixture was shaken and left at room temperature for several hours with intermittent shaking. After centrifugation, the supernatant extract was decanted and the residue was resuspended in 4.75 ml of methanol-chloroform-water (2:1:0.8, v/v); the mixture was then shaken and centrifuged. To the combined supernatant extracts were added 2.5 ml each of chloroform and water, and the mixture was either centrifuged or allowed to settle in a separatory funnel. The lower chloroform phase, containing the total lipids (Bligh and Dyer, 1959), was withdrawn and brought to dryness at 35 - 40°C in vacuo, with additions of absolute ethyl alcohol and/or benzene to remove traces of water. The red viscous lipid residue was dissolved in $CHCl_3$ to a known volume. The total lipid content was found to amount to 3.5 - 4.5% of the cell dry weight.

The red viscous total lipid residue, dissolved in a small volume of chloroform, was diluted with 10 volumes of cold acetone. The white precipitate of phospholipids, glycolipids, sulfolipids, etc. obtained was washed several times with small portions of cold acetone, dried *in vacuo*, weighed and finally dissolved in chloroform to a known concentration. The combined acetone supernatants containing the neutral lipid fraction (hydrocarbons, pigments, etc.) was evaporated *in vacuo* to a small volume and dissolved in acetone to a known volume. In general, the acetone insoluble lipids amounted to 91 - 95% of the total lipids.

V. Analytical Procedures

1. Protein Determination

The method of Lowry et al (1951) was employed to determine the protein concentration in the different preparations of cell fractions.

The total homogenate of H. cutirubrum cells, prepared as described above, was found to contain about 2 ± 0.2 mg protein/ml of suspension. The envelope fraction and the supernatant fraction contained 1.3 - 1.6 and 0.5 - 0.6 mg protein/ml, respectively.

2. Phosphorus Determinations

(a) Total lipid phosphorus

For samples of 20 - 80 µg , a modification of Allen's method (1940) was used throughout the present study, as follows. A chloroform solution containing 20 to 80 µg of lipid phosphorus (less than 2 mg lipids) in a straight-walled "Pyrex sugar tube" calibrated at 12.5 and 25 ml, was evaporated to dryness under a stream of air in a water-bath at 35°C. The residue was digested with 2.0 ml of 72% perchloric acid by heating for 5 - 10 minutes. The tube was allowed to cool to room temperature, washed down with 10 ml of distilled water and 2.0 ml of a freshly prepared 1%

amidol solution (1 g of 2, 4-diaminophenylhydrochloride and 20 g of sodium metabisulfite in 100 ml of water) and 1.0 ml of 8.3% aqueous solution of ammonium molybdate were then added. To avoid the production of molybdenum blue, care was taken not to touch the sides of the tube during amidol or ammonium molybdate solutions pipetting. The solution was mixed and left at room temperature for 20 min. to allow the color to develop; it was then diluted to the 25 ml mark with distilled water, mixed well, and the absorbency (optical density) was determined at 680 mμ against a reagent blank. A solution of potassium dihydrogen phosphate containing 10 μg P/ml was used as a standard. The optical density followed Beer's law in the range 10 - 100 μg P.

For samples containing less than 10 μg P, a modified microassay based on the methods of Fiske and Subba Rao (1925), Allen (1940) and Bartlett (1959) was used as follows:

A chloroform solution containing 1 to 5 μg of lipid phosphorus in a straight-walled 25 ml "Pyrex sugar tube" was evaporated to dryness under a stream of air. The residue was digested with 0.5 ml 10 N H_2SO_4 for 5 - 10 min. To the digest was added 4.1 ml of water, 0.2 ml of 5% ammonium molybdate aqueous solution and 0.2 ml of aminonaphtholsulfonic acid (Eastman Organic Chemicals) solution (0.25 g of purified 1-amino-2-naphthol-4-sulfonic acid in 100 ml of freshly prepared aqueous solution of 15 g anhydrous sodium bisulfite and 1.0 g of anhydrous sodium sulfite;

the solution was filtered before use). The solution was mixed well, heated in a boiling water-bath for 7 min., allowed to cool to room temperature and its absorbency at 830 mμ was determined against a reagent blank using a Beckman model-B photometer with the red sensitive phototube. A solution of KH_2PO_4 containing 10 μg P/ml was used as a standard.

(b) α-Glycerophosphate phosphorus

The microdtermination method of α- and β-glycerophosphates described by Burmaster (1946) and by Pande et al (1967) was modified for use in the present work. Essentially, the method was based on the conversion of α-glycerophosphate to glycolicaldehyde phosphate by reaction with periodate at room temperature. The excess periodiate and the iodate formed were destroyed with sodium sulfite, and the glycolicaldehyde phosphate was hydrolyzed with hot acid. The inorganic phosphate liberated was measured by a modification of the procedure of Bartlett (1959). The latter value, after correction for the inorganic phosphate liberated in a control in which periodate was omitted, was a measure of the α-glycerophosphate (α-GP-P) present (Pande et al, 1967 and Burmaster, 1946). Tests with an authentic sample of α-glycerophosphate showed that the conversion of stable phosphate of α-glycerophosphate (α-GP) to orthophosphate was completed, and that the release of orthophosphate in the absence of periodate was negligible.

In each of two straight-walled "Pyrex sugar tubes" were placed up to 3.5 ml of the sample (containining 1-10 μg P) to be analyzed (GP-samples prepared enzymatically were charcoal-treated to adsorb nucleotides), and 0.5 ml 10 N H_2SO_4. To one of the tubes was added 0.50 ml of 0.08 M freshly prepared sodium periodate and to the other 0.5 ml of water to serve as a blank. The tubes were covered with 20 ml beakers and heated for 50 min. in a boiling water-bath. Without cooling the tubes, 0.20 ml of 5% aqueous ammonium molybdate solution and 0.30 ml of the "amino-naphthol" reagent of Fiske and Subba Row (1925) (see micro-determination of phosphorus) were added. The tubes were returned to the boiling water-bath and heated for 10 min. The tubes were allowed to cool to room temperature and the absorbence of the stable blue color of the solutions was measured at 830 mμ against a reagent blank. The difference in absorbency between the periodate treated sample and the heated sample was taken as a measure of the α-glycerophosphate phosphorus.

3. Chromatography of Lipids

(a) Paper chromatography

Total lipids were chromatographed on silicic acid impregnated Whatman 3MM paper according to the procedure of Marinetti (1962 and 1964), using diisobutyl ketone-glacial acetic acid-water (40:25:5 v/v) for analysis of phosphatides (Marinetti, 1962 and 1964) and heptane-diisobutyl ketone 96:6(v/v) for analysis of neutral lipids, (Marinetti 1964). The chromatograms were stained with Rhodamine 6G for detection of all lipid components (Marinetti,

1962 and 1964), with ninhydrin to detect amino-lipids, with the phosphomolybdic acid-stannous chloride reagent (Levine and Chargaff, 1951) for choline-containing lipids, with the Schiff-Feulgen reagent for plasmalogens (Hack and Ferrans, 1959) and with a modified Periodate-Schiff reagent (Baddiley et al, 1956) to detect vicinal glycerol-containing lipids. ^{32}P-labelled lipids were located by autoradiography on Kodak no-screen X-ray film. The detailed procedures used were as follows (Kates, 1967):

(i) Impregnation of paper with silicic acid:

Filter paper - Whatman 3MM paper was cut into strips 11.5 x 45 cm. A pencil line was drawn 2 cm from one end, this space being used for handling.

Sodium silicate solution - 310 g silicic acid (Mallinckrodt, analytical reagent, 100 mesh) was added slowly with stirring (Teflon-coated magnetic stirrer) to 1 liter of a 7.2 N NaOH solution (288 g NaOH/liter). When the silicic acid had been dissolved, the solution was allowed to cool to room temperature and diluted to 1500 ml with distilled water. This amount of solution was sufficient for impregnation of up to 48 papers.

Procedure - The papers were dipped individually through the sodium silicate solution, in a Pyrex glass tray, and immediately hung to drain for 3 - 4 min.; the drippings at the lower edge of the paper were removed by blotting with filter paper. The papers were then immersed in 2 liters of 6N HCl (one liter of conc. HCl diluted with an equal volume of H_2O) in a Pyrex glass tray for 30 min., with intermittent rocking of the tray. Up to 12

papers may be placed in the acid bath at one time, taking the precautions described by Marinetti (1962 and 1964). The papers were then removed from the acid bath and washed under running tap water for 2 - 3 hours and finally in several changes of distilled water for a total period of 1 - 2 hr.; the last wash water should be free of chloride ion as tested with silver nitrate. The papers were hung to dry overnight and placed in batches of 20 to 24 in a closed cylindrical jar (15 x 45 cm) with the lower ends immersed in 200 ml of chloroform-methanol (1:1 v/v) until the solvent front rises to within a few inches of the top. This procedure served to remove traces of acid and lipids in the paper up to the solvent front. The papers were again hung to dry and finally pressed between glass plates for several hours, after which they could be used immediately. For storage, they were wrapped in filter paper and kept in a drawer for several months without affecting their chromatographic efficiency.

(ii) Application of lipids to the paper: The lipids were applied as spots on a starting line 5 cm above the line previously drawn, the spacing between the spots being not less than 1.5 cm; a maximum of seven spots could conveniently be applied. An aliquot of the lipid solution containing the total amount to be applied (4 - 5 μG lipid P per spot for total lipids, 1 - 2 μg lipid P for individual phosphatide) was placed in a 15 ml conical centrifuge tube and evaporated almost to dryness in a stream of nitrogen. The residue was immediately dissolved in chloroform to a concentration of 0.5 - 1 μg lipid P/μl, and the solution was

brought down completely into the tip of the tube by centrifugation for a few seconds; 5 - 10 µl of this solution was then applied per spot by micropipette using a pipette controller, under a gentle stream of nitrogen to give a small circular spot 5 - 6 mm in diameter. The bottom 2 cm was then cut off and the chromatogram was run immediately.

(iii) Solvent systems and chromatography chamber: The chromatograms were run ascending in the solvent system of Marinetti et al (1957) and Marinetti (1962, 1964); diisobutyl ketone-acetic acid-water 40:25:5 (v/v) at 23 - 25°C for 16 - 18 hours. Heptane-diisobutylketone 96:6 (v/v) was used for analysis of neutral lipids (Marinetti and Stotz, 1956; Marinetti et al, 1957).

The chromatographic jar consisted of an unlined Pyrex glass cylinder (15 x 45 cm) covered by a circular glass plate (15 cm in diameter) and sealed with masking tape; 2000 ml of solvent was placed in the jar and 18 - 20 hr. pre-equilibration of the solvent was required initially. The chromatogram was suspended in the jar by rolling the upper end of the paper around a glass rod and securing with a common paper clip or a stainless-steel spring clip. The lower end of the paper should be immersed in the solvent to a depth of 1 - 2 cm. The glass rod, 7 mm x 13.5 cm, had each end inserted into a piece of rubber tubing 3 cm long. The overall length was adjusted to fit tightly into the jar, the rubber ends serving to maintain the position of the holder without slipping.

(iv) Detection and identification of lipids: The following stains were used to detect and identify the lipid component separated on silicic acid-impregnated chromatograms. However, it should be emphasized that identification by means of these stains was only tentative and was confirmed by standard chemical procedures of isolation, analysis and identification of hydrolysis products.

Rhodamine 6G: - This was the most versatile and useful of the lipid stains, inasmuch as all lipid components were revealed and the color of the fluorescent spot was often an indication of the chemical nature of the lipid. The use of this stain was first introduced by Marinetti and Stotz (1956) and Marinetti et al (1957) and the staining procedure had been described in detail by Marinetti (1962 and 1964). A 0.12% stock solution of Rhodamine 6G (color index 752) (National Anailine Division, Allied Chemical Dye Corp., New York) was made by dissolving 1.2 g in 1 liter of distilled water; this solution was stable indefinitely when kept in the dark. Before use, the stock solution was diluted 1:100 with distilled water and transferred to a Pyrex glass tray. The paper chromatogram, after being dried in a hood for 1/2 - 1 hr., was immersed in the solution for 1 - 3 min.; the excess dye solution was rinsed off with distilled water and the wet chromatogram was viewed immediately under ultraviolet light (Mineralight Lamp, 366 mμ), the fluorescent spot being outlined with pencil. Acidic phosphatides and other acidic lipids gave blue or purple fluorescent

spots, whereas neutral lipids and neutral phosphatides gave yellow or orange spots; however, neutral phosphatides and other lipids might give bluish-gray spots when peroxidized and pigments also might give intensely blue spots. On dry chromatograms most of the lipids appeared as yellow fluorescent spots.

Ninhydrin: - This reagent was quite specific for phosphatides or lipids having a free amino group. Marinetti (1962 and 1964) has described a most convenient and effective procedure for staining silicic acid-impregnated chromatograms with ninhydrin:

The chromatograms were dried in a hood overnight, and sprayed with a 0.25% solution of ninhydrin in acetone-lutidine 9:1, v/v. After a few hours at room temperature, the amino lipids appeared on the chromatograms as mauve spots. The ninhydrin-positive spots were outlined in pencil and the chromatogram might then be stained with Rhodamine in order to locate the other lipid components.

Periodate-Schiff reagent: - This stain is specific for lipids that contain vicinal hydroxyl groups. The method depends on the cleavage of vicinal hydroxyl groups with periodate and staining of the resulting aldehydelipids with Fuchsin bisulfite (Schiff reagent). The procedure used was an adaption to silicic-acid-impregnated paper (Sastry and Kates, 1964) of the method devised by Baddiley et al (1956) for unimpregnated paper. The dried chromatogram was passed through a 0.25% solution of sodium meta periodate, hung for 15 - 20 min. at room temperature, and then passed repeatedly through a 1% solution of sodium metabisulfite until the released iodine was completedly decolorized.

The chromatogram was then passed through a Schiff reagent, whereupon the periodate positive lipids appeared within a few minutes as pink-mauve spots on a white background, which gradually became pink owing to reaction of the Schiff reagent with the paper. The chromatogram was then washed with distilled water, the positive spots outlined with pencil, and the paper stained with Rhodamine to locate the remaining lipids.

The Schiff reagent was prepared as follows: 1 g of basic Fuchsin and 10 g of sodium metabisulfite were dissolved in 10 ml of conc. HCl and 100 ml of water, and the solution was treated with charcoal for one hour, filtered, and made up to 500 ml with distilled water. This stock solution was colorless and could be stored for several months. Before being used, it was diluted 1:1 with 1% sodium metabisulfite solution and water.

Autoradiography: - The most reliable test for the presence of phosphatides and sulfolipids on chromatograms was the detection of ^{32}P and ^{35}S, respectively, in the separated spots. The chromatograms obtained with ^{32}P- or ^{35}S-labelled lipids were dried for several hours and stapled onto a sheet of Kodak no-screen X-ray film; the film was exposed for several hours to several days or weeks, depending on the amount of radioactivity per spot (1 day/1000 cpm). If sufficient material was applied to the paper, the chromatogram might then be stained with Rhodamine 6G and superimposed on the autoradiograph to determine the position of the radioactive spots relative to the unlabelled compounds.

The distribution of radioactivity among the spots was determined by cutting out each spot and counting it with an end-window GM-Counter; the results were expressed as a percentage of the total radioactivity counted on the spots.

(v) Quantitative Analysis of Phosphatides

A micromethod for quantitative analysis of phosphatide components separated on paper chromatograms, as described above, was used as follows:

A 3 cmwide longitudinal strip was cut out of the developed and Rhodamine-stained chromatogram, taking care that all the separated spots lay well within this strip. The paper strip was cut into rectangles each containing one of the separated spots as well as two blank rectangles 2 cm wide, one above the solvent front and the other below the starting line; the width of each of the rectangles was measured in centimeters.

Phosphorus was determined in each spot by a modification (Letters 1964) of the micromethods of Allen's (1940) and Bartlett's (1959) as follows: the rectangles of paper were chopped into small pieces and transferred quantitatively to Pyrex digestion tubes (1.7 x 19 cm); 0.5 ml of 72% perchloric acid and one drop of 5% aqueous solution of ammonium molybdate were added, and digestion was carried out by heating on a medium gas flame for 5 - 10 min. The ammonium molybdate acted as a catalyst for the oxidation of the paper, so that the digestion proceeded smoothly and rapidly. To the cooled digests were added a further 0.1 to 0.2 ml of 72% perchloric acid (to compensate for losses during digestion) and 6 ml of water; after mixing on a Vortex mixer, the silicic acid was centrifuged down. A 5 ml

aliquot of the clear supernatant was then removed, and the molybdenum blue color was developed by addition of 0.2 ml of 1% amidol solution (prepared as above) and 0.2 ml of 5% aqueous solution of ammonium molybdate and heating in a boiling water bath for 7 min. After 20 min. cooling, the optical densities were determined at 830 mμ in a Beckman Model B spectrophotometer. Standards containing 0.5 to 5 μg of P, as well as a reagent blank, were carried through the above procedure simultaneously. The blank corrections for P in the paper were calculated as follows:

Corrected μgP in component = (μg P found) - (average μg P in blanks x $\frac{W}{2}$) where W is the width of each paper rectangle, the width of the blanks being 2 cm. The results were then expressed as the percentage of the total blank minus corrected P in all the components.

(b) Thin-layer chromatography

Thin-layer chromatography (TLC) is one of the main analytical tools of current lipid research. Although many adsorbents are available, silicic acid without binder (and designated as silica gel H) has been the adsorbent of choice for TLC of lipids employing the ascending techniques.

A slurry of 50 g plain silica gel in 120 ml of 0.01% sodium carbonate was spread on micro-slides for rapid TLC analysis; for preparative purposes 40 g of silica gel were suspended in 90 ml of 0.01% sodium carbonate spread on 8 x 8 inch

glass plates (0.6 - 1 mm thick layers). The microslides and the plates were heat activated for 2 hrs. at 120°C. The amount of sample applied as a spot to a 8 x 8 inch plate with a micro-pipette ranged usually from 10 - 12 μg per single component or up to 10 mg as a streak. In the case of preparative TLC work, the silicic acid was washed prior to use with chloroform-methanol 2:1 (v/v) to remove contaminating lipids.

The solvent systems used for separation of neutral lipids were:

(a) heptane-benzene 9:1 (v/v); or

(b) petroleum ether-diethylether-acetic acid (90:10:1 (v/v);

(c) heptane;

and for separation of polar lipids:

(a) chloroform-acetone-methanol-acetic acid-water 50:20:10:10:5 (v/v/v/v/v) (Lepage, 1967);

(b) chloroform-ethylether 9:1 (v/v) and 20:1 (v/v);

(c) chloroform-methanol 1:1 (v/v).

The solvents were distilled before use to eliminate lipid contaminants and other impurities.

When samples were to be recovered, it was essential to locate the compounds by non-destructive agents such as exposure to iodine vapors, or by U.V. light (360 mμ), otherwise spraying with 20% H_2SO_4 in ethanol followed by heating in an oven or on a hot plate.

Radioactive spots were located by autoradiography, and individual components were eluted from the plate using $CHCl_3$-MeOH (1:1, v/v), and counted for radioactivity as described under "Radioisotopic Methods".

4. Paper Chromatography and Paper Electrophoresis of Water Soluble Phosphates

(a) Paper chromatography

Water-soluble phosphates were separated by paper chromatography as follows: aliquots containing 10 - 15 μg P or 500 - 1000 cpm (^{14}C or ^{32}P) per spot were applied to a strip of Whatman No. 1 paper (19 x 57 cm) and chromatography was carried out for 12 - 15 hours using the one-dimensional ascending technique in the following solvents: (i) saturated phenol-water (107 g phenol in 38 ml water), and (ii) n-butanol-acetic acid-water (5:3:1, v/v); or by the descending technique for 20 - 24 hours in (iii) t-butanol-picric acid-water (80:20:4, v/v/w) (Hanes and Isherwood, 1949). After autoradiography, the chromatograms were stained with sulfosalicylic acid-ferric chloride reagent by a modification (Vorbeck and Marinetti, 1967) of the Wade and Morgan (1953) procedure or with the ammonium molybdate "dip" reagent of Burrows et al (1952).

In Vorbeck and Marinetti's staining procedure, the thoroughly dried chromatograms were immersed in an acetone solution of ferric chloride (1.5 g $FeCl_3 . 6H_2O$ in 30 ml 0.3 N HCl diluted with 970 ml acetone). The chromatograms were dried in a hood for several minutes and then immersed in a 1.25% solution of sulfosalicyclic acid in acetone. The spots appeared deep yellow to brown on a white background.

In the staining procedure of Burrows et al, the dried chromatograms were dipped through a solution of ammonium molybdate made up as follows: a solution of 1.0 g ammonium molybdate in 8.0 ml water was diluted in succession with 3.0 ml concentrated HCl, 3.0 ml of 70% perchloric acid, and acetone to a final volume of 100 ml. The spots appeared deep blue on a light blue background.

(b) Paper electrophoresis

Aliquots of water soluble phosphate solutions containing 10 - 15 µg P or 500 - 1000 cpm (^{14}C or ^{32}P) per spot were applied to a strip of Whatman No. 1 paper (33.5 x 4.0 cm) 11.5 cm from the cathode end. Horizontal electrophoresis was carried out in a Gelman electrophoresis apparatus (Gelman Instrument Co., Ann Arbor, Michigan; size of the chamber 35 x 20 cm and the effective length of paper strip 24.0 cm) using 0.02 M sodium borate buffer of pH 9.1 at 415 volts (17.3 volts per cm) and 14 - 15 amps (Michelson and Letters, 1964; and Letters et Michelson, 1963). The time necessary to effect a satisfactory separation was

determined by using authentic inorganic phosphate, dihydroxy-acetone phosphate and rac-glycerol-3-phosphate (15 μg P) each applied to 4 strips; one strip was removed after 40, 70, 90 and 120 minute intervals and strained by the method of Burrows et al (1952). It was found that 90 minutes was the optimum time to effect a good resolution of these three phosphate compounds.

VI. Radioisotopic Methods

1. Labelled Precursors

(a) Commercially available compounds

2-^{14}C-malonic acid, DL-2-^{14}C-mevalonic acid dibenzyldiamine (DBED) salt, U- and 1,3-^{14}C-glycerol, 1-^{14}C-palmitic acid and malonyl-1,3-^{14}C-coenzyme A were products of the New England Nuclear Corporation. 1-^{14}C-acetate, ^{32}P-orthophosphate and ^{35}S-sulfate were obtained from Atomic Energy of Canada Limited. Glycerol-2-^{3}H and glycerol-1(3)-^{3}H were purchased from Amersham/Searle Corporation, Des Plaines, Illinois. The DBED salt of mevalonic acid was first treated with Amberlite IR-120 cationic exchange resin to remove the diamine, and then neutralized with 0.1 N NaOH. Aqueous solutions of the other labelled acidic precursors were neutralized with 0.1 N NaOH to pH 6 - 7.

(b) Synthesis of labelled glycerophosphates

(i) rac-3-Glycerolphosphate-^{32}P (GP-^{32}P): rac-3-GP-^{32}P was prepared by the procedure of McMurray et al (1957), modified by Sastry and Kates (1965) as follows.

A mixture of 2.0 mcuries of orthophosphate-^{32}P (purchased from Atomic Energy of Canada Ltd. as $H_3{}^{32}PO_4$ and neutralized to phenol red with 0.1 N NaOH), 80 μmoles of

3-chloro-1,2-propanediol in 0.16 ml of water was incubated at 37° for 20 hours. After the addition of 0.15 ml of 1 M tri-sodium phosphate, the mixture was neutralized with 0.2 ml of 1 N HCl and inorganic phosphate was precipitated by the addition of 0.8 ml of 0.5 M barium chloride and 0.5 ml of 0.1 M barium hydroxide (to pH 9). The precipitate was removed by centrifugation and washed with three 0.5 ml portions of water; the combined supernatant and washings were concentrated to about 1 ml at 40° in a stream of air and diluted with an equal volume of 99% ethanol. After several hours at 0°, the precipitate of barium GP-^{32}P was centrifuged and washed with 0.5 ml of ethanol-water (1:1, v/v); yield, 16 - 19 μmoles (20 - 24%); specific activity, ca. 11 - 14 x 10^6 c.p.m./μmole P (corrected for decay). The product contained 95 - 97% of organic-P and 3 - 5% inorganic phosphorus as determined by the Berenblum and Chain procedure (see below). The barium salt was converted to the sodium salt by treatment in 0.5 ml of water with Dowex-50 (H^+) resin, followed by neutralization with 0.1 N NaOH; the solution was finally made up to a volume of 2.0 ml.

(ii) sn-Glycerol-3-phosphate-1,3-^{14}C (GP-^{14}C): sn-3 GP-^{14}C was enzymatically synthesized following the procedure described by Kennedy (1962). Glycerol 1,3-^{14}C (New England Nuclear Corp., 10 μcuries) was phosphorylated with ATP in the presence of the enzyme glycerol kinase (from the yeast

Candida mycoderma). The incubation mixture contained in a final volume of 5.0 ml: Tris-HCl buffer pH 8.0 (250 μmoles), glycerol 1,3-^{14}C (1.1 μmoles, 10 μcuries), "cold" glycerol (18.8 μmoles), ATP (100 μmoles), $MgCl_2$ (62.5 μmoles), β-mercaptoethanol (50.0 μmoles), serum albumin (5 mg) and the enzyme glycerol kinase (0.05 mg). The mixture was incubated at 37°C for 3 hours, placed in a boiling water-bath for 5 minutes and centrifuged briefly. Earlier attempts to use trichloroacetic acid to terminate the enzymatic reaction by protein precipitation proved unsuccessful because of the adsorption of GP-^{14}C on the protein precipitate. The supernatant solution was then applied on a Dowex-1-formate column, to isolate the sn-3-GP-^{14}C.

Column chromatographic procedure for the isolation of α-GP: For the purpose of isolation of GP from enzymatic reaction mixture, the method of Bublitz and Kennety (1954 a and b) and Chang and Kennedy (1967 a) was used as follows.

A column of Dowex-1-chloride (50 - 100 mesh), 16 cm. in length and 1.3 cm in diameter, was washed with 4 M formic acid until no chloride ions could be detected in the effluent, and then finally with a few bed volumes of distilled water (Busch et al, 1952). The reaction mixture was then applied to the column after dilution to 50 ml with distilled water and 100 ml of distilled water was allowed to pass through the column (to wash out salts). Gradient elution was then begun with an arrangement of apparatus similar to that of Hurlbert et al (1954) with 250 ml of water in the lower mixing

chamber, and 4 N formic acid in the upper reservoir. The flow rate was 1.5 - 2.0 ml/minute. Fractions of 10 ml each were collected, and each fraction assayed for radioactivity. The fractions (tubes 25 - 49) containing radioactivity (glycerol-3-phosphate-^{14}C) were combined and taken to dryness under reduced pressure in a rotary evaporator. The bath temperature was maintained at about 10° during the evaporation in order to prevent the formation of any cyclic glycerophosphate in the gradually concentrated formic acid. The product was dissolved in 2.0 ml distilled water neutralized with ammonia diluted with 3.5 ml tris-HCl buffer pH 5.2, then shaken for 1 hour with 400 mg of acid washed animal charcoal (Norite) to remove any adenine nucleotides (Pande et al, 1967). The charcoal was filtered off and the clear supernatant was transferred to a 5.0 ml volumetric flask, neutralized with ammonium hydroxide and made up to volume with water and stored at 4° . Suitable aliquots were taken for counting of ^{14}C, for determination of total and inorganic phosphorus, for α-GP-P determination (Pande et al, 1967) and for enzymatic determination of sn-glycerol-3-phosphate (Kennedy, 1962; Bublitz and Kennedy, 1964 a and b). The purity of the preparation was also checked by autoradiography of paper chromatograms and paper electrophoregrams (see below). The overall yield from glycerol as determined by the periodate (Pande et al, 1967) or enzymatic (Kennedy, 1962) procedures was 70 - 85%; specific activity 6.1 - 8.3 x 10^5 cpm/μmole.

2. Determination of Water-soluble Organic and Inorganic ^{32}P (Berenblum and Chain Procedure)

A modification of the method of Berenblum and Chain (1938) for the determination of water-soluble ^{32}P-organic and inorganic phosphates was used throughout the present work. The method is based on the solubility of phosphomolybdic acid in isobutyl alcohol. The procedure is as follows.

To a 0.5 ml sample containing more than 500 cpm was added 0.5 ml H_2O, 1.0 ml of a mixture (1:1, v/v) of 5% ammonium molybdate and 2N H_2SO_4 and finally 2.0 ml of isobutanol. The mixture was shaken and then briefly centrifuged. 100 µl aliquots of the alcoholic and the aqueous phase were plated and counted for inorganic and organic ^{32}P-labelled phosphatides, respectively.

3. Counting of Labelled Material

Suitable portions of the chloroform solutions of the labelled lipids (containing 500 - 1000 r.p.m.) were plated on aluminum planchets and counted with an end-window GM counter. The counting efficiency was 5% for ^{14}C, 7% for ^{35}S and 28% for ^{32}P; all counts for ^{35}S and ^{32}P were corrected for decay. Tritiated samples (doubly labelled with ^{14}C glycerol) were counted in a liquid scintillation counter (Nuclear Chicago). Twenty-five µl aliquots of the labelled samples were mixed with 15 ml of toluene scintillation liquid containing 5 g 2,5-diphenyloxazole (PPO), and 0.3 g 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (POPOP), 130 ml methanol and 100 ml solubilize (Beckman Bio-solv, formula BBS-3) per liter. The counting efficiency was 50% for ^{14}C and 30% for ^{3}H.

VII. Experimental Procedures

1. Growth of Organism in Presence of Labelled Precursors

Cells of H. cutirubrum were grown in 50 ml of standard culture medium for extreme halophiles (Sehgal and Gibbons, 1960) containing 50 μc of either the ^{14}C labelled acetate, ^{14}C-malonate, ^{14}C-mevalonate, ^{14}C-glycerol or a mixture of 25 μc 1, 3-^{14}C-glycerol and 1 mc 2-^{3}H-glycerol or 1,(3)-^{3}H-glycerol as precursor; incubation was carried out in 250 ml Erlenmyer flasks. For the ^{32}P-labelling, cells were grown in one liter of medium (Sehgal and Gibbons, 1960) containing 2.0 μcuries of ^{32}P-orthophosphate, in a 4 liter flask. With ^{35}S-sulfate as precursor, the growth medium was modified, to avoid dilution of the tracer with "cold" sulfate, by replacing the $MgSO_4 . 7H_2O$ with equimolar amount of $MgCl_2 . 6H_2O$ (Dundas et al, 1963 and Onishi et al, 1965). The modified composition was as follows: NaCl, 25%: KCl, 0.5%; $MgCl_2 . 6H_2O$, 0.5%; yeast extract (Difco), 1%. 120 ml of this medium (in a 500 ml flask) containing 1.0 mc of ^{35}S-sulfate was inoculated with 3 ml of a culture of cells grown in the standard medium for 3 days. All incubations were carried out at 37°C with shaking on a rotary shaker at 120 r.p.m. Cells were harvested in their stationary phase (180 hr.) by centrifugation at 800 x g for 10 min. After removal of the supernatant medium, the cells were suspended in a solution of 4M NaCl + 0.2 M KCl to a volume of 3.2 ml for the measurement of ^{14}C, 24.0 ml for ^{32}P-phosphate and 8.0 ml for the ^{35}S-sulfate.

2. Extraction of Labelled Lipids

Lipids were extracted by a modification of the procedure of Bligh and Dyer (1959), as follows. To 3.2 ml of each of the above cell suspensions were added 8 ml of methanol and 4 ml of chloroform, and the mixture was shaken and left at room temperature for several hours. An additional 4 ml each of chloroform and water were then added, the mixture was centrifuged, and the chloroform layer was withdrawn, diluted with benzene, and brought to dryness under a stream of nitrogen. The residue was dissolved in 5.0 ml of chloroform, the solution was cleared by centrifugation if necessary, and used for the different analyses described below.

3. Lipid Degradation and Distribution of Radioactivity among Products

To determine the distribution of radioactivity between ether-soluble and the water-soluble moieties of the lipids, a portion of the total lipids was treated with 4.5 ml of 2.5% methanolic-HCl for 5 hr under reflux in a 50 ml flask with 5 ml side-tube (Kates et al, 1965; Kates, 1964). After addition of 0.5 ml of water, the glycerol diether fraction (including traces of glycerol monoether, pigments, hydrocarbons and any fatty acid methyl esters) was extracted with four 5 ml portions of petroleum ether (b.p. 30 - 60°). The combined extracts were concentrated under

a stream of nitrogen to a small volume and diluted to 5 ml with chloroform. The aqueous-methanolic phase (containing phosphate esters from the phosphatides and methyl glycosides of sugars from the glycolipids) was concentrated under a stream of nitrogen to 0.5 ml and diluted to 5 ml with methanol-water (1:1); portions of this solution were hydrolyzed in 1 N HCl, evaporated to dryness, neutralized with 0.1 N NH_4OH and chromatographed on Whatman No. 1 paper in pyridine-ethyl acetate-water (2:5:5, upper phase) for separation of sugars, and in tert-butanol-water-picric acid (80:20:4, v/v/w) for separation of phosphate esters. After autoradiography, the chromatograms were stained with the alkaline silver-nitrate reagent for sugars, and with the sulphosalicyclic acid-ferric chloride reagent (Vorbeck and Marinetti, 1965) for phosphate esters.

To separate any fatty acid methyl esters in the petroleum ether-soluble fraction, a portion of the latter was saponified in 5 ml of 0.7 N NaOH in 90% methanol under reflux for 1 hr, and the unsaponifiable material (glycerol diether and monoether, hydrocarbons, etc.) was extracted with four 5 ml portions of petroleum ether; after acidification of the aqueous phase with 6 N HCl, the fatty acids were extracted with petroleum ether and converted to methyl esters by refluxing in 2.5% methanolic-HCl, as described by Kates (1964). The extracts of the unsaponifiable material and of the fatty acids and their methyl esters were concentrated under a stream of nitrogen and brought to a volume of 5 ml with chloroform. Suitable portions of all fractions were plated and counted for radioactivity as described above.

The total petroleum ether-soluble fraction, the unsaponifiable material, and the fatty acid methyl esters were chromatographed on silicic acid-impregnated Whatman 3MM paper with diisobutyl ketone-heptane (6:96, v/v) as solvent (Marinetti et al, 1958) or on thin-layer plates of silica gel H (no binder) with chloroform-ethyl ether (20:1, v/v) as solvent. Radioactive spots were located by autoradiography and lipids were detected with Rhodamine 6G (silica gel paper chromatograms) or by exposure to iodine vapors (TLC plates).

^{14}C-labelled fatty acid methyl ester analysis was carried out in a Barber Colemann (Model 5000) gas-liquid chromatograph programmed from 145 to 190°C with argon gas as carrier under 200 pounds pressure. The flow rate was 60 ml per min. through a glass column of 6 feet and 1/4 inch internal diameter packed with diethyl glycol succinate. Of the samples injected, 1/10 was directed to the detector (hydrogen flame ionization detector) and the remainder to the counter where it was oxidized over a copper oxide furnace and put through a proportional counter system operated at 2500 volts. The output from the proportional counter was calibrated with authentic ^{14}C-methyl palmitate of known specific activity. The component peaks were identified by their retention time relative to authentic standard methylesters of palmitic, stearic, oleic, linoleic and linolenic acids (obtained from Applied Science Corporation, State College, Pa.).

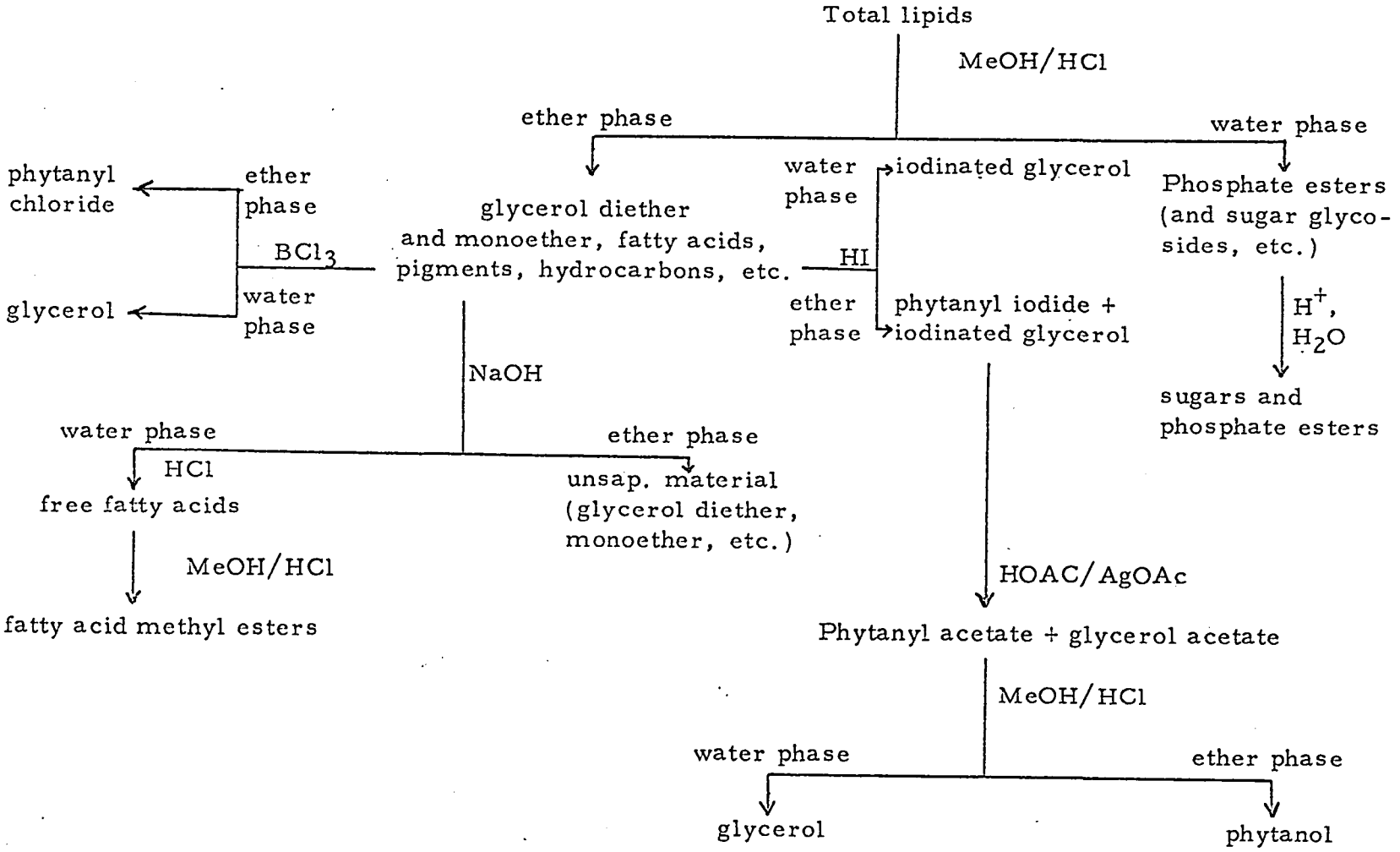
To determine ^{14}C activity in the phytanyl groups of the glycerol diether, a portion of the total petroleum ether-soluble fraction of the hydrolysate described above was heated under reflux in 1 ml of 47% HI for 5 hr in a flask with side-tube (Kates et al, 1965, 1966 and Kates, 1964). After dilution of the hydrolysate with 4 ml of water, the phytanyl iodide formed was extracted with several 5 ml portions of petroleum ether. The ether extracts were concentrated under a stream of nitrogen and made up to 10 ml with chloroform; the aqueous phase was diluted to 10 ml with water. Portions of the chloroform and of the aqueous solutions were plated on aluminum planchets and counted for ^{14}C-activity.

To prepare another phytanyl derivative and to check for the presence of any iodinated glycerol in the ether extract, the following reactions were carried out. A portion of the chloroform solution of the phytanyl iodide was concentrated to dryness under a stream of nitrogen, and the residue was dissolved in 5 ml of glacial acetic acid and stirred and heated under reflux with 0.3 g of silver acetate for 20 hr. The mixture was diluted with ethyl ether (10 ml), freed from silver salts by centrifugation, and the ether solution was evaporated to dryness in a stream of nitrogen. The residue was methanolyzed in 5 ml of 2.5% methanolic-HCl under reflux for 1 hr.; 0.5 ml of water was added and the mixture was extracted with petroleum ether. The ether extracts containing phytanol, and the water-methanol phase containing any glycerol were concentrated to a small volume in a stream of nitrogen and made up to 10 ml with chloroform and water, respectively; suitable aliquots were counted for ^{14}C-activity. Phytanyl iodide and phytanol were subjected to

thin layer chromatography on silica gel H using heptane, or chloroform-ethyl ether (9:1) as solvents. Glycerol was chromatographed on Whatman No. 1 paper with the pyridine-ethyl acetate-water solvent.

To obtain the glycerol moiety of the glycerol diether more directly, a portion of the diether fraction from the ^{14}C-glycerol precursor was treated with boron trichloride in chloroform solution at room temperature overnight (Kates et al, 1965). The mixture was evaporated almost to complete dryness under a stream of nitrogen and the residue was suspended in distilled water. The products were separated into ether-soluble (phytanyl chloride) and water-soluble (glycerol) fractions and each fraction was counted for ^{14}C-activity and chromatographed as described above. The procedures of degradation of the lipids are summarized in Scheme III.

SCHEME III. Degradation of Lipids of H. cutirubrum



4. Studies on Metabolism of α-Glycerophosphate

(a) Incorporation of ^{32}P from orthophosphate-^{32}P and rac-3-GP-^{32}P in H. cutirubrum cells

H.cutirubrum cells (0.8 mg dry weight suspended in 2.0 ml salt solution) were grown in 100 ml of the standard culture medium for extreme halophiles (Sehgal and Gibbons, 1960) containing 500 μcuries of either ^{32}P-orthophosphate or rac-3-GP-^{32}P (13 μmoles) (specific activity 10.7×10^6 cpm/μmole); incubation was carried out in 500 ml Erlenmyer side-arm flasks at 37° with shaking. The growth curve was followed by measuring the optical density (using Coleman Jr. Spectrophotometer at 660 mμ). Two-ml samples of the culture were withdrawn at different time intervals and the lipids were extracted by a modification of the method of Bligh and Dyer (1959) as described above. Total lipids were counted and chromatographed by Marinetti's procedure. Labelled spots on the chromatogram were located by autoradiography, cut out and counted. Water-soluble organic phosphate esters and inorganic phosphates in the methanol-water phase of the Bligh and Dyer extractions were estimated using the method of Berenblum and Chain (1938) as described above.

(b) Incorporation of ^{14}C and ^{32}P from sn-3-GP-^{14}C and rac-3-GP-^{32}P in H. cutirubrum cells

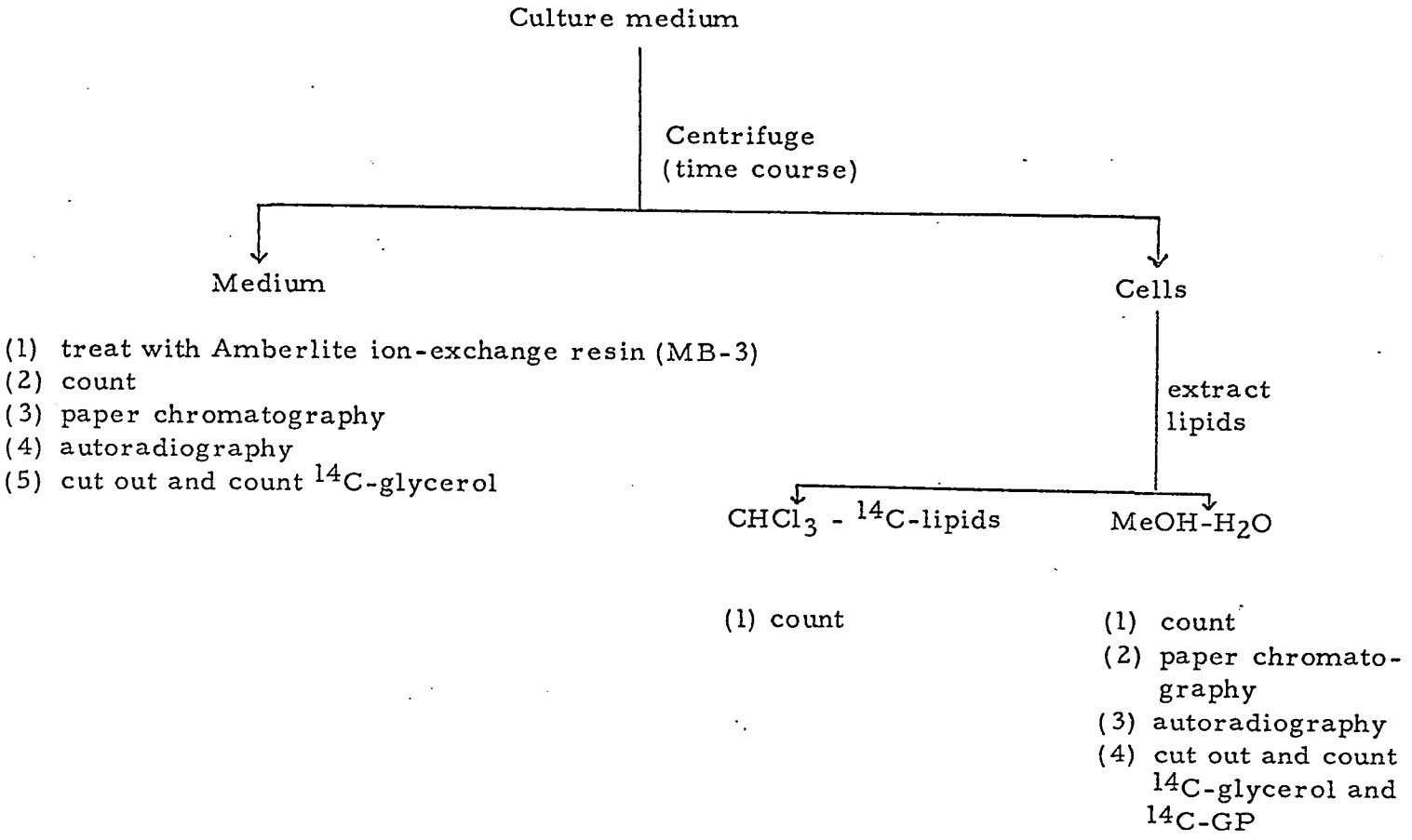
23.0 ml of extreme halophile culture medium (Sehgal and Gibbons, 1960) were inoculated with 1 ml of H. cutirubrum cell suspension prepared as described previously. Incubation was carried out at 37° in 250 ml side-arm Erlenmyer flasks in the presence of 2.0 μmoles

sn-3-GP-^{14}C (specific activity 4.5 x 10^5 cpm/μmole) or 8.0 μmoles rac-3-GP-^{32}P (specific activity 10.8 x 10^6 cpm/μmole). Growth was followed by measuring the optical density at 660 mμ on Coleman Jr. Spectrophotometer. For the different analytical measurements, samples of 2.0 ml were withdrawn at different time intervals and the cells were centrifuged down at 800 x g for 15 minutes at 0°C. The cells and supernatant (medium) were then treated as follows (see Schemes IV and V):

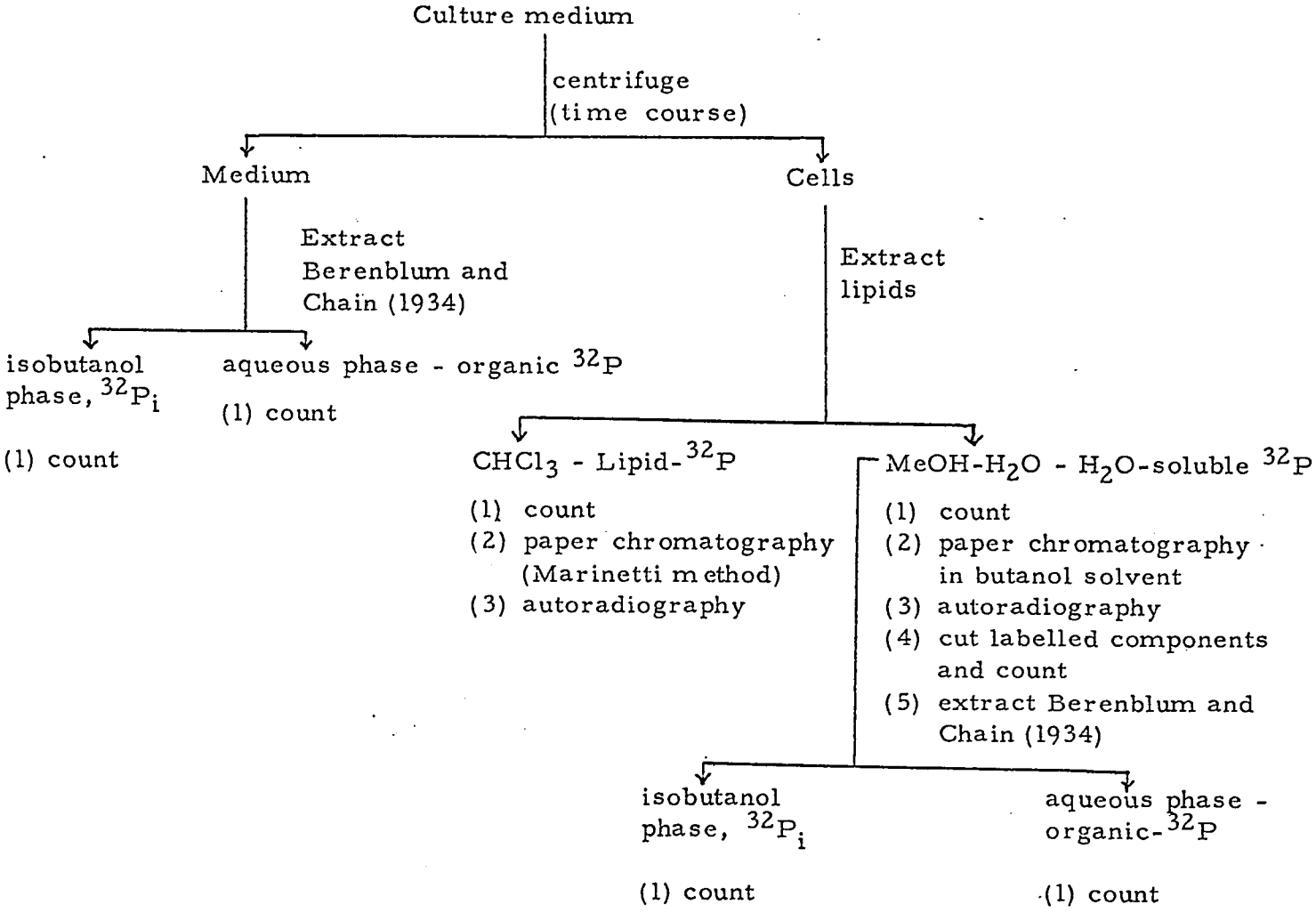
(i) With rac-3-GP-^{32}P as substrate: - Suitable aliquots of the supernatant (medium) were counted and assayed for organic water-soluble-^{32}P (^{32}P-GP) and inorganic-^{32}P as described above (Berenblum and Chain, 1934). The cells were extracted by a modification of the method of Bligh and Dyer (1959) (see page 48). Suitable aliquots of the chloroform layer containing the lipids were counted and the phosphatides were identified by paper chromatography and autoradiography. Aliquots of the methanol-water layer were counted, assayed for organic water-soluble-^{32}P and inorganic-^{32}P by the Berenblum and Chain procedure (1959), and by paper chromatography, autoradiography and counting the lablled components. The results represent intracellular glycerophosphate-P and inorganic-P, respectively.

(ii) With sn-3-GP-^{14}C as substrate: - A suitable aliquot of the supernatant (medium) after repeated treatment with mixed Amberlite anion-cation exchange resins (MB-3) to remove salts and GP-^{14}C, was counted for free ^{14}C-activity and chromatographed on paper in pyridine-ethyl-acetate-water 2:5:5 (v/v/v, upper phase) to

SCHEME IV. The Incorporation Studies of sn-3-GP-^{14}C by H. cutirubrum cells



SCHEME V. The Incorporation Studies of rac-3-GP-^{32}P in H. cutirubrum Cells



confirm the identity of ^{14}C-glycerol. The results represent extracellular free glycerol. The cells were extracted as described above and a suitable aliquot of the chloroform phase was counted for ^{14}C activity. Aliquots from the alcoholic-water layer were counted for total ^{14}C-activity and assayed for water soluble ^{14}C-GP and ^{14}C-glycerol by paper chromatography and autoradiography, and the radioactive components were cut out and counted; the results represent cellular ^{14}C-GP and ^{14}C-glycerol.

(c) Glycerophosphatase activity of H. cutirubrum cell fractions

(i) Optimum pH; - The pH optimum for the hydrolysis of rac-3-GP-^{32}P by H. cutirubrum cell fractions was determined by a method similar to that described by Kates (1954, 1955) as follows. 4.5 ml of each cell fraction [prepared as described in Section B, III, (b)], total cell free homogenate (containing 1.9 mg protein/ml), envelope fraction (containing 1.3 mg protein/ml) and supernatant fraction (containing 0.7 mg protein/ml), was incubated separately with 0.8 µmoles rac-3-GP-^{32}P (specific activity 10.8×10^6 cpm/µmole) for 2 hours at 37° in 0.2 M Tris-HCl buffer at pH 4.0, 4.5, 5.0, 5.5, 6.0 and 7.0 in a final volume of 5.0 ml. At the end of the incubation period, 1 ml aliquots were withdrawn and assayed for inorganic phosphate-^{32}P by the method of Berenblum and Chain (1948) as described above. Tubes containing substrate and buffer but with 4M salt solution replacing the cell fractions served as controls. It was

found that the optimum pH for the hydrolysis of rac-3-GP-^{32}P was pH 5.0 for all cell fractions (Fig. 4).

(ii) Time course: - The reaction mixtures and experimental procedures described for determining the optimum pH were used for the time studies except that the incubation period varied from 0.5 to 2.0 hours, and the pH was kept at the optimum pH, 5.0.

(d) Glycerokinase activity of H. cutirubrum

(i) Assay of glycerokinase activity in total cell-free homogenate of H. cutirubrum: - The glycerokinase activity was assayed enzymatically using a method essentially similar to that of Bublitz and Kennedy (1954 a and b), Wieland and Suyter (1957 a and b) and Kennedy (1962). The method is based on spectrophotometric determination of NADH produced in the oxidation process of sn-3-glycero-phosphate to dihydroxyacetone phosphate in the presence of NAD and catalyzed by the enzyme α-GP dehydrogenase at an alkaline pH. The detailed assay procedure was as follows. Total cell-free homogenate of H. cutirubrum cells (3.75 ml, 1.9 mg protein per ml), was incubated with 62.5 μmoles $MgCl_2$, 125 μmoles KF, 250 μmoles Tris-HCl buffer pH 7.5, 20 μmoles glycerol, 50 μmoles dithiothreitol (Cleland's reagent) and 100 μmoles ATP neutralized with NaOH in a final volume of 5.0 ml. A control experiment was carried out simultaneously using the same reaction mixture except that ATP was replaced by the 4M salt solution. The reaction tubes were incubated at 35°C in a water bath with gentle shaking. One ml aliquots were taken at different time intervals and pipetted into 1 ml of 0.2 N cold metaphosphoric acid and the precipitated

protein was removed by centrifugation. As a zero hour control 0.90 ml of the mixture was pipetted into 1 ml of 0.2 N metaphosphoric acid; then 100 µl of a solution containing 20 µmoles ATP was added.

The glycerophosphate formed was assayed as follows. 1.0 ml of clear supernatant was neutralized with 1.0 ml of 0.1 N NaOH; 0.5 ml of the neutralized solution was incubated for one hour at room temperature with 1.5 ml 1.0 M hydrazine-hydrazine hydrochloride buffer of pH 9.4, 100 µl of 25 mM NAD, 150 µl of 1.0 M KF, 150 µl of 1% nicotinamide-sodium carbonate buffer and 40 µl of the enzyme, α-glycerophosphate dehydrogenase (from rabbit muscle - A grade - Calbiochem. 170 IU/ml-4 mg protein/ml). The optical density was recorded at 340 mµ on a Cary recording spectrophotometer. The concentration of sn-3-glycerophosphate in the reaction mixture was calculated from standard curves using synthetic sn-3-GP. It was found that about 0.9 µmoles sn-3-GP was produced within 90 minutes under the above reaction conditions (Fig. 6).

(ii) Synthesis of α-glycerophosphate (α-GP) by H. cutirubrum cell fractions: - The enzymatic procedure was essentially the same as that described by Bublitz and Kennedy (1954, a and b) and Chang and Kennedy (1967). 3.5 ml of each of the H. cutirubrum cell fractions (total homogenate, 7.35 mg protein; envelope, 4.6 mg protein; supernatant, 2.45 mg protein) was separately incubated for 3.5 hours at 37° with 100 µmoles ATP, 250 µmoles Tris-HCl buffer of pH 7.5; 62.5 µmoles $MgCl_2$, 125 µmoles KF, 1.1 µmole 1,3-^{14}C-glycerol (10 µcuries), 20 µmoles "cold" glycerol, and 50 µmoles

β-mercaptoethanol in a final volume of 5.0 ml. The incubation period was terminated by placing the reaction tubes in a boiling water-bath for 5 minutes and then they were centrifuged briefly. The clear supernatant was applied to a Dowex-1-formate column and the radioactive α-GP was eluted as described above for standard synthetic sn-3-GP-^{14}C. The combined radioactive fractions were taken to dryness under reduced pressure and the residues were dissolved in a few milliliters of distilled water. The solutions were neutralized with ammonia, treated with acid washed charcoal to remove nucleotides and filtered as described above and finally made up to 5.0 ml with distilled water. Suitable aliquots were taken for counting, determination of total and inorganic phosphorus, α-GP-phosphorus and enzymatic determination of sn-glycero-3-phosphate.

(iii) Identification of the glycerophosphate isomer(s) produced by H. cutirubrum cell fractions: - To identify the isomer(s) of ^{14}C-GP formed, 0.5 ml aliquots of the glycerophosphate solutions were assayed for total α-GP-P by the periodate procedure (Pande et al, 1967) and for sn-glycerol-3-phosphate by the specific enzymatic procedure of Kennedy (1962); Bublitz and Kennedy (1954, a and b) as follows: 0.5 ml of the charcoal treated GP solutions (containing 0.3 - 0.4 μmole GP) was incubated with 2.4 mmoles hydrazine-HCl buffer pH 9.8, 10 μmoles NAD, 2 mg bovine serum albumin, 25 μl sn-3-GP-dehydrogenase (from rabbit muscle - 170 IU/ml and 4 mg protein/ml, Calbiochem Corp.) in a final volume of 1.50 ml. The reaction mixture was incubated for 20 minutes at room temperature, then 1.5 ml of 95% ethanol was added and the tubes were centrifuged briefly. The

absorbency of the clear supernatant solutions at 340 mμ, due to NADH formation, was recorded against a control, which received identical treatment except that α-GP dehydrogenase was added after the ethanol.

For further identification of the ^{14}C-GP isomer(s), aliquots of the GP-dehydrogenase reaction mixtures, after dilution with ethanol, were paper chromatographed in t-butanol-water-picric acid (80:20:4, v/v/w) and the chromatograms were autoradiographed.

(e) α-Glycerophosphate dehydrogenase activity of H. cutirubrum cell fractions

Glycerophosphate dehydrogenate activity in H. cutirubrum cell fractions was detected by a modification of the procedures of Bublitz and Kennedy (1954, a and b) and Kennedy (1962) as follows. Aliquots of H. cutirubrum total cell-free homogenate, 0.25 ml (2.0 mg protein/ml), envelope fraction, 0.25 ml (1.7 mg protein/ml) and supernatant fraction, 0.25 ml (0.5 mg protein/ml) were separately incubated at room temperature for 2 hours with 2.4 mmoles hydrazine-HCl buffer of pH 9.7, 10 μmoles NAD and 0.2 μmoles rac-3-GP-^{32}P (specific activity 11.4×10^6 cpm/μmole) in a final volume of 1.5 ml. In another experiment, sn-3-GP-^{14}C (specific activity 6.5×10^5 cpm/μmole) was used instead of rac-3-GP-^{32}P, in the same system, to examine the stereospecificity of glycerophosphate dehydrogenase. The incubation period was terminated by the addition of 6.0 ml of 95% ethanol and the tubes were centrifuged briefly. Control experiments were carried out in which authentic α-GP dehydrogenase (from rabbit

muscle) was used instead of the H. cutirubrum cell fractions. "Zero time" tubes received identical treatment except that the additions of H. cutirubrum cell fractions or authentic α-GP dehydrogenase was made after that of ethanol. Suitable aliquots of the ethanol-water supernatants (3.0 ml) were evaporated to a small volume, centrifuged briefly and paper chromatographed with tert. -butanol-water-picric acid (80:20:4, v/v/w); the radioactive spots located by autoradiography were cut out and counted.

5. Fatty Acid Synthetase Activity in H. cutirubrum Total Cell-free Homogenate

Fatty acid synthetase system in H. cutirubrum total cell-free homogenate prepared as described in Section B, III, (a) was tested by the procedure described by Lennarz et al (1962) and Norris et al (1964) as follows. 0.15 ml of total cell-free homogenate containing 2 mg protein/ml was incubated with 1 μmole NAD, 3 μmoles glucose-6-phosphate, 5 μmoles β-mercaptoethanol, 2μl of glucose-6-phosphate dehydrogenase (from Calbiochem. Corp., 1 mg or 200 enzyme units/1 ml), 50 μmoles phosphate buffer pH 7, 75 μmoles acetyl CoA, and 25 μmoles ($5.5. \times 10^6$ dpm) 1, 3-^{14}C-malonyl-CoA (from New England Nuclear Corp.) in a final volume of 0.5 ml. The final salt concentration was about 1.6 M. A similar experiment was carried out at a salt concentration of 3.6 M achieved by preparing the phosphate buffer in 4 M salt solution. The reaction mixtures were incubated at 30° for 2 hours. The reaction was stopped by the addition of 1.25 ml of methanol followed by 0.75 ml of $CHCl_3$ and the lipids were extracted by a modification of the procedure

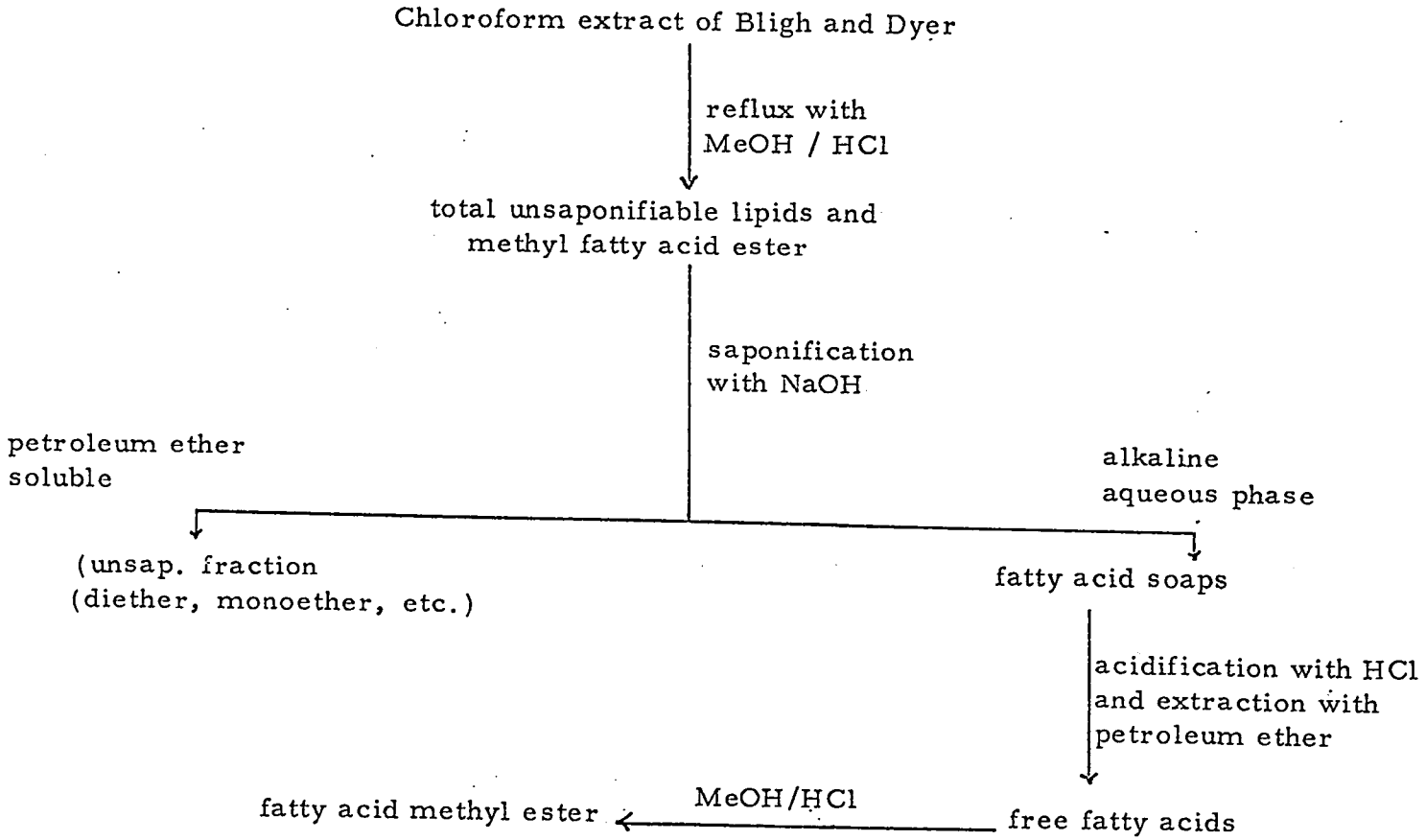
of Bligh and Dyer (1959) as described in Section B, IV. Control tubes contained all the components except the total cell-free homogenate which was added after the addition of methanol and chloroform.

The lipid fraction obtained by the Bligh and Dyer extraction procedure was refluxed for one hour with 4.5 ml of 2.7% methanolic HCl in a 50 ml Erlenmyer flask with side-arm tube. The mixture was made alkaline by addition of 0.5 ml of 7 N NaOH and refluxing was continued for 1 hour. The total unsaponifiable lipids were extracted with petroleum ether, counted for ^{14}C-radioactivity and subjected to TLC and autoradiography. The free fatty acids were extracted after acidification of the aqueous alkaline solution from the saponification process with 6 N HCl; they were then counted and subjected to TLC and autoradiography. The methyl fatty acid esters were prepared by refluxing the free fatty acids in 4.5 ml of 2.7% methanolic HCl for one hour in the same side-arm Erlenmyer flask, 0.5 ml of H_2O was added and the methylesters were extracted with petroleum ether, counted and chromatographed. Scheme VI outlines the above reaction procedures.

6. Short Term Labelling of H. cutirubrum Cells with ^{32}P-orthophosphate

Cells of H. cutirubrum grown in 50 ml of standard complex medium for halophiles (as described above) were harvested at the stationary phase (3 days) by brief centrifugation and washed twice with 4M salt solution. The cells were resuspended in 250 ml side-arm Erlenmyer flasks containing 48.5 ml of modified (low phosphate) complex medium composed of (g/l): yeast extract 5, casamino acids 3.7,

SCHEME VI. For Separation and Isolation of Unsaponifiable Lipids and Fatty Acids



sodium citrate 3, KCl 2, $MgSO_4 . 7H_2O$, 20, NaCl 250, and 0.05 $FeSO_4 . 7H_2O$ and the pH was adjusted at 6.7. 1.5 mc of ^{32}P-orthophosphate in 1.5 ml H_2O neutralized to phenol red was added to the cell suspension and the culture was incubated at 37° with shaking. Growth was followed by measuring O.D. at 660. Samples of 1.0 ml were withdrawn at zero time, at 1 min, and at 2, 5, and 10 minutes and immediately added to 3.75 ml of methanol-chloroform (2:1, v/v). After several hours, 1.25 ml each of chloroform and water was added, mixed and centrifuged. Suitable aliquots from the chloroform layer were counted and chromatographed on silicic acid-impregnated paper with Marinetti's solvent. Labelled components located by autoradiography were cut out and counted.

C. RESULTS

I. Radioisotopic Studies on the Biosynthesis of the Glyceryl Diether Lipids of Halobacterium cutirubrum

1. Overall Incorporation of Radioisotopes into Total Lipids

When cells of H. cutirubrum were grown in the ^{14}C-labelled precursors given in Table 1, it was found that mevalonate showed the highest degree of incorporation, amounting to 8% of the tracer supplied. Acetate and glycerol each showed about 2% incorporation, but only traces of ^{14}C appeared in the lipids when malonate was supplied. The ^{14}C-specific activities of the lipids were also in the same order (Table 1).

Incorporation of ^{32}P-orthophosphate to the extent of 1.5% of that supplied was observed under growth conditions comparable to those for the ^{14}C precursors (Table 1). Considering that the complex growth medium contained inorganic phosphate which diluted the ^{32}P-orthophosphate, the observed incorporation of ^{32}P was quite reasonable. The ^{35}S-sulfate, however, was very poorly incorporated when cells were grown in the standard culture medium (Sehgal and Gibbons, 1960), because of dilution of the tracer with "cold" sulfate from the $MgSO_4$ in the medium. When the $MgSO_4$ was replaced by $MgCl_2$, incorporation of ^{35}S into the lipids was increased about forty-fold, but was still low on a percentage basis (Table 1).

TABLE 1

Incorporation of Radioisotopes from Various Labelled Precursors into the Total Lipids of *H. cutirubrum*

Precursor supplied*		μC per ml culture	Radioisotope incorporated into total lipids		Total lipids		Specific Activity	
			mμC per ml culture	% of activity supplied	μg lipid-P per ml culture	μg lipid per ml culture†	mμC per μg lipid-P	mμC per mg lipid
1-^{14}C-Acetate		1.0	19	1.9	0.79	20	-	950
2-^{14}C-Malonate		1.0	0.9	0.	0.66	17	-	50
2-^{14}C-Mevalonate		1.0	82	8.2	0.75	19	-	4300
U-^{14}C-Glycerol		1.0	20	2.0	0.92	23	-	870
^{32}P-Orthophosphate		2.0	30	1.5	1.4	35	22	-
^{35}S-Sulfate **	(a)	5.0	0.2	0.004	-ϕ	-ϕ	-	-
	(b)	8.0	8	0.1	-ϕ	-ϕ	-	-

* Cells were grown in the presence of the indicated amount of labelled precursor in the standard culture medium for extreme halophiles at 37° to their stationary phase, except for experiments with ^{35}S-sulfate (see footnote **). For detailed experimental procedure see Materials and Methods Section.

† Calculated from lipid-P content thus: μg lipid/ml culture = μg lipid-P/ml culture x 25

** Values for (a) obtained when cells were grown in standard medium containing $MgSO_4$; values for (b) were obtained when $MgSO_4$ was replaced by $MgCl_2$.

ϕ Not determined.

2. Distribution of Radioisotopes among Individual Lipid Components

The distribution of ^{14}C among the lipid components was found to vary with each of the precursors supplied (Plate I, Table 2). Almost half of the ^{14}C derived from mevalonate was associated with the major lipid phosphatidyl glycerophosphate (Spot 6 and 7, Plate I), while the pigments and neutral lipids (Spot 9, Plate I), the glycolipid sulfate (Spot 2, Plate I), and phosphatidyl glycerol (Spot 8, Plate I) had much lower (and about equal) proportions of ^{14}C activity. In contrast, the highest proportions of ^{14}C derived from either acetate or glycerol were associated with the glycolipid sulfate, and progressively lower proportions appeared in phosphatidyl glycerophosphate, phosphatidyl glycerol and the pigment and neutral lipid fractions. It may also be noted that the unidentified component (Spot 4, Plate I) was relatively heavily labelled with acetate or glycerol but less so with mevalonate.

The distribution of the traces of ^{14}C incorporated when malonate was used as precursor was almost the same as that with acetate (Table 2). This finding suggests that the incorporation observed with malonate is probably due to traces of acetate impurity.

The incorporation of ^{32}P-orthophosphate closely paralleled the distribution of lipid phosphorus among the components (Table 2). Thus, the major phosphatide, phosphatidyl glycerophosphate (Spot 6 and 7, Plate I) and the minor phosphatide, phosphatidyl glycerol

(Spot 8, Plate I) accounted for 77% and 8%, respectively, of the total ^{32}P incorporated into the lipids, values which are almost identical with the respective proportions of lipid-P in these components. The other minor phosphatide (Spot 4) which has not yet been identified, appears to be relatively strongly labelled with ^{32}P as well as ^{14}C, and also seems to contain some ^{35}S. This spot (Spot 4, Plate I) probably consists of a phosphatide and a sulfolipid which are not resolved by the chromatographic system used. It should be noted that the traces of ^{32}P found associated with Spots 1, 2 and 3 (Plate I) are probably due to streaking of the major phosphatide Spot 6, and are not considered to be part of these lipid molecules.

When ^{35}S-sulfate was used as precursor, 80% of the radioisotope incorporated into the lipids appeared in the glycolipid sulfate ester component (Spot 2, Plate I), and about 15% was associated with a slow-moving unidentified component (Spot 1, Plate I). The remaining 5% appeared to be associated with the minor unidentified component, Spot 4 (Plate I) mentioned above.

PLATE I

Chromatograms and autoradiographs of total lipids of H. cutirubrum cells grown in the presence of ^{32}P-orthophosphate, ^{14}C-labelled glycerol, mevalonate, acetate, or malonate, and ^{35}S-sulfate. Chromatograms were stained with Rhodamine 6G and viewed under ultraviolet light (abbreviations: B, blue; O, orange; Y, yellow, G, grey; w, weak; m, moderate; s, strong). Identity of spots: 1, unidentified sulfolipid; 2, glycolipid sulfate; 3, glycolipid; 4, unidentified phospholipid + sulfolipid; 6 + 7, phosphatidyl glycerophosphate; 8, phosphatidyl glycerol; 9, pigments + neutral lipids.

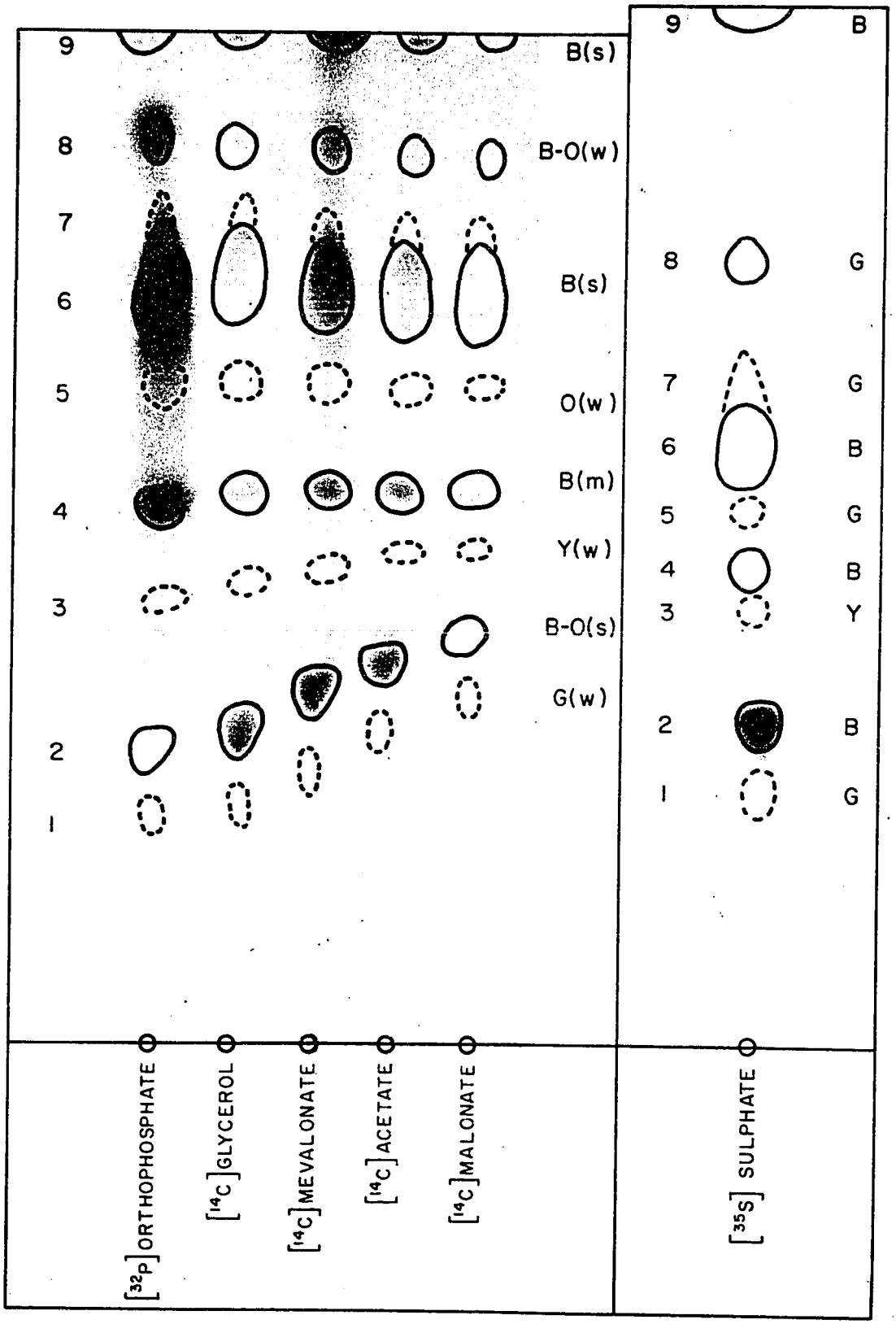

9
8
7
6
5
4
3
2
1
B(s)
B-O(w)
B(s)
O(w)
B(m)
Y(w)
B-O(s)
G(w)
[32P] ORTHOPHOSPHATE
[14C] GLYCEROL
[14C] MEVALONATE
[14C] ACETATE
[14C] MALONATE
9
8
7
6
5
4
3
2
1
B
G
G
B
G
B
Y
B
G
[35S] SULPHATE

TABLE 2

Distribution of Radioisotopes among Lipid Components

Spot No. *	Component	% of total radioisotope in total lipids					
		^{14}C-acetate	^{14}C-malonate	^{14}C-mevalonate	^{14}C-glycerol	^{32}P-phosphate	^{35}S-sulfate
Origin	-	3	0	2	2	0.3	0.2
1	Unidentified sulfolipid	3	3	1	4	0.4	13
2	Glycolipid sulfate	37	35	13	44	0.5	80
3	Glycolipid	1	1	1	2	0.4	0
4	Unidentified	15	14	7	13	11	6
5	Unidentified	2	3	3	1	2	0
6 + 7	Phosphatidyl glycero-phosphate	27	30	44	24	77	0
8	Phosphatidyl glycerol	7	8	13	6	8	0
9	Pigments + neutral lipids	6	7	16	4	0.5	0

* See Plate I. Distribution of lipid-P among components: Spots 1, 2, 3, 9, traces; Spot 4, 6%: Spot 5, 1%: Spot 6 and 7, 83%: Spot 8, 5%.

3. Distribution of Radioisotopes among the Lipid Moieties

Methanolysis of the total lipids of H. cutirubrum in 2.5% methanolic-HCl results in complete conversion of lipid-P and lipid-bound sugars to water-soluble forms, with concomitant liberation of the diphytanyl glycerol ether moiety which is soluble in petroleum ether (Kates et al, 1965; Bligh and Dyer, 1959). The distribution of ^{14}C among the water-soluble and petroleum ether-soluble products after methanolysis of the ^{14}C-labelled lipids (Table 3) showed that with all the precursors, except glycerol, more than 95% of the ^{14}C was incorporated into the petroleum ether-soluble fraction and only a few percent appeared in the water-soluble fraction. With glycerol as precursor, a considerable proportion (29%) of the ^{14}C was found in the water-soluble fraction, but most of the activity was still associated with the petroleum ether soluble fraction. The water-soluble ^{14}C was found mostly in the sugars (glucose, galactose, mannose) and phosphate esters (Plate II, A and B).

Autoradiograms of chromatograms of the petroleum ether-soluble fractions (Plates III and IV) showed that with each of the ^{14}C-labelled precursors almost all of the radioactivity was associated with the diphytanyl glycerol ether moiety of the lipids, but small to trace amounts of ^{14}C appeared to be associated with spots corresponding to monophytanyl glycerol, hydrocarbons [mostly squalene (Tornabene et al, 1969)] and fatty acid methyl esters.

TABLE 3

Distribution of Incorporated ^{14}C among Water-soluble and Ether-soluble Hydrolysis Products of Total Lipids of H. cutirubrum

Precursor	Distribution of ^{14}C, % of ^{14}C in total lipids		
	Ether-soluble fraction *	Water-soluble fraction **	Recovery
1-^{14}C-Acetate	97	1	98
2-^{14}C-Malonate	95	2	97
2-^{14}C-Mevalonate	98	2	100
^{14}C-Glycerol	74	29	103

* Contains mostly di-O-phytanyl glycerol with small to trace amounts of monophytanyl glycerol, pigments, hydrocarbons, and fatty acid methyl esters (see Plate III).

** Contains phosphate esters (glycerol diphosphate and glycerophosphate) derived from the diether phosphatides, and sugars (glucose, galactose, mannose) derived from the glycolipid sulfate (see Plate II).

PLATE II

Chromatogram and autoradiograph of water-soluble moieties of ^{14}C-labelled lipids from the ^{14}C-glycerol precursor. Material chromatographed: 1, water-soluble products of methanoic-HCl hydrolysis of total lipids, after further hydrolysis with aqueous HCl; 2, water-soluble products of BCl_3 cleavage of di-O-phytanyl-glycerol diether. Solvent: A, pyridine-ethyl acetate-water (2:5:5, upper phase); B, tert.-butanol-water-picric acid (80:20:4, v/v/w).

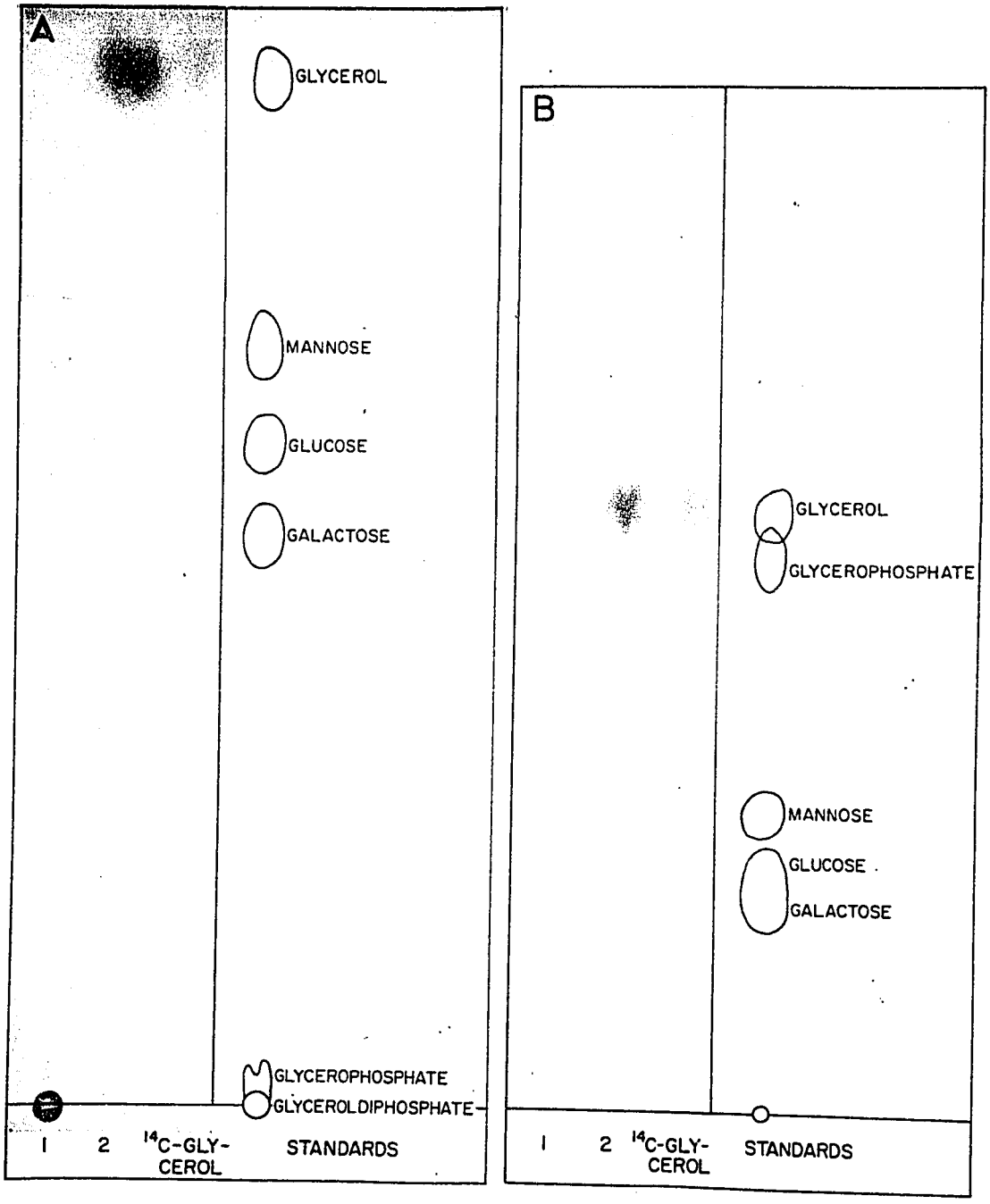

A
GLYCEROL
MANNOSE
GLUCOSE
GALACTOSE
GLYCEROPHOSPHATE
GLYCEROLDIPHOSPHATE
1
2
14C-GLY-CEROL
STANDARDS
B
GLYCEROL
GLYCEROPHOSPHATE
MANNOSE
GLUCOSE
GALACTOSE
1
2
14C-GLY-CEROL
STANDARDS

PLATE III

A thin-layer chromatogram and autoradiograph of ether-soluble products of methanolic-HCl hydrolysis of ^{14}C-labelled total lipids from the following precursors: 1, 1-^{14}C-acetate; 2, 2-^{14}C-malonate; 3, 2-^{14}C-mevalonate; and 4, U-^{14}C-glycerol; standard, synthetic α, β-di-O-phytanyl glycerol. Solvent, chloroform-ethyl ether (20:1, v/v). Identity of spots: a, monophytanyl glyceryl ether; b, diphytanyl glycerol; c, fatty acid methyl esters; d, hydrocarbons and pigments.

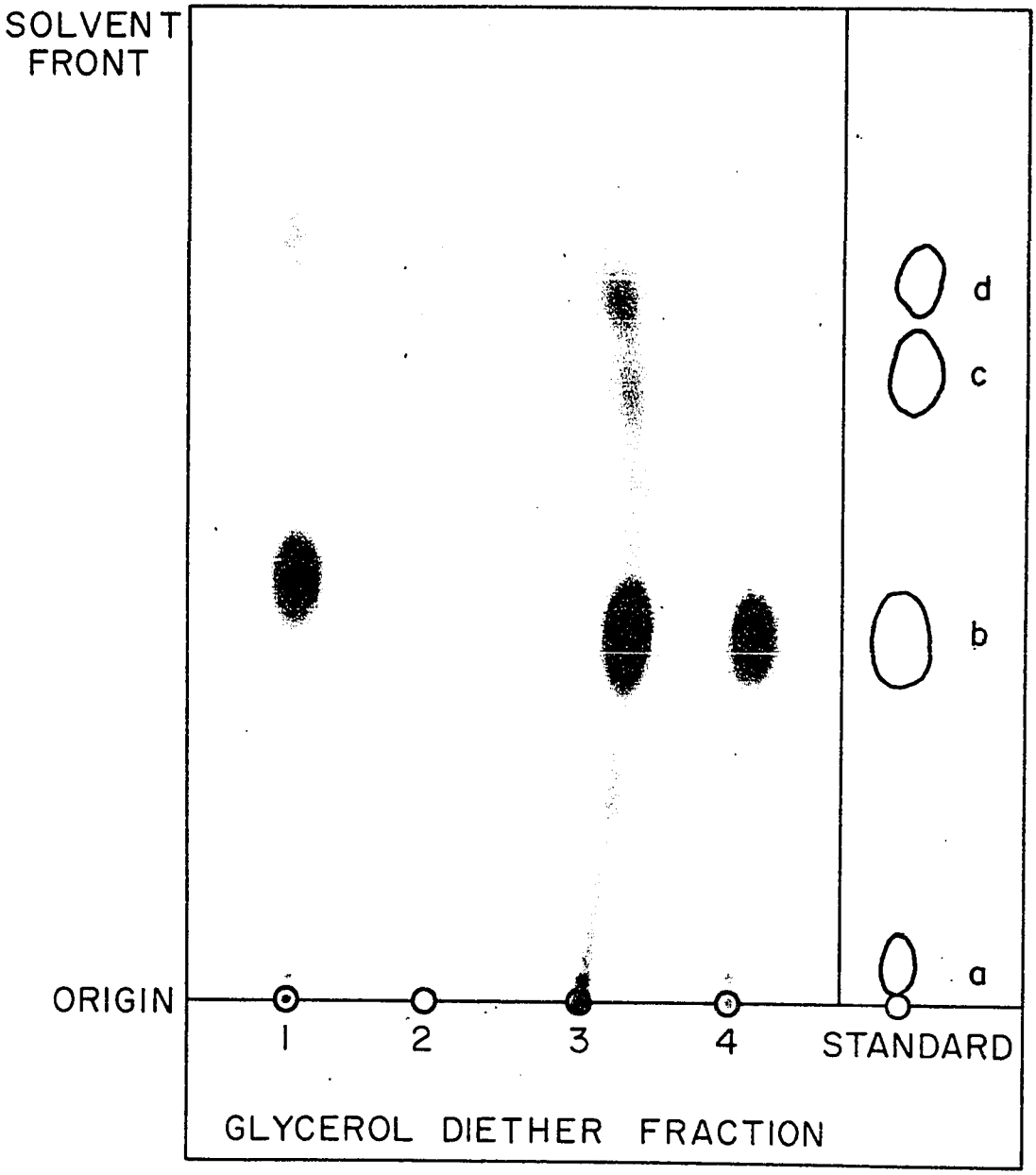
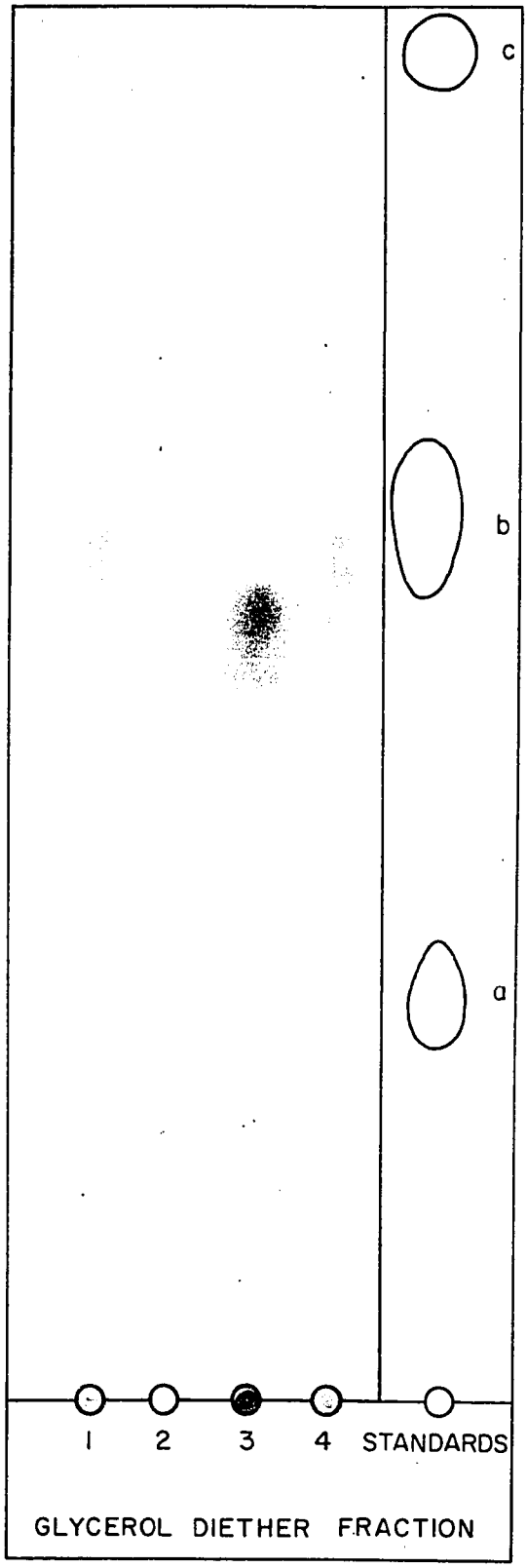

SOLVENT FRONT
d
c
b
a
ORIGIN
1
2
3
4
STANDARD
GLYCEROL DIETHER FRACTION

PLATE IV

Chromatogram and autoradiograph of ether-soluble products of methanolic-HCl hydrolysis of total ^{14}C-lipids on silicic acid impregnated paper with heptane-diisobutyl ketone (96:6) as solvent. Material applied, as in Plate III; identity of spots: a, fatty acids; b, diphytanyl glycerol; c, fatty acid methyl esters, hydrocarbons and pigments.


c
b
a
1
2
3
4
STANDARDS
GLYCEROL DIETHER FRACTION

4. ^{14}C-Labelling of the Phytanyl and Glycerol Moieties of the Diether

After hydrolysis of the glycerol diether fractions with HI and separation of the cleavage products into ether-soluble (phytanyl iodide) and water-soluble fractions, the proportions of the ^{14}C in the diether fractions found in the ether-soluble products were 97, 94 and 76% for the acetate, mevalonate and glycerol precursors, respectively (Table 4). With the malonate precursor, insufficient activity was available for accurate counting. When the ether-soluble products were chromatographed by thin-layer chromatography, most of the ^{14}C was found associated with the phytanyl iodide spot (Plate V). To verify the identity of the phytanyl iodide, the ether-soluble products from the mevalonate and acetate precursors were treated with silver acetate followed by methanolysis. After thin-layer chromatography of the resulting products, a new spot, corresponding to phytanol, contained most of the ^{14}C activity (Plate VI). Any iodinated glycerol which might have been present in the ether-soluble cleavage products would have been converted to free glycerol during the silver acetate-methanolysis treatment. When the water-soluble products of the latter treatment were examined chromatographically, however, no glycerol was detected and no ^{14}C appeared in the position on the chromatogram expected for glycerol. Examination of the HI-water phase for glycerol by paper chromatography was inconclusive because of the high concentration

TABLE 4

Distribution of Incorporated ^{14}C Water-soluble and Ether-soluble Hydrolysis Products of the Diether from H. cutirubrum Total Lipids

Precursor	Distribution of ^{14}C, % of ^{14}C in diether		
	Ether-soluble fraction *	Water-soluble fraction **	Recovery
1-^{14}C-Acetate	97 ***	traces	97
2-^{14}C-Mevalonate	94 ***	traces	94
U-^{14}C-Glycerol	76 ***	n.d.	-
	76 ****	24 ****	100

* Contains mostly phytyl group

** Contains mainly glycerol

*** Cleavage by HI

**** Cleavage by BCl_3

PLATE V

Thin-layer chromatogram and autoradiograph of ether-soluble products of HI cleavage of diphytanyl glycerol fraction from the following ^{14}C-labelled precursors: 1, acetate; 2, malonate; 3, mevalonate; 4, glycerol; standard, synthetic phytanyl iodide. Solvent: heptane; identity of spots: a, phytanyl iodide; b, c, unidentified.

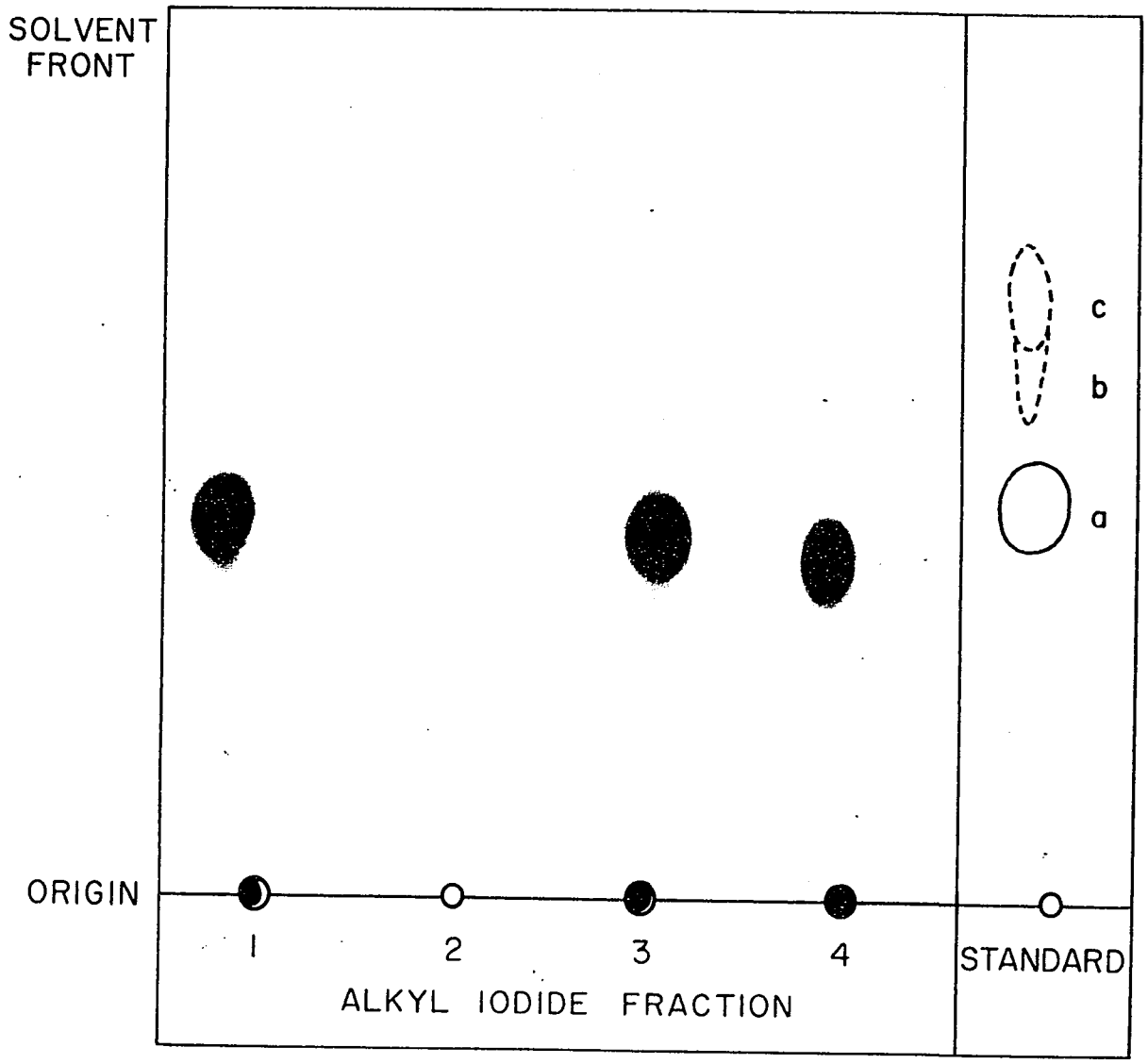
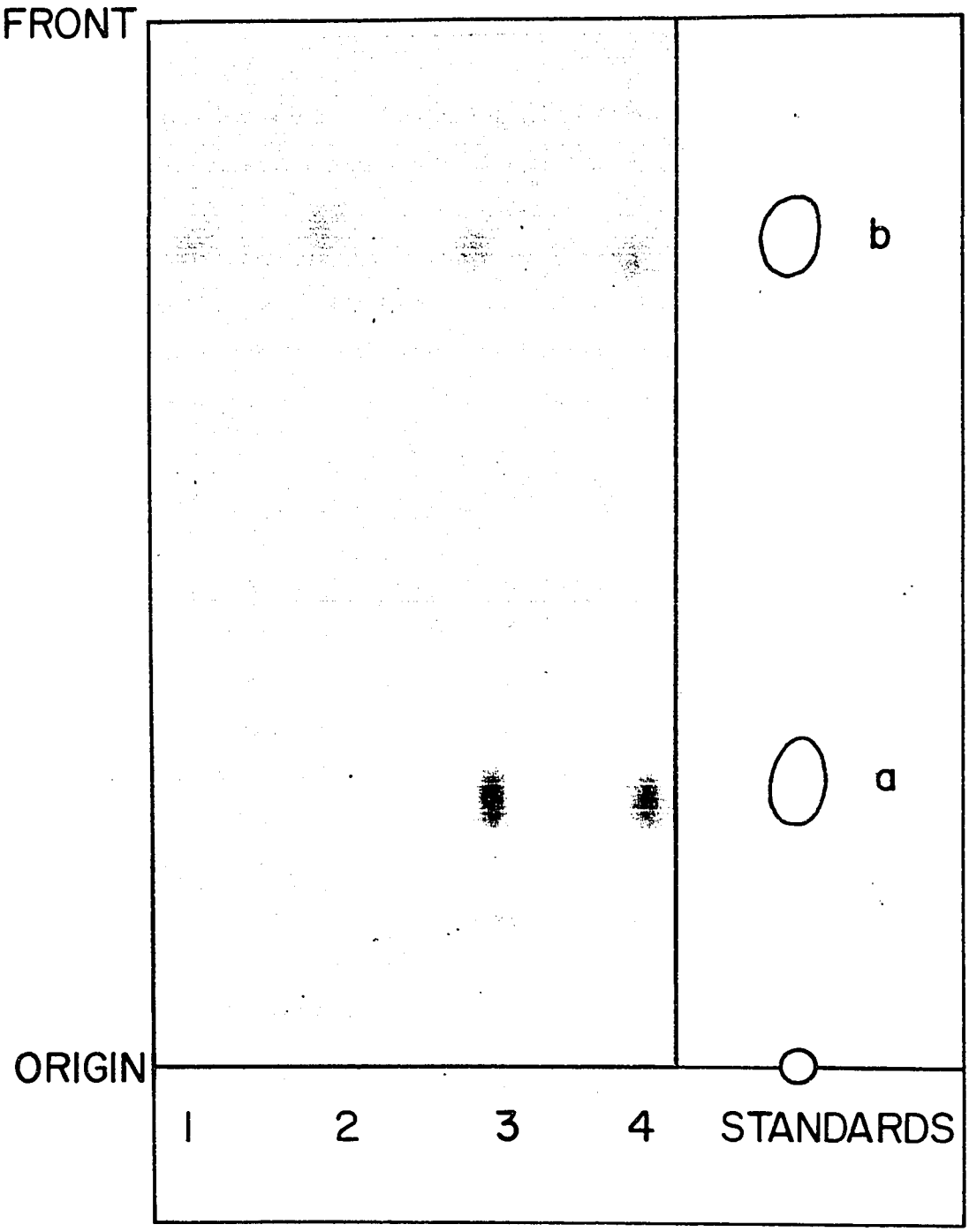

SOLVENT FRONT
c
b
a
ORIGIN
1
2
3
4
STANDARD
ALKYL IODIDE FRACTION

PLATE VI

Thin-layer chromatograph and autoradiograph of phytanyl iodide and phytanol derived from lipids labelled with ^{14}C-acetate or mevalonate. Material applied: 1, 2, phytyl iodide from mevalonate and acetate, respectively; 3, 4, phytanol from mevalonate and acetate, respectively; standards; (a) synthetic phytyl iodide and (b) phytanol. Solvent: chloroform-ether (20:1).


FRONT
b
a
ORIGIN
1
2
3
4
STANDARDS

of HI present. However, when the diether labelled with ^{14}C from the glycerol precursor was cleaved with boron trichloride, 76% of the radioactivity was found in the ether-soluble products (mostly phytanyl chloride) and the remainder (24%) was found in the water-soluble products (Table 4). Chromatography of the water-soluble products followed by autoradiography showed that the ^{14}C was associated entirely with glycerol (Plate II).

5. ^{14}C-Labelling of Fatty Acids

To check specifically for the presence of fatty acid methylesters, the petroleum ether-soluble methanolysis products from the lipids labelled with ^{14}C-acetate were saponified and the products separated into neutral (unsaponified) and fatty acid fractions, the latter being converted to methylesters by refluxing in methanolic-HCl (see Methods Section). Almost all of the activity (98%) remained with the unsaponifiable fraction and was associated mostly with the diphytanyl glycerol diether, but about 1.5% appeared in the fatty acid fraction. While much of the latter was associated with traces of glycerol diether carried over from the unsaponifiable fraction, a faint but definite radioactive spot, having the same mobility as methyl ^{14}C-palmitate was detected on thin-layer chromatography of the fatty acid methyl esters fraction (Plate VII). Gas-liquid chromatography of the methyl esters (Fig.1)

PLATE VII

Thin-layer chromatogram and autoradiograph of ether-soluble products of methanolic-HCl hydrolysis of total lipids labelled with ^{14}C-acetate precursor. Material applied: 1, total ether-soluble products; 2, unsaponifiable fraction (diphytanyl glycerol); 3, fatty acid fraction; 4, methyl esters of fatty acid fraction; 5, palmitic acid; 6, methyl palmitate. Solvent, chloroform-ethyl ether (9:1). Identity of spots: a, fatty acids and monophytanyl glycerol; b, diphytanyl glycerol; c, fatty acid methyl esters.

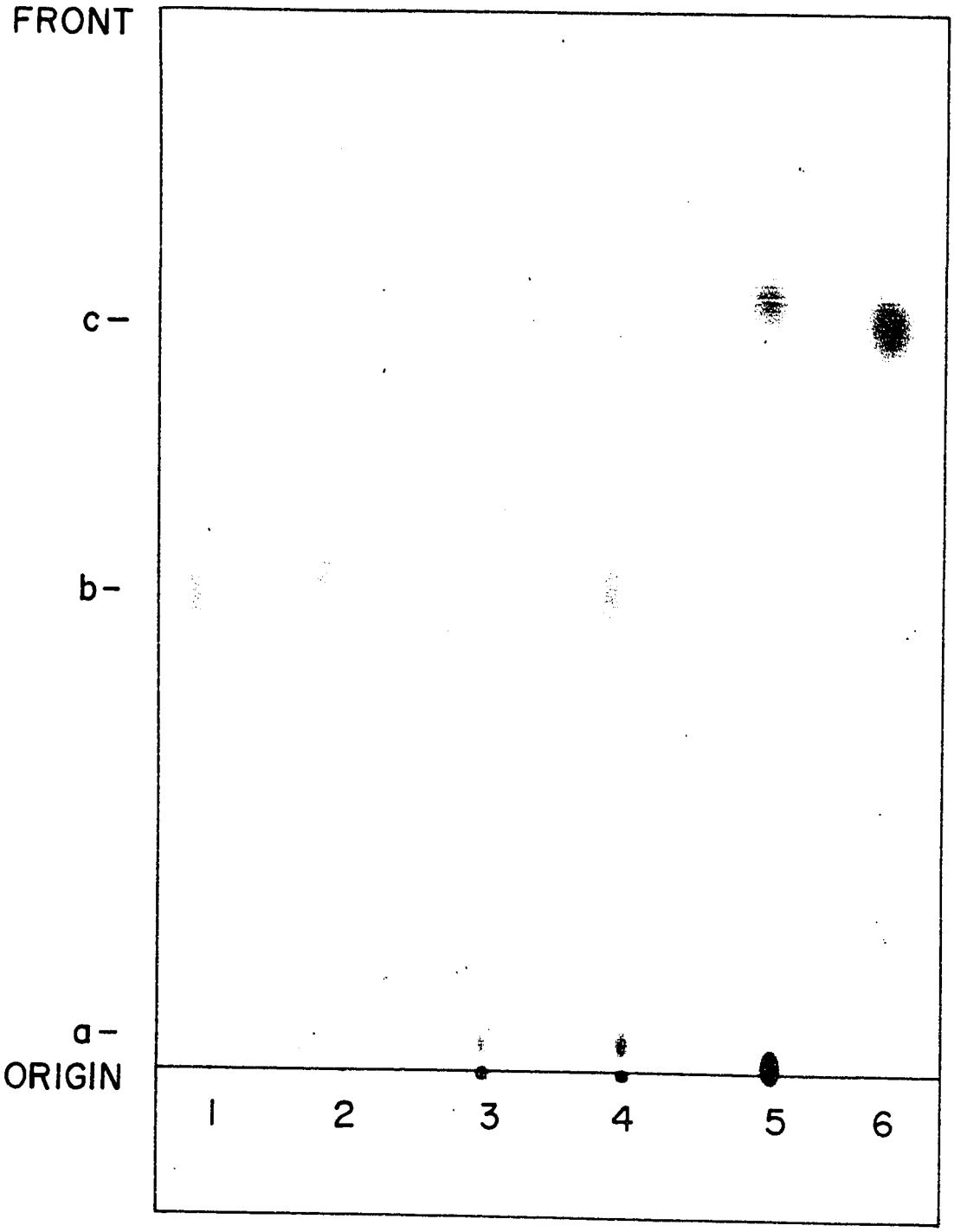

FRONT
c –
b –
a –
ORIGIN
1
2
3
4
5
6

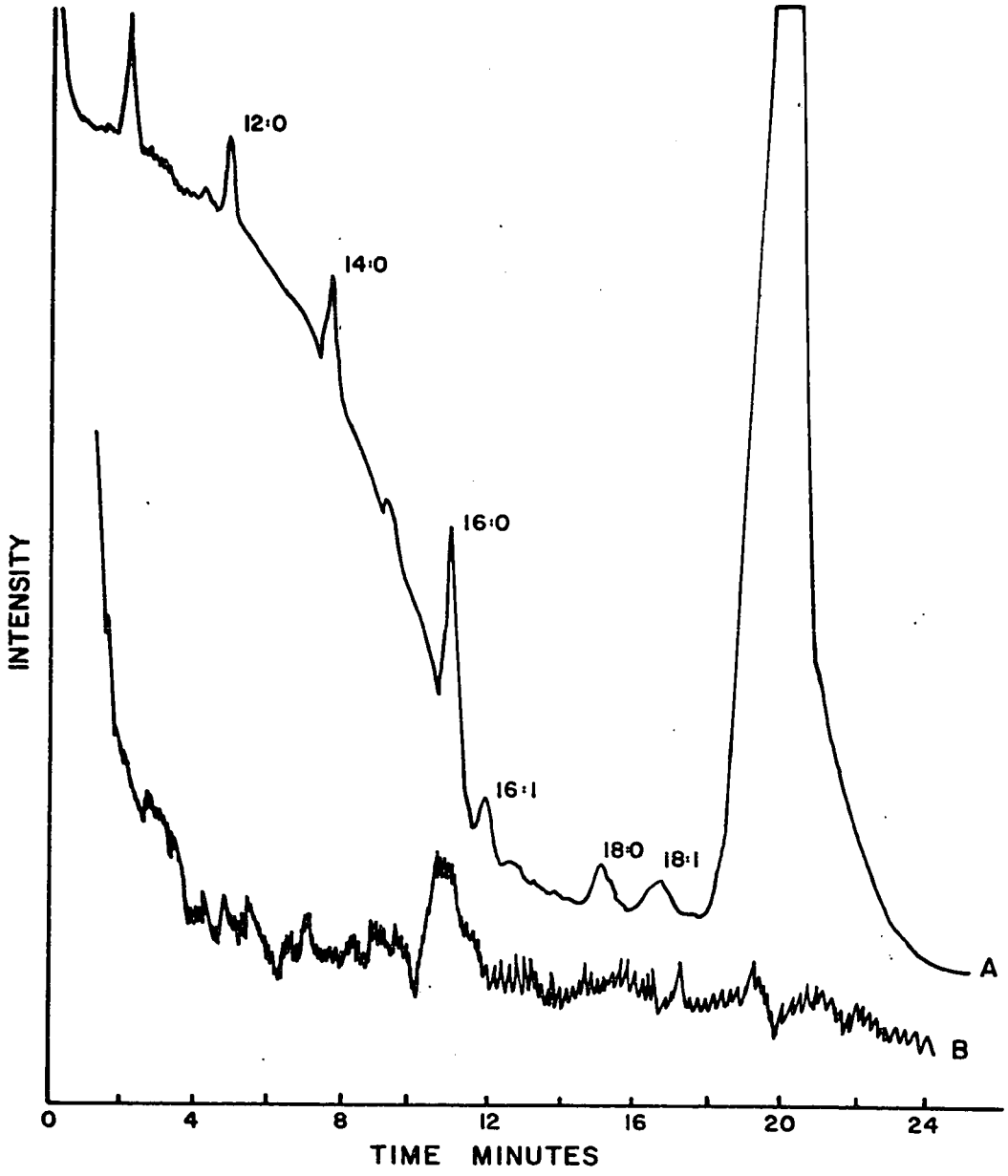
FIGURE 1

Gas-liquid radiochromatogram of methyl fatty-acid esters synthesized by H. cutirubrum using ^{14}C-acetate as a precursor. Identification of the components was based on their retention time relative to standard methyl fatty acid esters.

(A) is the mass record of peaks.

(B) is the radioactive record of peaks.

It should be noted that the radioactivity corresponds only to the palmitic acid peak (16:0). Extracts of the uninoculated halophiles complex growth medium had the same pattern of peaks but were not labelled (Kates et al, 1966).


12:0
14:0
16:0
16:1
18:0
18:1
A
B
INTENSITY
0
4
8
12
16
20
24
TIME MINUTES

showed that most of the ^{14}C was in palmitic acid. This finding is the first direct evidence that fatty acids are synthesized by H. cutirubrum, but it must be emphasized that the ^{14}C associated with the fatty acids eluted from the thin-layer chromatographic plate amounts to no more than about 0.5% of the ^{14}C incorporated into the total lipids with acetate as precursor.

The above results thus indicate that labelled acetate and labelled mevalonate were incorporated almost exclusively (more than 95% of the ^{14}C label) into the phytanyl groups of the diether moieties and only traces appeared in the glycerol and water-soluble moieties. In contrast, with radioactive glycerol about 56% of the total ^{14}C incorporated appeared in the phytanyl groups and the remainder (44%) was present in the glycerol moieties of the phosphatidyl glycerol and in the sugar groups of the sulfolipid. Only with ^{14}C-acetate was any ^{14}C observed in fatty acids, and the amount incorporated was only ca. 0.5% of the total ^{14}C in total lipids.

II. Biosynthesis of Fatty Acids

Demonstration of a Fatty Acid Synthetase System in H. cutirubrum

The finding that traces of labelled fatty acids were definitely formed with 1-^{14}C-acetate as precursor (Plate VII) suggested that a pathway for synthesis of fatty acid might be operative in H. cutirubrum. It was hoped to obtain more definite evidence on this point by the use of 2-^{14}C-malonate as precursor, but only traces of ^{14}C were incorporated into the lipids (Table 1) and were distributed among the lipid components and moieties in the same way as was the ^{14}C-activity from the acetate precursor (Table 2). Malonate itself being a dicarboxylic acid was apparently not taken up by the cells and the ^{14}C-activity obtained was probably due to incorporation of an acetate impurity. In order to settle the question of the existence of a malonyl-CoA fatty acid synthetase system, studies on the biosynthesis of fatty acids were carried out using a cell-free system, with ^{14}C-labelled malonyl-CoA as precursor, as described in Section B, VII, 5.

As shown in Table 5, 0.4 - 0.6% of the ^{14}C-labelling was found to be present in the fatty acid fraction, while the neutral unsaponifiable fraction (containing diether, monoether, etc.) contained no detectable radioactivity. It should be noted that lowering the salt concentration (from 3.8 M to 1.8 M) caused about 30% increase in the incorporation of ^{14}C activity in the fatty acids fraction (Table 5). Autoradiograms of chromatograms of the neutral fraction,

fatty acids fraction and their methyl esters (Plate VIII) confirmed that there was no radioactivity in the unsaponifiable fraction and that labelling was associated almost entirely with the fatty acids (and/or their methyl esters).

The trace incorporation of ^{14}C-activity into fatty acids either from 1-^{14}C-acetate (Fig. 1, Plate VII) or 1,3-^{14}C-malonyl-CoA (Table 5 and Plate VIII) as precursors, suggests that H. cutirubrum does contain a malonyl-CoA system for fatty acid biosynthesis. However, the fatty acid synthetase system is operating, obviously, at a low level of activity and it might be partially depressed by high concentrations of salt (Table 5).

TABLE 5

Biosynthesis of Fatty Acids by H. cutirubrum *

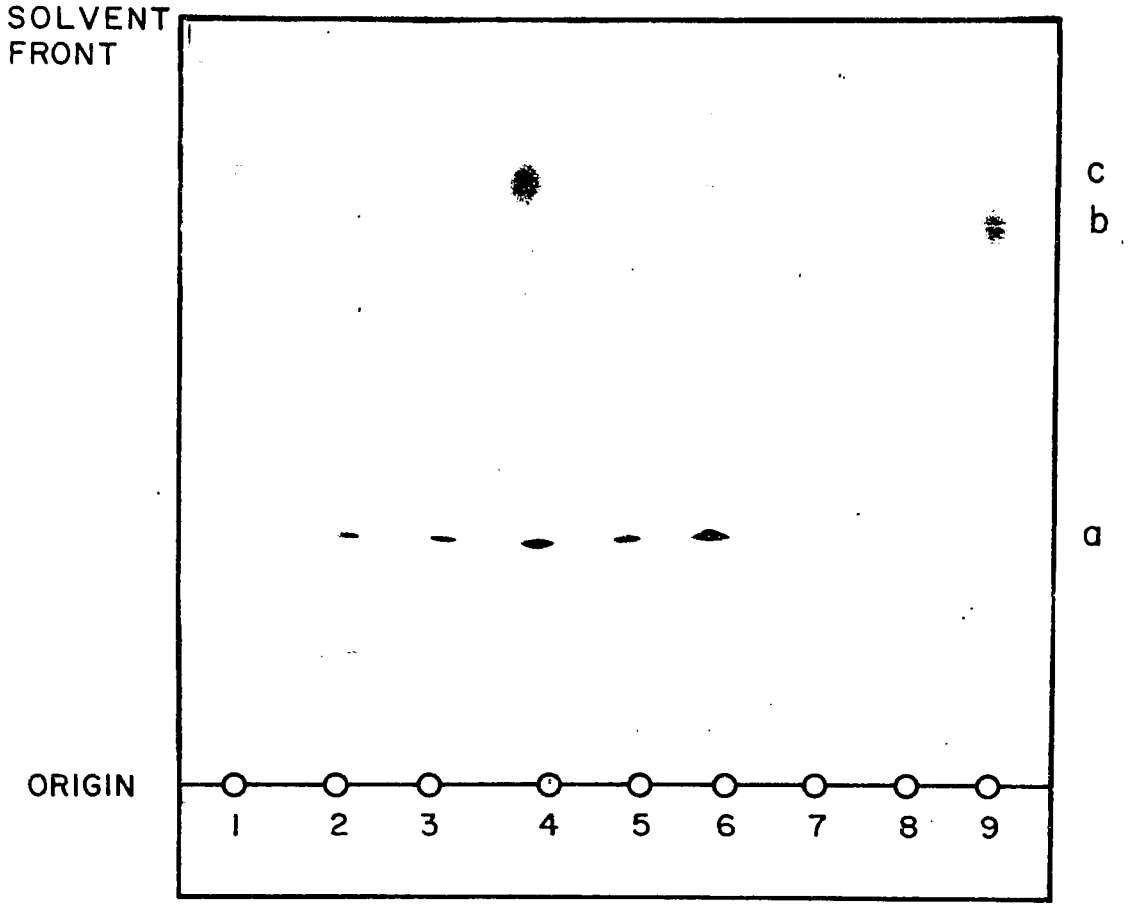
Salt concentration	Total Fatty Acids		Neutral unsap. Fraction	
	cpm/tube	% ≠	cpm/tube	% ≠
3.8 M	2.2×10^3	0.4	10	0
1.8 M	3.1×10^3	0.6	10	0

* Complete assay reaction mixture for the biosynthesis of fatty acids as described in the Materials and Methods Section.

≠ Expressed as % of cpm in 1, 3-^{14}C-malonyl-CoA precursor used in each tube

PLATE VIII

Thin-layer chromatogram and autoradiograph of fatty acids and methyl fatty acid esters synthesized by H. cutirubrum total cell-free homogenate and labelled 1, 3-^{14}C-malonyl-CoA. Material applied at high and low salt concentrations respectively: 2, 3-methyl fatty acid esters; 5, 6 - "free" fatty acids; 7, 8 - neutral unsaponified lipids. Standards: 1, methyl palmitate (Spot c), 4, palmitic acid (Spot a) and 9 - α, β-di-O-phytanyl glycerol (Spot b). Solvent: chloroform-acetone-methanol-acetic acid-H_2O (50:20:10:10:5).


SOLVENT
FRONT
c
b
a
ORIGIN
1
2
3
4
5
6
7
8
9

III. Distribution of ^{14}C and ^{3}H Labelling among Lipid Components of H. cutirubrum Cells grown in the Presence of 1,(3)-^{3}H-and 1,3-^{14}C-Glycerol Mixture and 2-^{3}H-and 1,3-^{14}C-glycerol mixture

The ratio of ^{3}H to ^{14}C activities in lipids of H. cutirubrum cells was found to vary with the position of the ^{3}H label in the precursors supplied (Table 6). Cells were grown in the presence of 1,(3)-^{3}H-and 1,3-^{14}C-glycerol mixture (Precursor I; initial $^{3}H/^{14}C$ ratio of counts, 27.3) and in 2-^{3}H- and 1,3-^{14}C-glycerol mixture (Precursor II; initial $^{3}H/^{14}C$ ratio of counts, 17.3). Total lipids from cells grown in Precursor I showed a $^{3}H/^{14}C$ ratio of 21.6, indicating a retention of 79% of the ^{3}H; while those cells grown in Precursor II showed a $^{3}H/^{14}C$ ratio of 0.84. After methanolysis of total lipids in 2.5% methanolic-HCl, the petroleum ether-soluble (diphytanyl glycerol ether) fraction was found to have a $^{3}H/^{14}C$ ratio of 19.1 (70% retention of ^{3}H) for cells grown in Precursor I and 0.53 for cells grown in Precursor II; the water-soluble fraction (sugar moieties and phosphate esters) had a $^{3}H/^{14}C$ ratio of 18.2 (67% retention of ^{3}H) and 0.97,for cells grown in Precursors I and II respectively. After adsorption of the phosphate esters from the methanolic-HCl fraction, for cells grown in Precursor I by treatment with Amberlite MB-3 resin, the remaining sugar components showed a lower $^{3}H/^{14}C$ ratio of 14.5 (53% retention of ^{3}H). The $^{3}H/^{14}C$ ratio of the glycerol phosphates is thus probably greater than 14.5, indicating that here too the glycerol retained most of its ^{3}H.

When the diphytanyl glycerol ether fraction was cleaved with boron trichloride, the ether-soluble products (mostly phytanyl chloride) for cells grown in Precursor I showed a $^{3}H/^{14}C$ ratio of 13.8 (53% retention of ^{3}H) which is about half that of the precursor's activity. On the other hand, the $^{3}H/^{14}C$ ratio of the diphytanyl chloride fraction for cells grown in Precursor II was very low, namely 0.46. The water-soluble moiety of the diether (glycerol) from cells grown in Precursor I showed a $^{3}H/^{14}C$ ratio of 27.8 (102% retention of ^{3}H) which is similar to that of the precursor's initial activity; while the glycerol moiety of the diphytanyl ether fraction had a very low $^{3}H/^{14}C$ ratio of 0.67 for cells grown in Precursor II.

These results show that when H. cutirubrum cells were allowed to grow in the presence of 1,(3)-^{3}H glycerol, almost all of the ^{3}H was retained in the glycerol moiety of the diphytanyl glycerol ether analogue, but only 50% was retained in the phytanyl groups and in glycosidic moieties. In marked contrast, with the 2-^{3}H-glycerol precursor, the ^{3}H content of either the phytanyl groups or the glycerol moiety of the diphytanyl glycerol ether analogue was almost completely lost.

TABLE 6

Incorporation of ^{14}C and ^{3}H glycerol into Lipids of H. cutirubrum *

Lipid Component	1, 3-^{14}C and 1, 3-^{3}H glycerol precursor				1, 3-^{14}C and 2-^{3}H glycerol precursor			
	Counts/min.** ^{3}H x 10^{-3}	^{14}C x 10^{-3}	Ratio $^{3}H/^{14}C$	Retention of ^{3}H, % of initial precursor	Counts/min.** ^{3}H	^{14}C	Ratio $^{3}H/^{14}C$	Retention of ^{3}H, % of initial precursor
Total lipids	82.01	3.79	21.6	79.2 ± 1.6	95	114	0.84	4.8
Diphytanyl ether fraction	68.63	3.60	19.1	70.0 ± 1.4	105	197	0.53	3.6
Water-soluble moieties (sugars and phosphate esters)	52.31	2.87	18.2	66.8 ± 1.3	716	740	0.97	5.6
Sugar moieties	42.27	2.92	14.5	53.0 ± 1.1	-	-	-	-
Phytanyl group	345.1	25.0	13.8	50.5 ± 1.0	4005	8566	0.46	2.7
Glycerol moiety	64.42	2.31	27.8	102 ± 2.0	413	628	0.67	3.7
Initial precursor***	66.10	2.42	27.3	100	7683	443	17.3	100

* For detailed experimental procedure, see Section B, VII, (1).

** Counts per 25 µl aliquot were corrected for background and quenching but not for efficiency of counting. 17% of the ^{14}C counts were subtracted from the ^{3}H to correct for the machine channel contamination.

*** The initial composition of the precursor 1, 3-^{3}H and 1, 3-^{14}C-glycerol mixture was: 1.0 mcurie, 46.0 µM of 1, 3-^{3}H-glycerol (specific activity 436 mcuries/mmole); and 25.0 µcuries, 50µM of 1, 3-^{14}C-glycerol (specific activity 110 mcuries/mmole). $^{3}H/^{14}C$ µcuries ratio of 40. The initial composition of the precursor 2-^{3}H and 1, 3-^{14}C-glycerol mixture was: 1.0 mcurie, 40.0 µM of 2-^{3}H-glycerol (specific activity 500 mcuries/mM); and 25.0 µcuries 50 µM of 1, 3-^{14}C-glycerol (specific activity 110 mcuries /mmole). $^{3}H/^{14}C$ µcuries ratio of 40.

IV. Studies on Metabolism of α-Glycerophosphate

1. Incorporation of ^{32}P from rac-3-GP-^{32}P and ^{32}P-orthophosphate into Lipids by Whole Cells

The time course curves presented in Fig. 2 showed that when H. cutirubrum cells were incubated in the presence of rac-3-GP-^{32}P, no ^{32}P activity was detected in the lipids until the late linear phase of growth. Further investigation showed that labelled inorganic phosphate was being liberated at a rate paralleling the growth curve (Fig. 2B), up to a maximum near the mid-linear phase and declining thereafter to a minimum at the stationary phase. No ^{32}P incorporation into lipids occurred until this period of decrease in ^{32}P-inorganic phosphate. Thereafter, ^{32}P incorporation into the lipids increased at a rate similar to that for decrease in ^{32}P-P_i.

These observations suggested that the extra-cellular glycerophosphate was not being used directly by H. cutirubrum cells for phospholipid synthesis, but was being hydrolyzed to inorganic phosphate and glycerol which were then utilized for lipid synthesis. This suggestion was strengthened by the fact that when the cells were incubated with ^{32}P orthophosphate alone, the latter was immediately taken up by the cells and ^{32}P incorporation into the phosphatides exactly paralleled the growth curve. It is of interest that ^{32}P incorporation into the PGP-diether analogue paralleled that into the total lipids.

Fig. 2 and Tables 7 and 8 also show the distribution of ^{32}P activity among lipid components of H. cutirubrum cells with rac-3-GP-^{32}P and P_i-^{32}P as precursors. It should be noted that with both precursors, PGP-diether accounted for ca. 60% of the total lipid-^{32}P in the early stages of growth increasing to a final value of about 80%. This increase in PGP-diether labelling appeared to occur at the expense of two unidentified components, namely Spots number 3 and 4 (Plate I, Tables 7 and 8). These results suggest that the latter components may be related biosynthetically to PGP-diether analogue.

FIGURE 2

Time course of uptake of ^{32}P by growing cultures of *H. cutirubrum* cells in the presence of:

(A) rac-3-GP-^{32}P, and

(B) ^{32}P-orthophosphate.

See Tables 7 and 8 and Section B, VII, 4 (a) for detailed procedure. Counts were corrected for "zero time" values.

Abbreviations:

PGP Phosphatidylglycerolphosphate-diether form.

P_i-^{32}P-water soluble: ^{32}P-inorganic phosphate assayed by the Berenblum and Chain procedure.

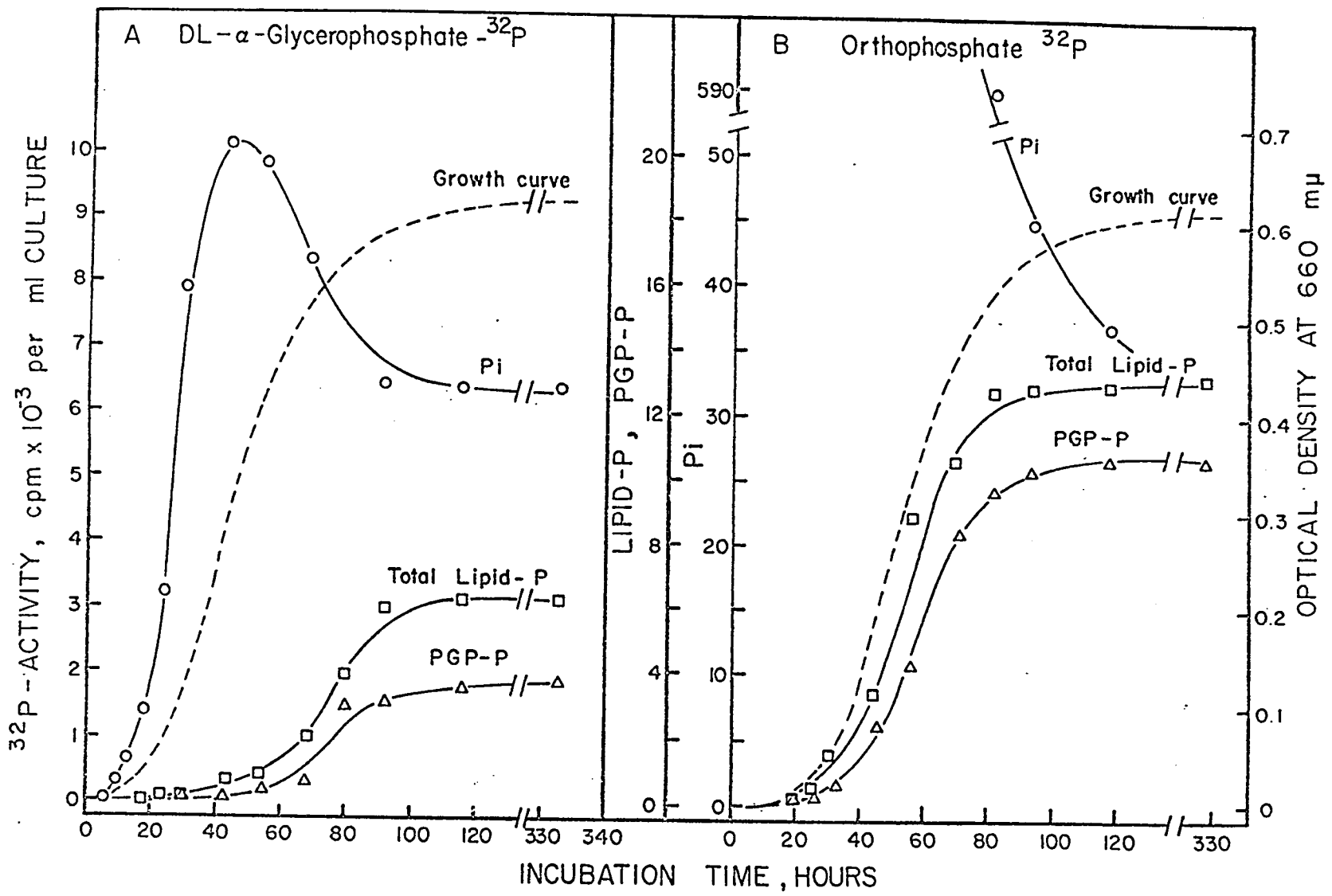

A DL-α-Glycerophosphate -32P
B Orthophosphate 32P
32P-ACTIVITY, cpm x 10-3 per ml CULTURE
LIPID-P, PGP-P
Pi
OPTICAL DENSITY AT 660 mμ
Growth curve
Pi
Total Lipid-P
PGP-P
INCUBATION TIME, HOURS

TABLE 7

Distribution of ^{32}P Activity among Lipid Components of H. cutirubrum grown in the Presence of ^{32}P-orthophosphate *

Time, hours	Total lipid-^{32}P, cpm per 1 ml culture	Distribution of ^{32}P among Individual Lipid Components, %								
		Origin	Unidentified sulfolipid Spot 1	Glycolipid sulfate Spot 2	Glycolipid Spot 3	Unidentified phospholipid Spot 4	Unidentified phospholipid Spot 5	PGP-diether Spot 6 and 7	PG-diether Spot 8	Pigments plus neutral lipids Spot 9
30	1,595	6.0	2.8	3.6	7.0	3.0	3.0	64.0	9.0	1.0
43	3,500	1.5	1.0	2.0	3.5	5.0	4.5	73.0	8.0	0.50
54	8,860	1.4	0.90	1.3	3.0	6.0	5.0	75.0	7.0	0.40
68	10,650	1.4	0.80	1.0	2.2	5.5	6.0	76.0	7.0	0.35
80	11,500	0.5	0.75	0.5	1.6	5.0	7.0	78.0	6.5	0.30
92	12,200	0.9	0.60	0.4	1.5	3.0	6.0	80.0	7.0	0.20
116	12.940	1.0	0.55	0.4	1.0	2.0	3.0	92.0	8.0	0.30
130	15,240	0.8	0.60	0.4	0.7	1.0	2.0	83.0	8.0	0.45
2 wks.	15,730	0.7	0.70	0.3	0.5	0.8	1.9	83.0	7.8	0.50

* H. cutirubrum cells were grown in the presence of ^{32}P-orthophosphate. Two ml culture samples were withdrawn at the indicated time and total lipids were chromatographed on silica-impregnated paper (Marinetti's procedure, 1962, 1965 and Kates, 1967) and the radioactive components, located by autoradiography were cut out and counted. Total phosphorus for PGP-diether and PG-diether was determined and their specific activity was found to be constant (1055 cpm/µgP for PGP-diether and 94 cpm/µgP for PG-diether). See Section B, VII, 4 (a) for detailed procedure, and Plate I for numbering of spots. For O.D. of cultures refer to Fig. 2B.

TABLE 8

Distribution of ^{32}P Activity among Lipid Components of H. cutirubrum grown in the Presence of rac-3-glycerolphosphate-^{32}P

Time, hours	Total lipid-^{32}P, cpm per 1 ml culture	Distribution of ^{32}P among Individual Lipid Components, %								
		Origin	Unidentified sulfolipid Spot 1	Glycolipid sulfate Spot 2	Glycolipid Spot 3	Unidentified phospholipid Spot 4	Unidentified phospholipid Spot 5	PGP-diether Spot 6 and 7	PG-diether Spot 8	Pigments plus neutral lipids Spot 9
43	200	1.5	1.0	4.0	15.0	7.0	4.0	53.0	7.8	5.0
54	500	2.0	2.0	2.2	13.0	6.0	3.6	59.0	6.0	4.0
68	1,000	1.8	2.1	2.0	10.0	5.0	3.0	63.0	5.5	3.5
80	1,500	2.1	2.0	1.8	7.0	4.2	2.5	72.0	6.0	3.0
92	2,700	1.0	1.8	1.5	6.0	4.0	2.3	79.8	7.8	2.5
116	2,800	1.5	1.4	1.0	3.8	2.8	2.0	79.0	7.5	2.0
130	2,900	1.0	0.8	0.5	2.4	2.4	1.0	80.0	7.9	1.8
2 wks.	3,000	0.8	0.5	0.3	1.0	2.0	0.8	81.0	8.0	1.0

* H. cutirubrum cells were grown in the presence of rac-3-glycerolphosphate-^{32}P. Two ml culture samples were withdrawn at the indicated times and total lipids were chromatographed on silica-impregnated paper (Marinetti's procedure 1962, 1965 and Kates, 1967) and the radioactive components, located by autoradiography, were cut out and counted. Total phosphorus for PGP-diether and PG-diether spots was determined and their specific activity was found to be constant (211 cpm/μgP for PGP-diether and 21 cpm/μgP for PG-diether). See Section B, VII, 4 (a) for detailed procedure, and Plate I for numbering of spots. For O.D. of cultures refer to Fig. 2 A.

2. Incorporation of ^{32}P and ^{14}C from rac-3-glycerol-phosphate-^{32}P and sn-glycerol-3-phosphate-^{14}C by Whole Cells

The results of the previous experiment suggested that α-glycerolphosphate was being hydrolyzed to inorganic phosphate which was then incorporated into the phospholipids. The question now arose whether α-GP penetrated the cell first or whether it was hydrolyzed outside the cells. To answer this, H. cutirubrum was grown in the presence of rac-3-GP-^{32}P and in the presence of sn-3-GP-^{14}C, cultures were centrifuged and the cells and the media, in both experiments were tested for labelled organic and inorganic components. Both P_i-^{32}P and ^{14}C-glycerol were found in the extracellular medium at a rate paralleling the growth curve (Fig. 3A and B); the liberation reached a maximum in the late linear phase and began to decrease thereafter. During the period of decrease in the extracellular inorganic-^{32}P and ^{14}C-glycerol, intracellular ^{32}P- and ^{14}C-glycerophosphate, respectively, were formed only after about a 40-hour lag period and remained at lower concentration levels than those of intracellular ^{32}P- or ^{14}C-glycerophosphates (Fig. 3A and B). At the same time, intracellular P_i-^{32}P and ^{14}C-glycerol were not detected until a lag period of about 40 hours (Fig. 3A and B, Plate IX).

These results show that α-GP, as such, is not taken up by H. cutirubrum cells, but that it is first hydrolyzed extracellularly into glycerol and inorganic phosphate which then pass into the cells and are recombined into α-GP.

FIGURE 3

Time course of metabolism of α-GP by H. cutirubrum cells.

(A) Cells grown in the presence of rac-3-GP-^{32}P. (DL-α-GP-^{32}P)

(B) Cells grown in the presence of sn-3-GP-^{14}C. (L-α-GP-^{14}C)

Counts are corrected for "zero-time" values.

For detailed procedures see Section B, VII, 4 (b).

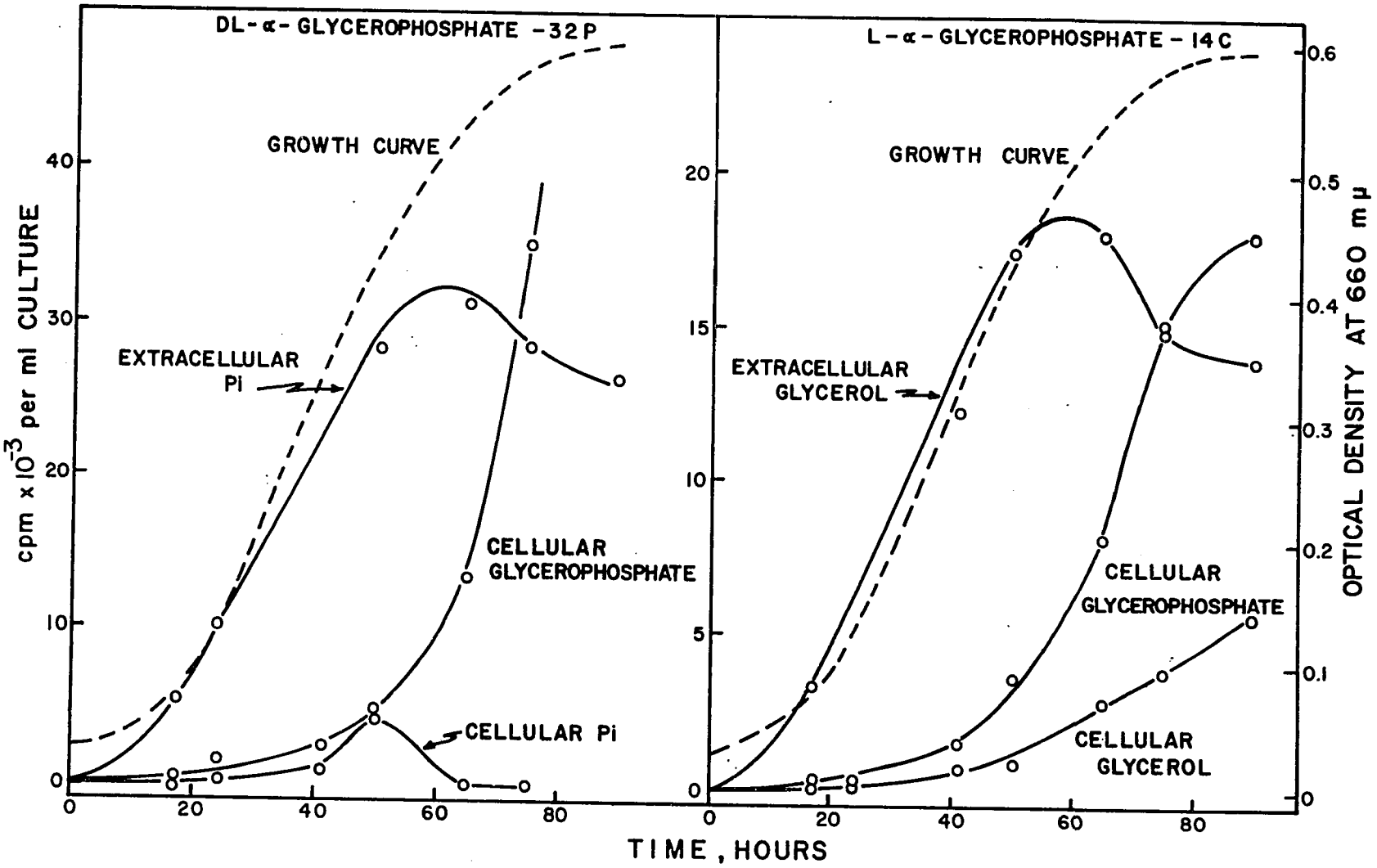
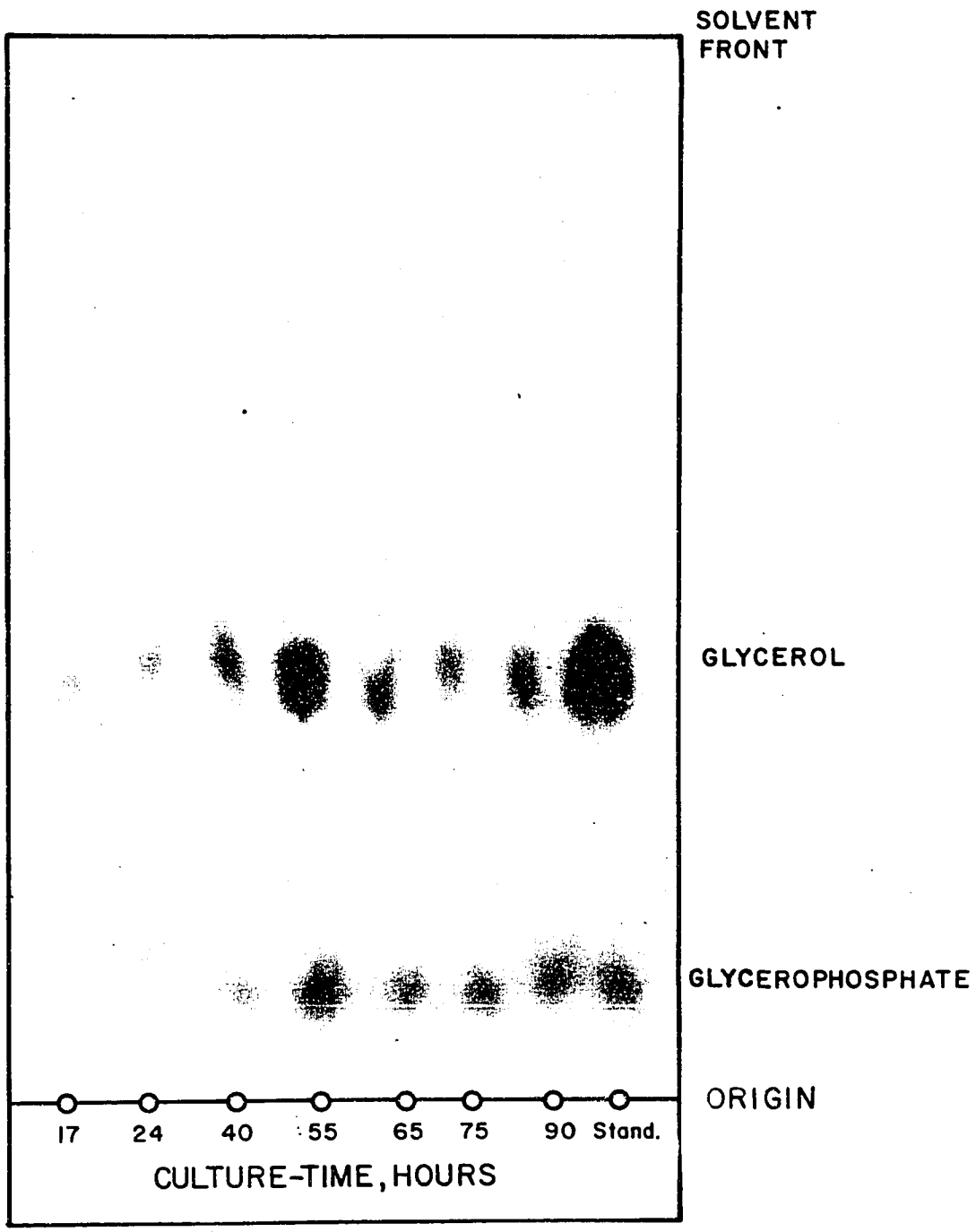

DL-α-GLYCEROPHOSPHATE -32P
GROWTH CURVE
EXTRACELLULAR Pi
CELLULAR GLYCEROPHOSPHATE
CELLULAR Pi
L-α-GLYCEROPHOSPHATE - 14C
GROWTH CURVE
EXTRACELLULAR GLYCEROL
CELLULAR GLYCEROPHOSPHATE
CELLULAR GLYCEROL
cpm x 10^-3 per ml CULTURE
OPTICAL DENSITY AT 660 mμ
TIME, HOURS
0
10
20
30
40
5
15
0.1
0.2
0.3
0.4
0.5
0.6
20
40
60
80

PLATE IX

Chromatogram and autoradiograph of intracellular water-soluble components of H. cutirubrum cells grown in the presence of sn-3-GP-^{14}C.
Radioactive components were cut out and counted.
Values used in Fig. 3B.
Solvent: Butanol-acetic acid-water (5:3:1) (v/v).
For detailed procedure see Section B, VII, 4 (b).


SOLVENT FRONT
GLYCEROL
GLYCEROPHOSPHATE
ORIGIN
17
24
40
55
65
75
90
Stand.
CULTURE-TIME, HOURS

3. Glycerophosphatase Activity in H. cutirubrum Cell Fractions

The results of the previous experiment indicated the existence of a glycerophosphatase probably located on the periphery of the cell. H. cutirubrum cell fractions were then investigated for phosphatase activity. Total cell-free homogenate, envelope fraction and supernatant fraction, prepared as described in Section B, III, (b), were incubated with rac-3-GP-^{32}P at different pH values. It was found that the substrate was hydrolyzed by both envelope and supernatant fractions, the optimum pH being 5.0. At this optimum pH, the sum of the glycerophosphatase activities in the two fractions accounted for the activity of the total cell-free homogenate indicating complete recovery of the enzyme in these two fractions (Fig. 4). Also, it should be noted that most of the phosphatase activity (about two-thirds) was associated with the envelope fraction. The remaining activity may represent water-soluble (cytoplasmic) phosphatase since the latter fraction was centrifuged at 100,000 x g.

The phosphatase activities of H. cutirubrum cell fractions calculated on per mg of total protein basis were found to be very similar (Table 9). Because each fraction is still very crude, this agreement is probably fortuitous.

These results are consistent with the hypothesis that α-glycerophosphate is hydrolyzed by a glycerophosphatase at the surface of the cell.

FIGURE 4

pH-Activity curve for the hydrolysis of α-glycerophosphate-^{32}P by a phosphatase enzyme present in cellular fractions of H. cutirubrum. For reaction conditions, preparation of cell fractions and estimation of % GP-hydrolysis, see Materials and Methods Section, B, VII, 4 (c).

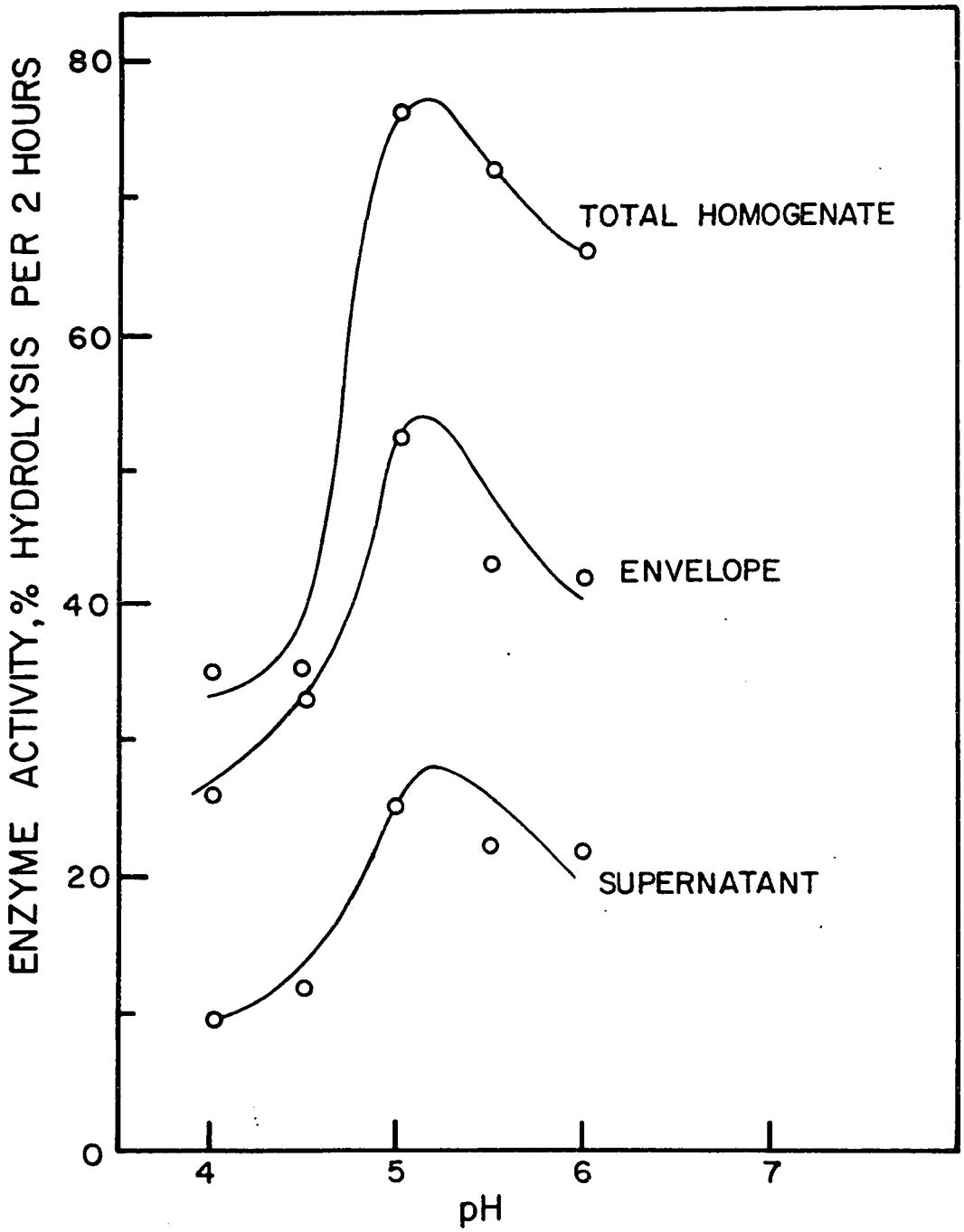

ENZYME ACTIVITY, % HYDROLYSIS PER 2 HOURS
80
60
40
20
0
4
5
6
7
pH
TOTAL HOMOGENATE
ENVELOPE
SUPERNATANT

FIGURE 5

Time course for the hydrolysis of α-glycerophosphate-^{32}P by a phosphatase enzyme present in H. cutirubrum cell fractions. For detailed reaction conditions, see Materials and Methods Section, B, VII, 4 (c).

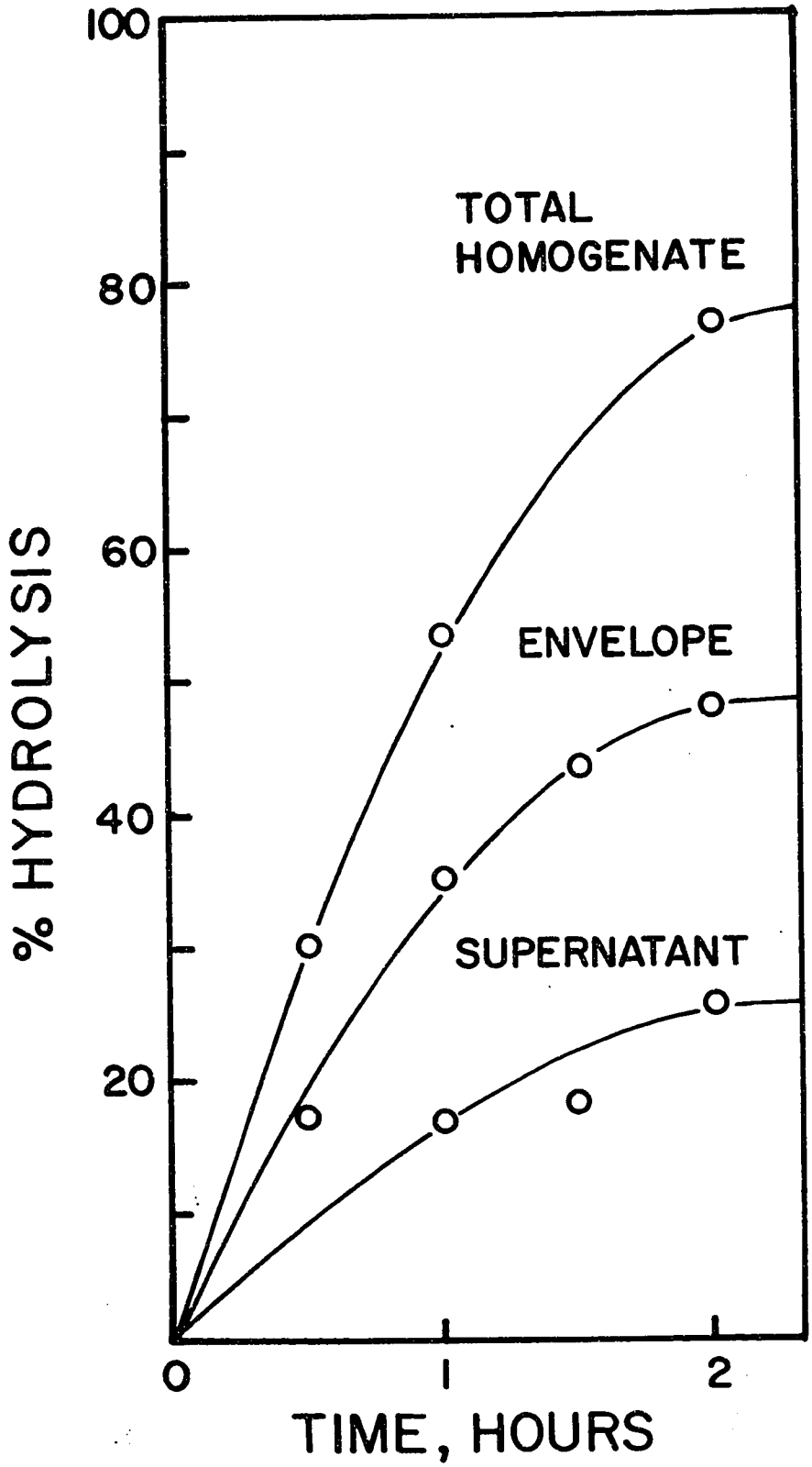

100
80
60
40
20
0
1
2
% HYDROLYSIS
TIME, HOURS
TOTAL
HOMOGENATE
ENVELOPE
SUPERNATANT

TABLE 9

Glycerophosphatase Activity in H. cutirubrum Cell Fractions *

Cell Fraction**	Protein mg/ml reaction mixture	Enzyme activity mμmoles/ 10 min.***	Distribution of enzyme activity, %	Specific activity mμmoles/10 min. /mg protein
Total homogenate	1.7	17	100	10
Envelope fraction	1.1	11	65	10
Supernatant fraction	0.6	5	30	8

* Incubation mixtures (1.0 ml) contained 0.16 μmoles rac-3-GP-^{32}P (sp. activity 10.8×10^6 cpm/μmole), 0.9 ml cell fraction (in 4M salt solution), 0.02 ml of 1M Tris buffer (pH 5.0), and 4M salt solution to volume; incubation was carried out at 37°.

** Cell fractions have been prepared as described in Section B, III, (b).

*** Values obtained from Fig. 5. For detailed experimental procedure, see Section B, VII, 4 (c).

4. Glycerolkinase in H. cutirubrum

(a) Demonstration of glycerolkinase activity:

The previous experiments showed that α-glycerolphosphate could be hydrolyzed extracellularly by a glycerophosphatase, to glycerol and inorganic phosphate, which then passed independently into the cells where they appeared to be recombined to α-GP (Figs. 2, 3 and 4; Table 9, and Plate IX). To test for the presence of a glycerol kinase; total cell-free homogenate was incubated with glycerol in the presence of ATP and the reaction mixture was assayed for sn-3-GP spectrophotometrically. Rapid synthesis of sn-3-glycerolphosphate was observed in the presence of ATP whereas in the absence of ATP no glycerophosphate was produced (Fig. 6).

This experiment shows that H. cutirubrum contains an active glycerolkinase which requires ATP for the phosphorylation of glycerol and the formation of α-GP.

(b) Cellular distribution and stereospecificity of H. cutirubrum glycerolkinase:

To determine the cellular distribution and stereospecifictiy of the glycerol kinase, H. cutirubrum cell fractions were incubated with ^{14}C-glycerol in the presence of ATP, and the labelled α-GP was isolated by chromatography on Dowex formate. With all cell fractions, a single radioactive peak was obtained which gave a single radioactive spot corresponding to authentic sn-3-GP on paper electrophoresis (Plate X) and paper chromatography (Plate XI). Recovery of

FIGURE 6

Incubation of total cell-free homogenate with glycerol in the absence and the presence of ATP. sn-3-GP produced was assayed enzymatically by the specific enzyme L, α-GP dehydrogenase.
Values were corrected for "zero time".
For detailed reaction conditions and assay procedures, see Materials and Methods Section, B, VII, 4 (d).

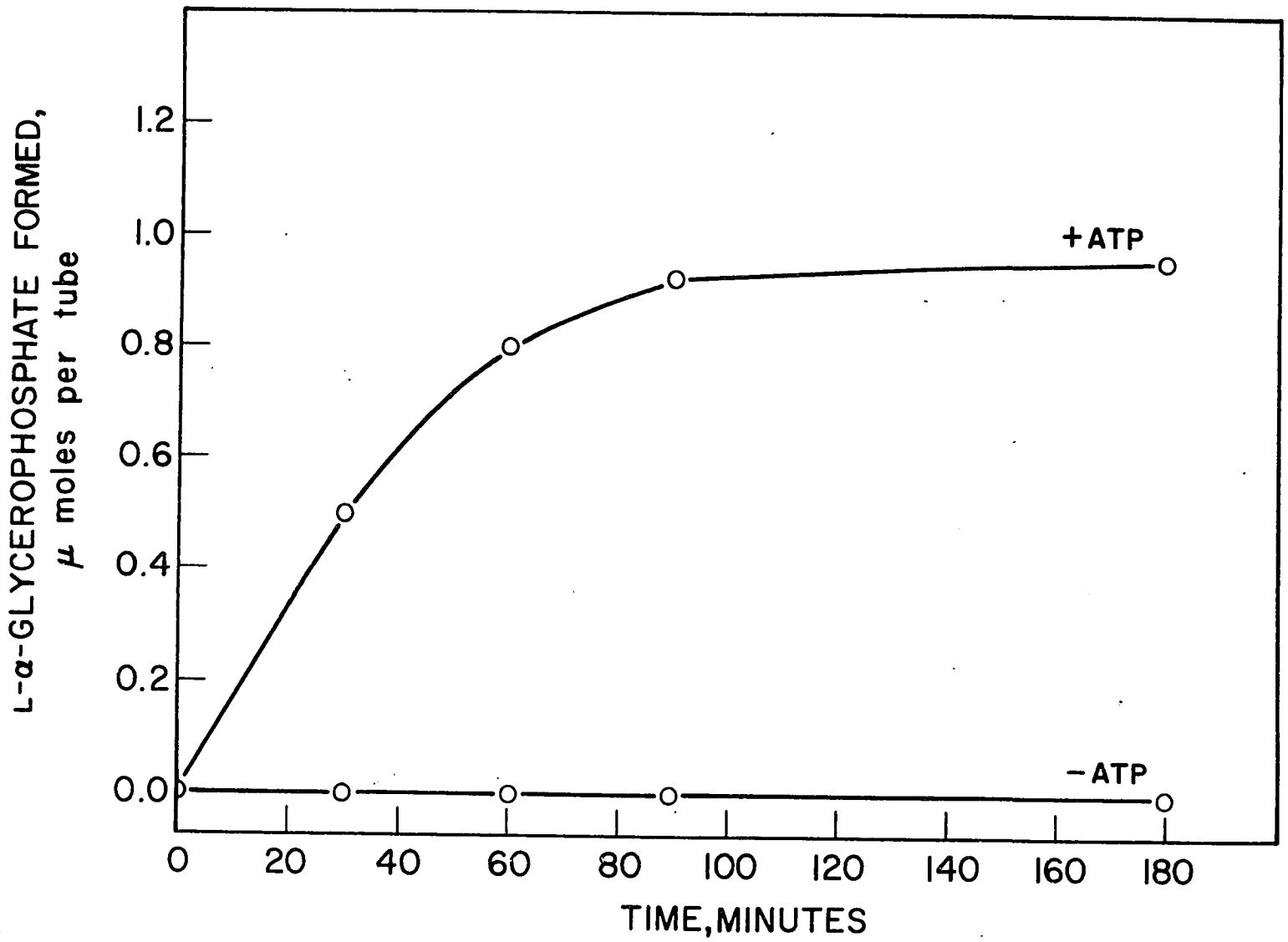

L-α-GLYCEROPHOSPHATE FORMED,
μ moles per tube
1.2
1.0
0.8
0.6
0.4
0.2
0.0
+ATP
−ATP
0
20
40
60
80
100
120
140
160
180
TIME, MINUTES

PLATE X

Autoradiograph of paper elctrophorogram of chromatographically isolated α-glycerolphosphate-^{14}C produced by glycerol kinase in H. cutirubrum cell fractions:

(1) total cell-free homogenate;

(2) supernatant fraction;

(3) envelope fraction.

Standards: authentic unlabelled dihydroxyacetone phosphate (DHAP) and sn-3-glycerolphosphate (L, α-GP).

Electrophoresis was carried out in borate buffer at pH 9.1, at 415 volts and 14-15 amps. for 1-1/2 hours.

For detailed procedure, see Section B, V, 4 (b).

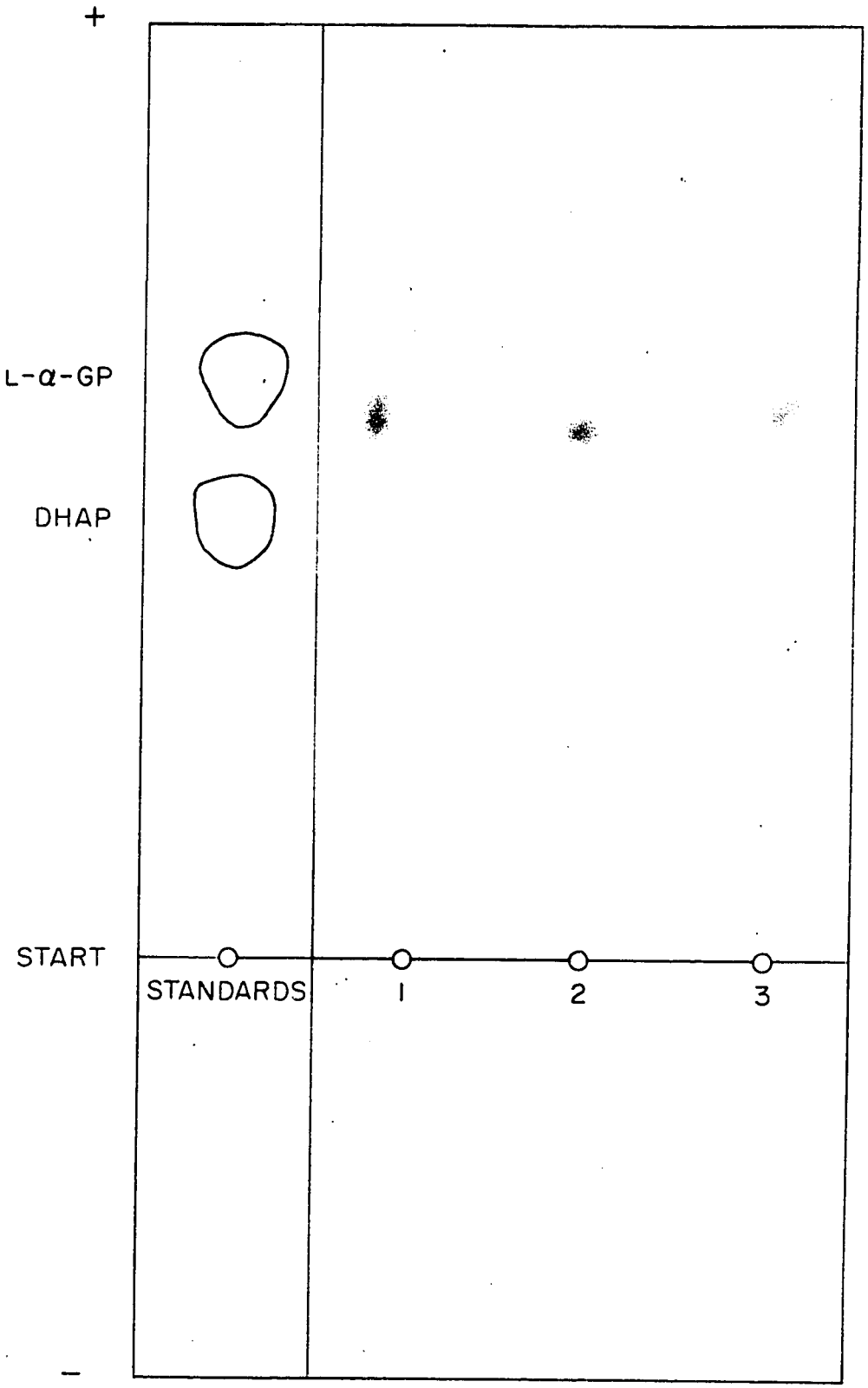

+
L-α-GP
DHAP
START
STANDARDS
1
2
3
−

PLATE XI

Chromatogram and autoradiograph of chromatographically isolated glycerolphosphate-^{14}C produced by glycerol kinase in H. cutirubrum cell fractions:

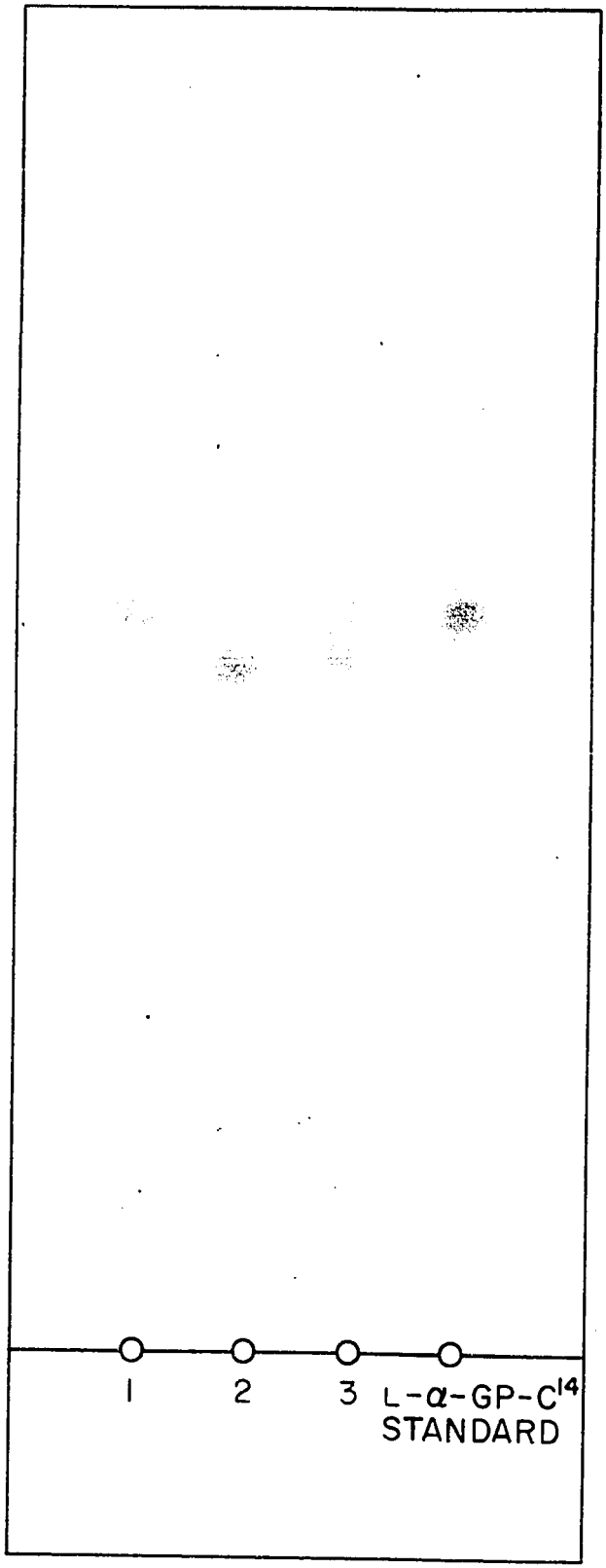
(1) total cell-free homogenate;

(2) supernatant fraction;

(3) envelope fraction.

Solvent: n-butanol-acetic acid-water (5:3:1, v/v).

For detailed procedure, see Section B, V, 4 (a).


1
2
3
L-α-GP-C14
STANDARD

^{14}C in the isolated α-GP as well as determination of total-P and α-GP-P showed that 86 - 91% of the ^{14}C-glycerol substrate was converted to ^{14}C-glycerophosphate with a specific activity of $4.3 \pm 0.3 \times 10^5$ cpm/μmole (see Table 10).

Assay for the sn-3-GP (L, α-GP) content in the α-GP synthesized by H. cutirubrum cell fractions using the specific enzyme L, α-GP-dehydrogenase showed that α-GP synthesized by all fractions was 98 - 100% in the L, α-form (Table 10). To confirm these results, the products of the specific dehydrogenase reaction were subjected to paper chromatography and autoradiography. The results (Plate XII) showed that all of the α-GP synthesized was completely converted into a new compound having an R_f value identical with that of dihydroxyacetone phosphate.

It should be noted that the glycerol kinase activity of the total homogenate appeared to be almost equally distributed between the envelope and supernatant fractions. However, since the supernatant fraction used here was not centrifuged at 100,000 x g to remove envelope fragments, its high kinase activity may be due to contamination with these envelope particles.

The results of these experiments show clearly that H. cutirubrum contains a glycerol kinase which is probably associated with the cell envelopes and which catalyzes the phosphorylation of glycerol in the presence of ATP to form specifically the sn-glycerol-3-phosphate isomer.

TABLE 10

Identification of End Product of Glycerol Phosphorylation by the Glycerol Kinase in *H. cutirubrum* *

Enzyme Preparation	^{14}C-glycerol added		Total ^{14}C-α-GP		sn-3-GP ***	
	µmoles	µcuries	µmoles**	%	µmoles	%
Total homogenate	21.1	10	19.2	91.2	18.8	98.0
Envelope fraction	21.1	10	18.1	85.8	18.1	100.0
Supernatant fraction	21.1	10	18.3	86.8	17.9	98.0

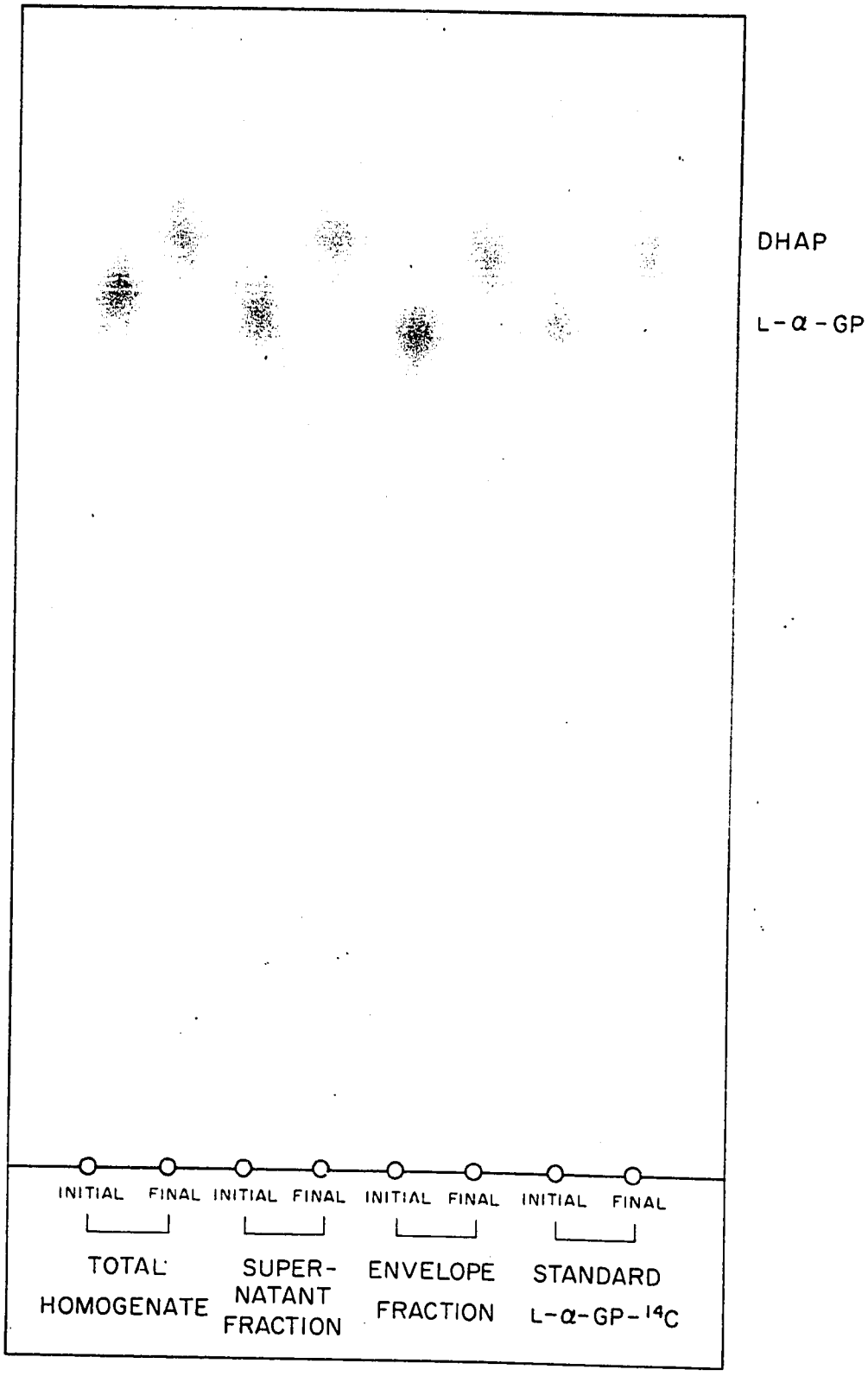
* See Section B, VII, 4(d) for experimental details

** Estimated by periodate-oxidative procedure.

*** Estimated by the specific L-α-GP-dehydrogenase procedure (Kennedy, 1962).

PLATE XII

Chromatogram and autoradiograph of reaction mixture of α-GP-^{14}C produced by H. cutirubrum cell fractions. Phosphorylation of ^{14}C-glycerol, and the specific enzyme L, α-GP dehydrogenase (from rabbit muscle).
Solvent: t-butanol-water-picric acid (20:20:4, v/v/w).
For each fraction spotted, "initial" designates the reaction mixture at "zero time" and "final" designates the reaction mixture at the end of the incubation period.
See Section B, VII, 4 (d) for detailed reaction procedure.


DHAP
L-α-GP
INITIAL
FINAL
INITIAL
FINAL
INITIAL
FINAL
INITIAL
FINAL
TOTAL HOMOGENATE
SUPER-NATANT FRACTION
ENVELOPE FRACTION
STANDARD L-α-GP-14C

5. L, α-Glycerolphosphate Dehydrogenase Activity in H. cutirubrum

To determine whether α-GP could be formed in H. cutirubrum by reduction of dihydroxyacetone phosphate catalyzed by the enzyme L-α-GP dehydrogenase, authentic sn-3-GP-^{14}C and rac-3-GP-^{32}P were incubated separately with H. cutirubrum cell fractions in the presence of NAD. Autoradiograph of chromatograms of the reaction mixtures showed that with the sn-3-GP-^{14}C as a substrate, complete conversion to dihydroxyacetone phosphate occurred (Plate XIII, B). On the other hand, with rac-3-GP-^{32}P as a substrate, approximately half of the α-GP was transformed into dihydroxyacetone phosphate (Plate XIII, A). By cutting out and counting the labelled spots, it was found that 92 - 98% of the sn-3-GP-^{14}C was converted to labelled dihydroxyacetone phosphate (DHAP) and 43 - 48% of the rac-3-GP-^{32}P was found to be converted to DHAP (Table 11).

These results show that H. cutirubrum cell particulate fractions contain an L-α-glycerolphosphate dehydrogenase which catalyzes the formation of sn-3-GP by reduction of DHAP. Thus it would appear that sn-3-GP is the only isomer synthesized by H. cutirubrum.

PLATE XIII

Chromatogram and autoradiographs of reaction mixture of *H. cutirubrum* cell fractions incubated with:

(A) rac-3-GP-^{32}P (DL-a-GP), and

(B) sn-3-GP-^{14}C (L-a-GP).

Solvent: t-butanol-water-picric acid (80:20:4, v/v/w).

For each fraction spotted, "initial" designates the reaction mixture at "zero time", and "final" designates the reaction mixture at the end of the incubation period.

For detailed reaction procedure, see Section B, VII, 4 (e).

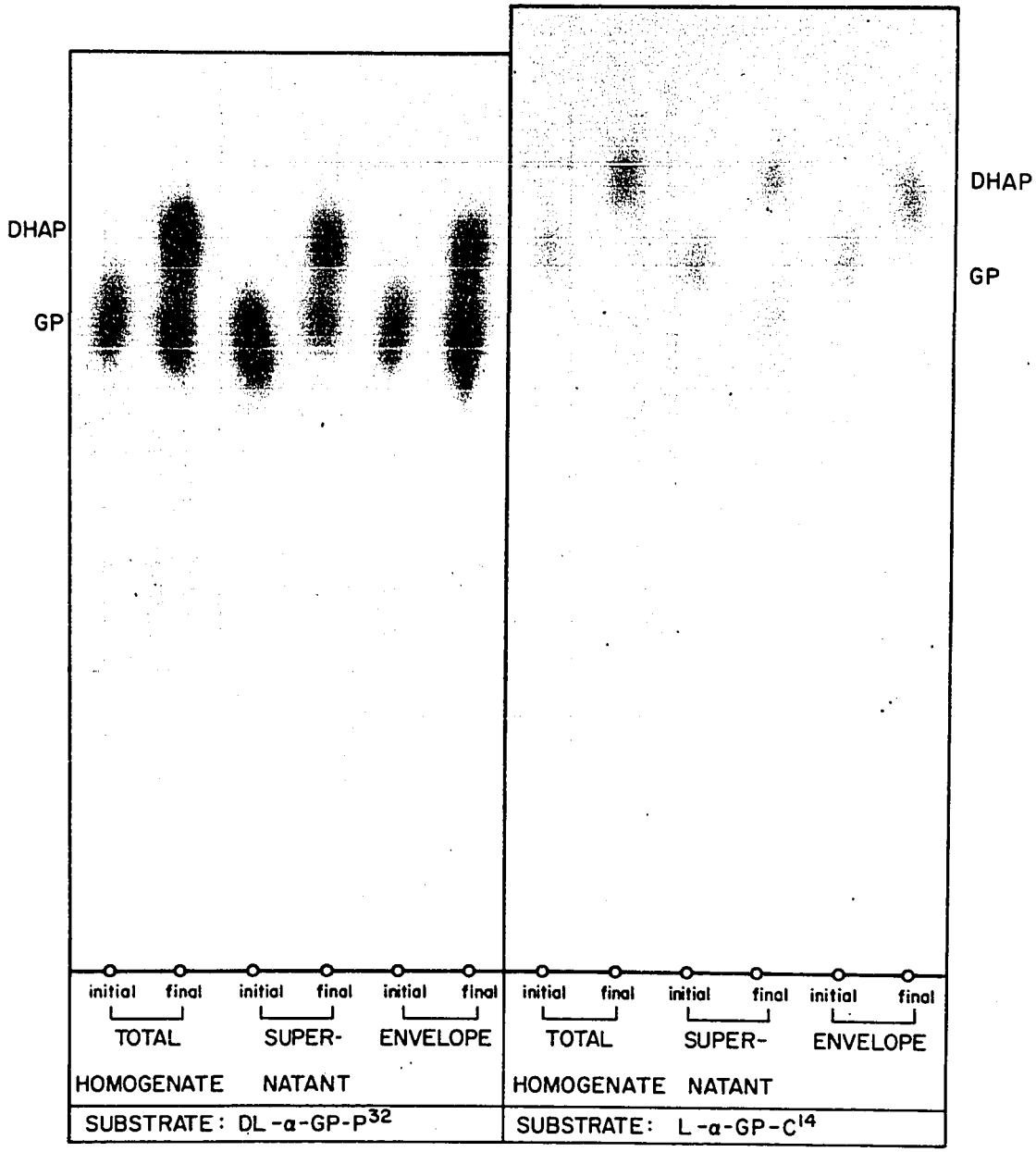

DHAP
GP
DHAP
GP
initial
final
initial
final
initial
final
initial
final
initial
final
initial
final
TOTAL
SUPER-
ENVELOPE
TOTAL
SUPER-
ENVELOPE
HOMOGENATE
NATANT
HOMOGENATE
NATANT
SUBSTRATE: DL-α-GP-P32
SUBSTRATE: L-α-GP-C14

TABLE 11

Distribution of Radioactivity among Labelled Components isolated by Paper Chromatography in the Incubation Mixture of α-GP and H. cutirubrum Cell fractions *

Enzyme Preparation	rac-3-GP-^{32}P			sn-3-GP-^{14}C		
	Initial cpm	% GP unreacted	% GP conversion to DHAP	Initial cpm	% GP unreacted	% GP conversion to DHAP
Total homogenate	2722	46	54	582	8	92
Envelope fraction	2540	45	55	577	2	98
Supernatant fraction	2000	48	52	470	4	96

*
rac-3-GP-^{32}P and sn-3-GP-^{14}C incubated with H. cutirubrum cell fractions in the presence of NAD. The incubation mixture was paper chromatographed and the labelled components were cut out and counted (see Plate XIII).
For detailed experimental procedure see Section B, VII, 4(e).

V. Short Term Labelling of Total Lipids with ^{32}P-Orthophosphate

Autoradiograms of chromatograms of total lipids of H. cutirubrum cells incubated for short periods of time in the presence of ^{32}P-orthophosphate showed the appearance, within one minute, of a single major ^{32}P-labelled phospholipid (Spot 6, Plate XIV) located between the PGP-diether analogue (Spot 5) and the PG-diether analogue (Spot 7) and having an R_f value of 0.51; it was designated as Compound X_3. A minor ^{32}P component (Spot 1; X_1) was also detected during the early periods of incubation, but its degree of labelling was inconsistent. Neither the PGP, nor the PG-diether analogues, nor any of the other phospholipids were labelled during the first hour of incubation. However, it should be noted that as the incubation time increased, the radioactivity of Spot 6 (Component X_3) decreased with a concomitant increase in the labelling of PGP-diether (Spot 5, Plate XIV). When the labelled components were cut out from the chromatogram and counted, the percent distribution of ^{32}P activity of Spot 6 (Component X_3) was found to be high (ca. 55%) during the first 10 minutes of incubation, thereafter declining to small values. At the same time, the percent ^{32}P activity in PGP-diether (Spot 5) showed a corresponding increase, indicating a precursor-product relationship between it and Component X_3 (Figure 7).

The percent activity of ^{32}P in the PG-diether analogue (Spot 7) was very low up to 1 hour, increasing thereafter at a rate paralleling that of PGP-diether, suggesting that the PGP-diether analogue is the precursor of the PG-diether analogue.

It should be noted that another unidentified phospholipid component (X_2, Spot 4) appeared to be labelled within the first hour of incubation and increased thereafter at a rate paralleling that of the PGP-diether. Also, the percent activity of ^{32}P in Component X_2 was found to be higher than that of PG-diether analogue. However, since labelling of PG-diether and Component X_2 occurred almost simultaneously, it is most probable that PG-diether is not the precursor of Component X_2.

PLATE XIV

Chromatograms and autoradiographs of total lipids of H. cutirubrum cells grown in a low phosphate, modified complex medium in the presence of ^{32}P-orthophosphate. Incubation was carried out at 37°C on a rotary shaker and samples were withdrawn at the indicated time. Chromatograms were stained with Rhodamine 6G and viewed under ultraviolet light. Identity of spots:

1. unidentified phospholipid (X_1);
2. glycolipid sulfate;
3. glycolipid;
4. unidentified phospholipid + sulfolipid (X_2);
5. phosphatidyl glycerophosphate diether analogue;
6. unidentified phospholipid (X_3);
7. phosphatidyl glycerol-diether analogue.

For detailed experimental procedure see Section B, VII (6).

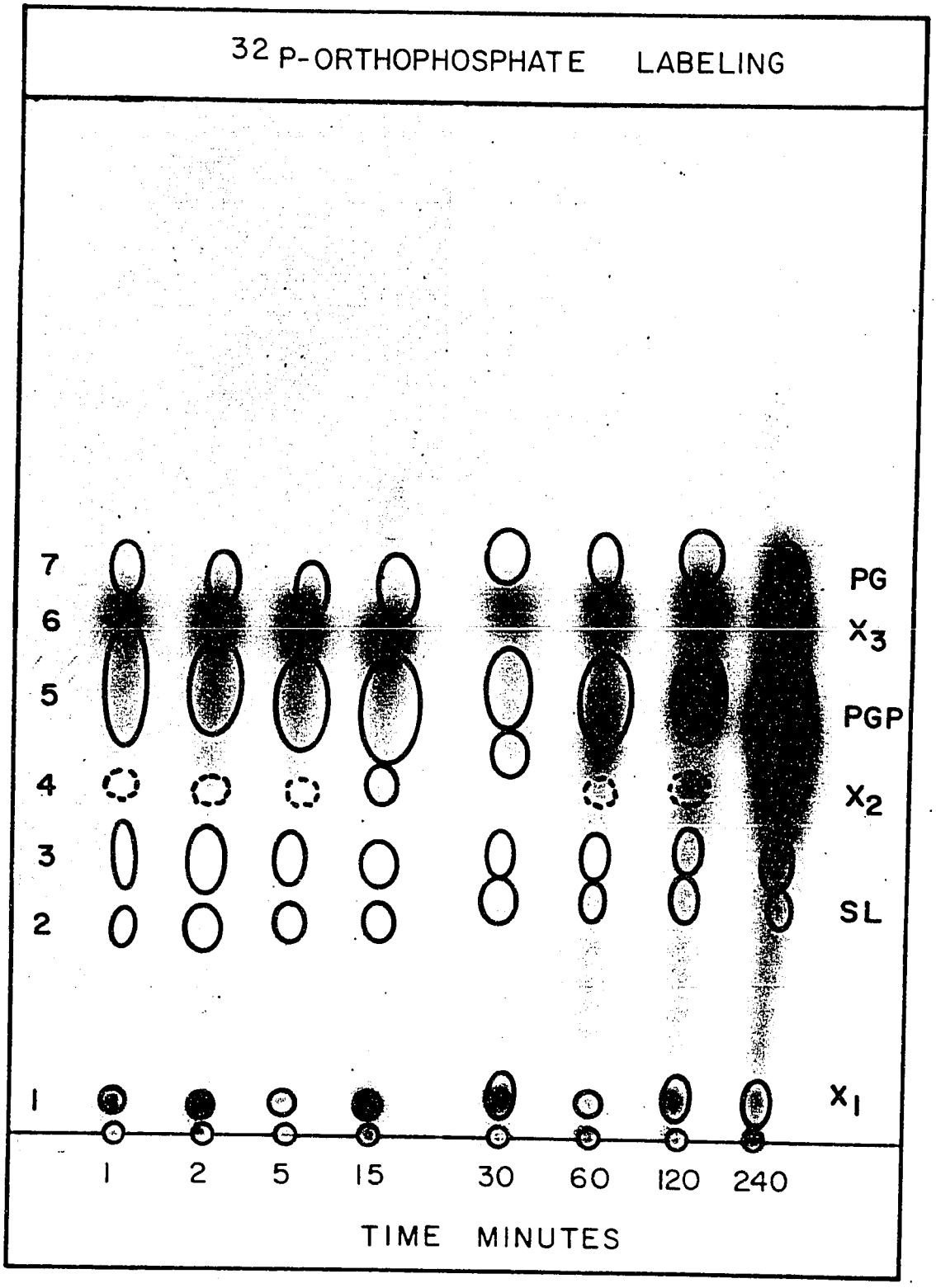

32 P-ORTHOPHOSPHATE LABELING
7
6
5
4
3
2
1
PG
X3
PGP
X2
SL
X1
1
2
5
15
30
60
120
240
TIME MINUTES

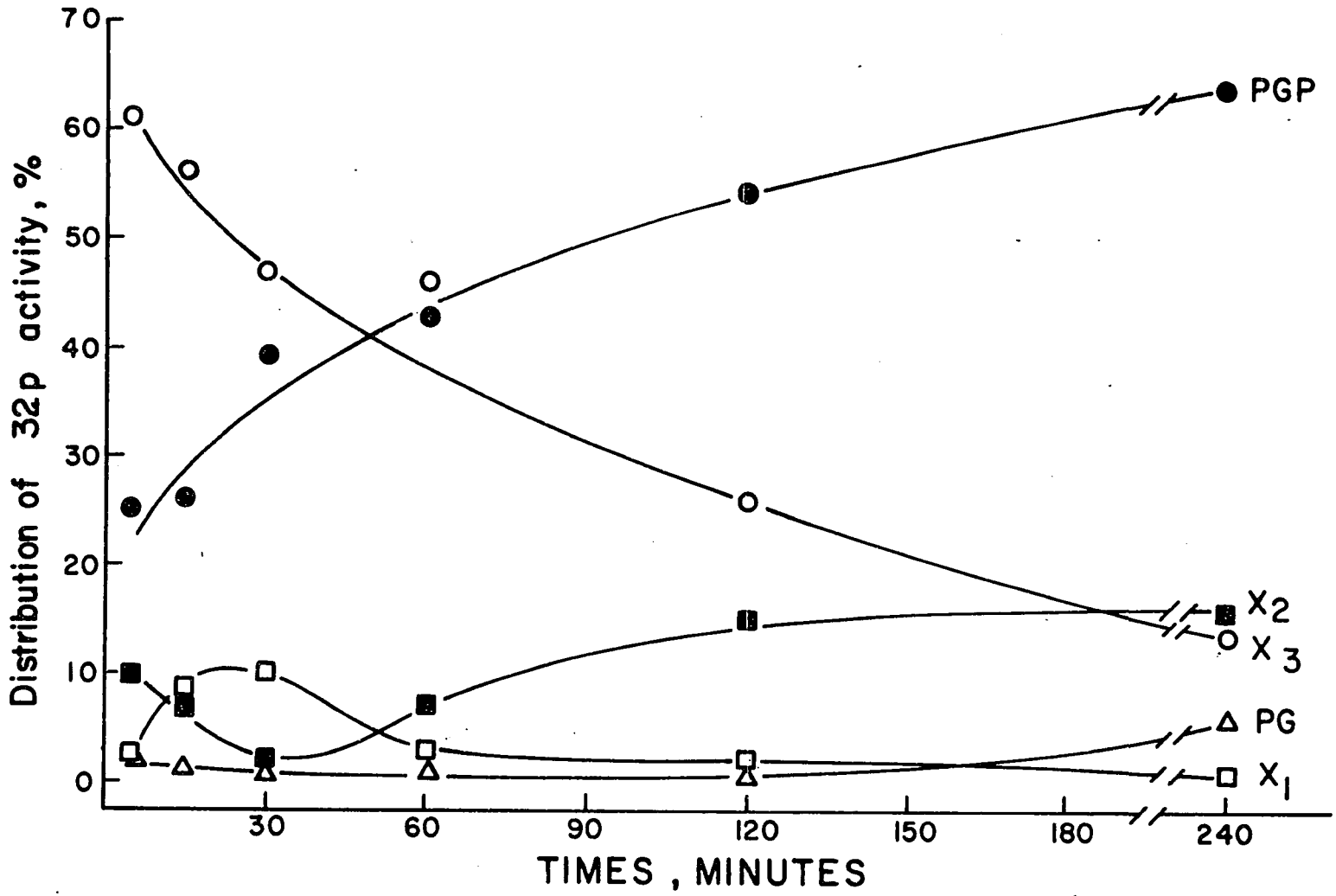
FIGURE 7

Percent distribution of ^{32}P activity among individual phospholipid components of H. cutirubrum cells incubated for short periods of time with ^{32}P-orthophosphate.

X_1, X_2 and X_3 are unidentified phospholipids.

PG and PGP are the diether analogues of phosphatidylglycerol and phosphatidyl glycerophosphate respectively (see Plate XIV).

For detailed experimental procedure, see Section B, VII (5).

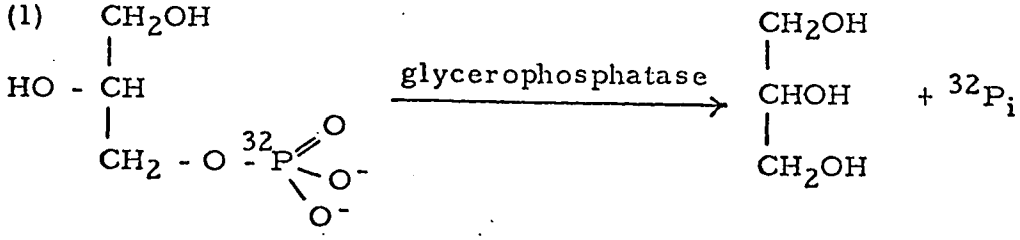

70
60
50
40
30
20
10
0
Distribution of 32p activity, %
30
60
90
120
150
180
240
TIMES , MINUTES
PGP
X2
X 3
PG
X1

D. DISCUSSION

I. Metabolism of Glycerolphosphate

The studies carried out on the metabolism of α-GP showed that intact H. cutirubrum cells do not take up and use α-GP as such directly for the synthesis of phospholipids (Fig. 2A). Instead, α-GP is hydrolyzed extracellularly by a phosphatase associated with the envelope (Fig. 5), and the resulting glycerol and inorganic phosphate diffuse into the cell independently and are then used for lipid synthesis (Fig. 3, Tables 7 and 8). This view is supported by the finding that when the cells were incubated in the presence of $^{32}P_i$ as a precursor, the latter was immediately incorporated into the phosphatides in a fashion paralleling the growth curve (Fig. 2B). Further investigation on α-GP uptake indicated that H. cutirubrum contains an active glycerophosphatase, associated mostly with the cell envelopes, that catalyzes the hydrolysis of α-GP into glycerol and P_i (Figs. 4 and 5). Each of the latter two components passes into the cells and is recombined into α-GP which is then used for lipid synthesis (Fig. 3A and B; Plate IX). Presumably, the sequence of reactions for α-GP hydrolysis and formation is as follows:

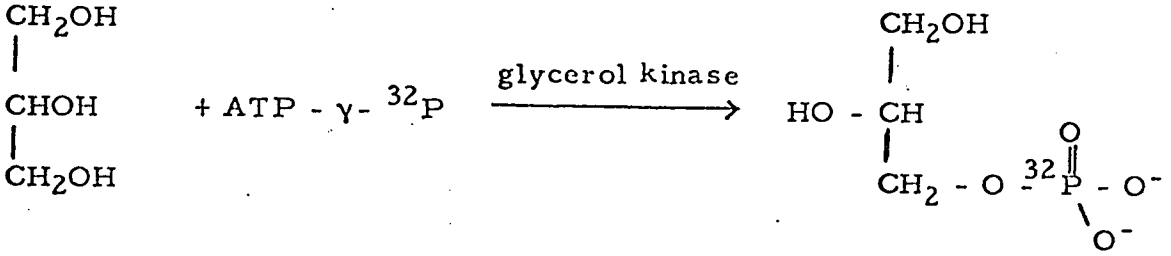
(1)



α-GP-^{32}P — on the cell surface — glycerol

(2) $ADP + {}^{32}P_i \longrightarrow ATP - \gamma - {}^{32}P$

(3)



glycerol — intracellularly — sn-glycerol-3-phosphate

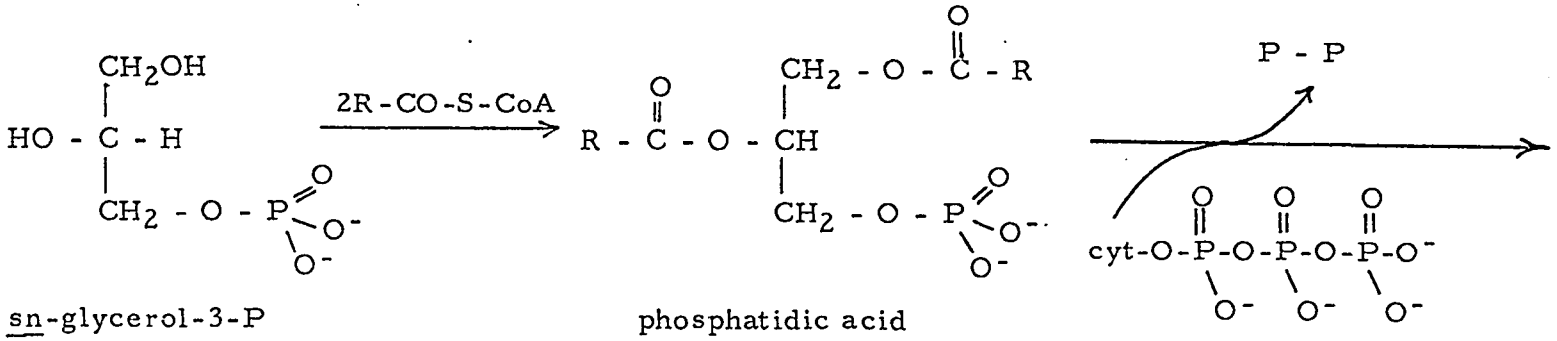
The finding that α-GP is not taken up by H. cutirubrum cells is not unexpected in view of the well-known impermeability of membranes towards di-ionized phosphates (Davis, 1958). On the other hand, mechanisms are known to exist in some organisms to facilitate the uptake of essential organic phosphates when these provide a selective advantage to the cells. For instance, mechanisms for the uptake of glucose-6-phosphate by an E. coli mutant that has a deficient transport system for free glucose has been demonstrated (Hagihira et al, 1963). Also, Hayashi et al (1964) have shown that certain mutant strains of E. coli possess a specific transport system for sn-glycerol-3-phosphate so that this compound can be taken up by living cells without prior hydrolysis to glycerol and orthophosphate. However, it is clear from the evidence presented here that H. cutirubrum is not an exception to the general rule of impermeability to phosphorylated compounds containing a net negative charge of more than one (Davis, 1958).

Diffusion of free glycerol (Hayashi and Lin, 1965) and inorganic-P (Mitchell, 1953) across the cell membrane probably occurs by facilitated transport, most likely as follows: The ADP-ATP phosphotransferase on the inner surface of the cell membrane utilizes the diffused P_i-^{32}P to form ATP-γ-^{32}P, according to the proposal of Mitchell (1953). The ^{32}P-ATP is then used by the glycerol kinase, also present on the inner membrane surface, to phosphorylate the diffused glycerol to α-glycerophosphate. This hypothesis is supported by the observation that at all times, the intracellular concentration of

^{32}P- or ^{14}C-α-glycerophosphate was much higher than that of either intracellular P_i-^{32}P or ^{14}C-glycerol (Fig. 3). Furthermore, as will be shown below, glycerol kinase activity is associated with the cell envelopes of H. cutirubrum. However, further studies are required to demonstrate the presence of ADP-ATP phosphotransferase activity in the cell envelopes. For final verification of the above mechanism, it will be necessary to establish the presence of the glycerol kinase and the phosphotransferase on the inner membrane surface and of the glycerophosphatase on the outer surface.

In H. cutirubrum, sn-glycerol-3-phosphate was the only α-GP isomer synthesized by the cells particulate fractions. sn-3-GP was demonstrated to arise by phosphorylation of glycerol catalyzed by the enzyme glycerol kinase (Table 10, Fig. 6, Plates X, XI and XII), and/or by the reduction of dihydroxyacetonephosphate catalyzed by the enzyme L-α-glycerophosphate dehydrogenase (Table II and Plate XIII). In this respect, H. cutirubrum does not differ from other microorganisms (or plants or animal tissues).

Studies carried out in many laboratories have firmly established the central role of sn-glycerol-3-phosphate in de novo phospholipid biosynthesis (McMurray et al, 1957; Kennedy, 1961; Kiyasu et al, 1963; Kanfer and Kennedy, 1963 a and b; Chang and Kennedy, 1967 a, b and c; Possmayer and Strickland, 1967 and Possmayer et al, 1968). The following sequence of reactions (Scheme VII) has been shown to occur in non-halophiles for the biosynthesis of phosphatidyl glycerol phosphate and phosphatidyl glycerol from sn-glycerol-3-phosphate (Kennedy, 1961):



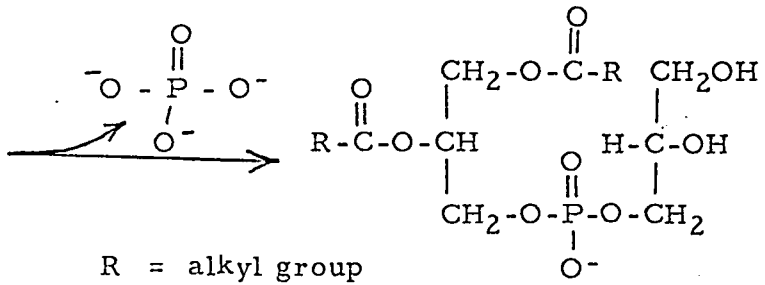
sn-glycerol-3-P

phosphatidic acid

cytidinediphosphate diglyceride

sn-glycerol-3-P

phosphatidyl glycerolphosphate (PGP)
1, 2-diacyl-sn-glycerol-3-phosphoryl-1'-sn-glycerol-3'-phosphate



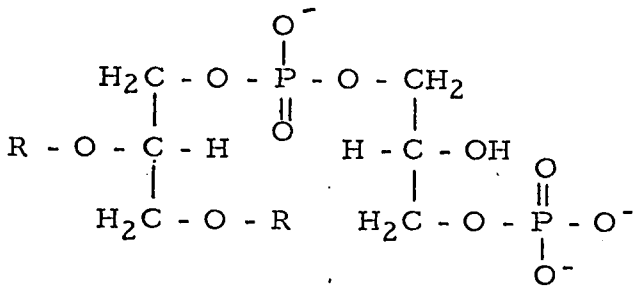
R = alkyl group

phosphatidyl glycerol (PG)
1, 2-diacyl-sn-glycerol-3-phosphoryl-1'-sn-glycerol

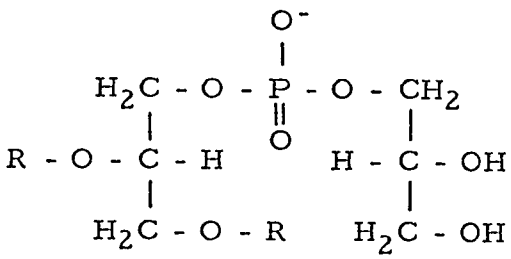
SCHEME VII. Kennedy's Pathway for the de novo Biosynthesis of Phosphatides in Non-halophilic Organisms

In Kennedy's pathway for the biosynthesis of phospholipids diester form (see Scheme VII), sn-3-GP (L-α-GP) is alkylated with 2 RCO-S-CoA to form phosphatidic acid which reacts with CTP to form CDP-diglyceride. Phosphatidyl glycerophosphate (PGP) is then formed by the transfer of the phosphatidic acid portion of CDP-diglyceride to sn-3-GP (L-α-GP). PGP then undergoes enzymatic dephosphorylation to phosphatidyl glycerol (PG). It should be emphasized that both α-GP involved in the above reactions have to be the sn-3-GP (L-α-) isomer and that the configuration of the products, PGP and PG diesters, are 1,2-diacyl-sn-glycerol-3-phosphoryl-1'-sn-glycerol-3-phosphate and 1,2-diacyl-sn-glycerol-3-phosphoryl-1'-sn-glycerol, respectively.

In contrast, the configuration of PGP and PG diether analogues in the extremely halophilic organisms were found to be opposite to those in non-halophilic organisms (Kates, et al, 1965; Joo and Kates, 1968, 1969 and Joo et al, 1968) namely: 2,3-di-O-(phytanyl)-sn-glycerol-1-phosphoryl-3'-sn-glycerol-1'-phosphate and 2,3-di-O-(phytanyl)-sn-glycerol-1-phosphoryl-3'-sn-glycerol, respectively:



PGP-diether analogue



PG-diether analogue

To synthesize the phospholipids of H. cutirubrum by Kennedy's pathway, assuming no inversion during O-alkylation, then the a-GP isomer utilized would have to be sn-1-GP(D-a-GP). However, the present studies show conclusively that sn-glycerol-1-phosphate is not formed by H. cutirubrum, but rather the usual sn-glycerol-3-phosphate is the only isomer synthesized (Table 10, Plates XII and XIII, and Fig. 6).

Even if sn-3-GP were utilized directly by the halophilic bacteria for biosynthesis of phospholipids and assuming alkylation of the α-GP were to take place with inversion of configuration, then only one glycerol residue (namely that in the phosphatidyl group) will have the configuration opposite to that found in non-halophiles. It therefore appears, on the basis of the present investigation, that sn-3-GP cannot be involved directly in the biosynthesis of phospholipids in H. cutirubrum according to Kennedy's pathway. Alternative pathways for biosynthesis of these phosphatides will be discussed in the following section.

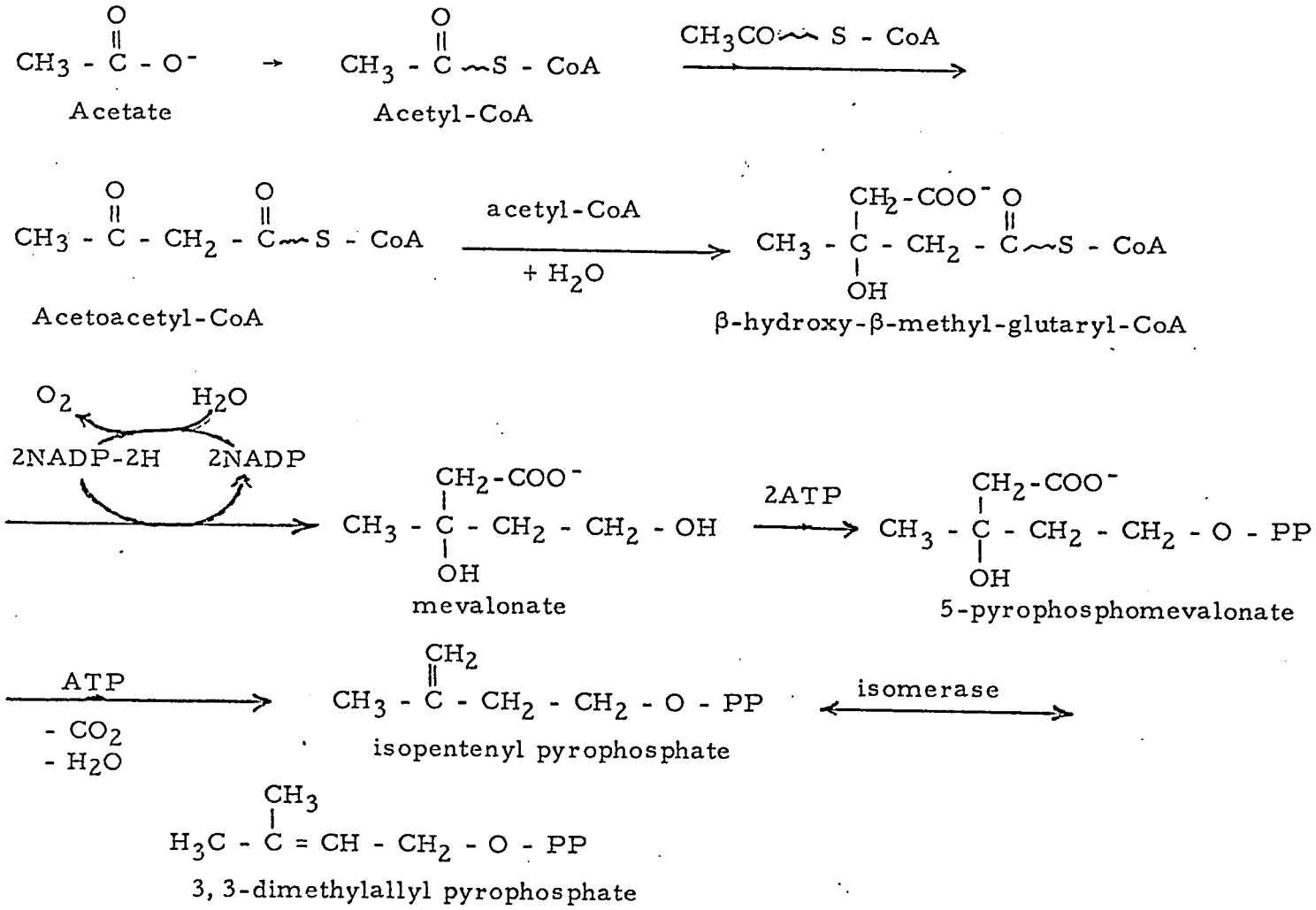
II. Biosynthesis of Lipid Moieties

The studies on the biosynthesis of the glyceryl diether lipids using ^{14}C-labelled acetate, malonate, mevalonate and glycerol as precursors of long-chain hydrocarbon groups, showed that both acetate and mevalonate were incorporated almost exclusively into the phytanyl chains (Plates III and IV) and that the specific activity of the lipids was much higher with mevalonate than with acetate (Table 1). These findings suggest that the predominant biosynthetic route for the lipid chains in H. cutirubrum involves mevalonic acid, a conclusion which is not unexpected in view of the isoprenoid nature of the halophile lipids. This conclusion is consistent with the finding of Ellingboe and Karnovsky (1967) that

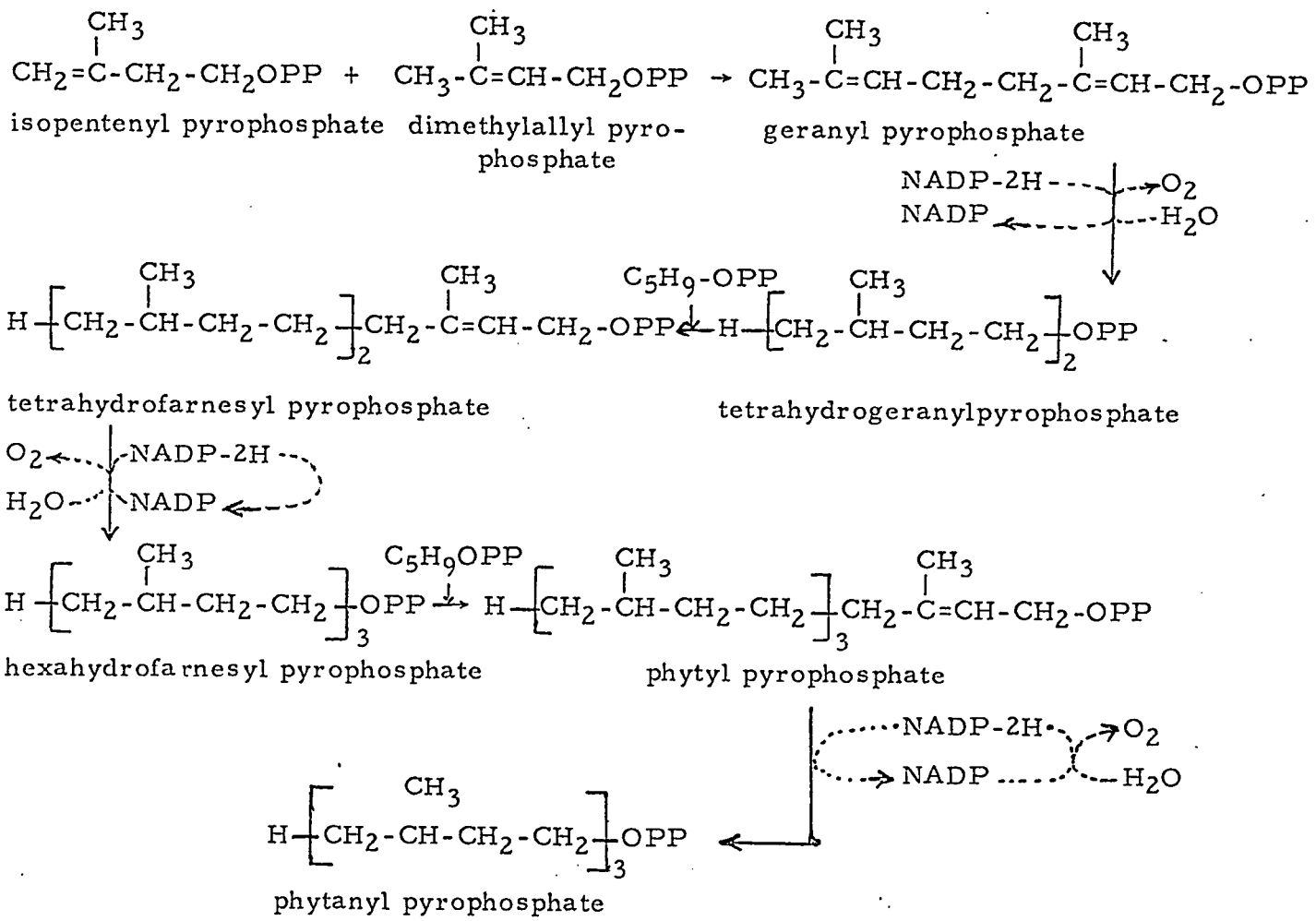
mevalonic acid is the only highly incorporated precursor in the alkyl monoether side chains of starfish lipids. Although the results of the present study can have no bearing on the individual biosynthetic steps in H. cutirubrum leading from acetate to mevalonate to isopentenyl pyrophosphate (or dimethylallyl pyrophosphate), the pathway shown in Scheme VIII, which is based on present knowledge of the mevalonate pathway for biosynthesis of carotenoids and steroids (Liaaen Jensen, 1965 and Bloch, 1965) may be postulated. However, the individual steps in this pathway would have to be confirmed by further studies with cell-free or purified enzyme systems and labelled intermediates.

The route from dimethylallyl pyrophosphate to the fully saturated C_{20} isoprenoid (phytanyl) group, about which nothing is known, is of particular interest. Presumably it is similar to the pathway, also unknown, for the biosynthesis of phytol in photosynthetic organisms. One might envisage a mechanism wherein reduction of the isoprenoid double bonds occurs at each step in the pathway, as shown in Scheme IX.

Alternatively, the biosynthesis of the phytanyl group from dimethylallyl pyrophosphate might be envisaged as taking place through the condensation of 4 isoprenoid units to form geranyl geranyl pyrophosphate in which the double bonds are subsequently hydrogenated to the fully saturated phytanyl pyrophosphate. Kates et al (1967) have established the absolute configuration of the asymmetric carbons of the phytanyl groups in H. cutirubrum lipids as



SCHEME VIII. Biosynthetic Mevalonate Pathway (Bloch, 1965).



SCHEME IX. Proposed Biosynthetic Pathway for Phytanyl Pyrophosphate

3R, 7R and 11R. Therefore, all that can be stated with certainty now about the above hypothetical mechanisms is that the reduction of the double bonds must be stereospecific and give rise to the R- absolute configuration at the asymmetric carbon atoms 3, 7 and 11.

Of great interest also was the finding that traces of labelled fatty acids were definitely formed with 1-^{14}C-acetate as precursor (Fig. 1, Plate VII), indicating that the malonyl-CoA pathway for fatty acid synthesis may be operative in H. cutirubrum. It was hoped to obtain further evidence on this point by the use of 2-^{14}C-malonate as precursor, but unfortunately, this dicarboxylic acid was apparently not able to penetrate the cell envelope. Since the traces of ^{14}C that were incorporated into the lipid were distributed among the lipid components and among the lipid moieties in the same way as was the activity from labelled acetate, it is assumed that the malonate used either had traces of acetate impurity or was decarboxylated to acetate either spontaneously in solution or enzymatically at the cell surface.

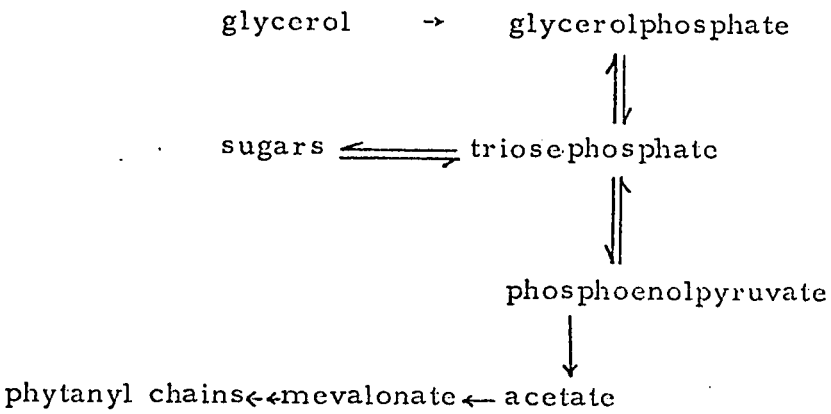
However, studies carried out on the biosynthesis of fatty acids using a cell-free system with ^{14}C-malonyl-CoA as precursor showed that about 0.4 to 0.6% of the ^{14}C-label was found to be present in the fatty acid fraction, while the neutral unsaponifiable fraction (containing diether, monoether analogues etc.) contained no detectable radioactivity (Table 5). It should be noted that lowering the salt concentration from 3.8 M to 1.8 M caused about 30% increase in the incorporation of ^{14}C activity in the fatty

acids fraction (Table 5). However, carrying out the experiment in the complete absence of NaCl does not increase the degree of ^{14}C incorporation in the fatty acids (Sauer, E. private communication). Autoradiograms of chromatograms of the neutral fractions, fatty acids fraction and their methyl esters (Plate VIII) confirmed that there was no radioactivity in the unsaponifiable fraction and that labelling was associated almost entirely with the fatty acids.

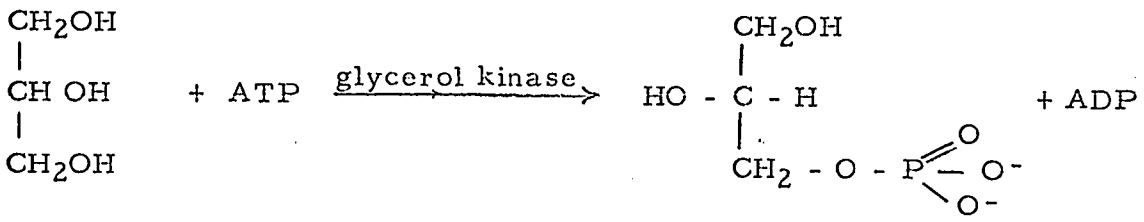
The trace incorporation of ^{14}C-activity into fatty acids either from 1-^{14}C-acetate (Fig. 1, Plate VII) or 1, 3-^{14}C-malonyl-CoA (Plate VIII and Table 5) as precursors, suggests that H. cutirubrum does contain a malonyl-CoA system for fatty acid biosynthesis. However, the fatty acid synthetase system must be operating, obviously, at a low level of activity, probably as a result of the inhibition by the high salt concentrations (Table 5). On the other hand, the depressed biosynthesis of the fatty acids could be just an evolutionary carry-over since fatty acid synthetase system may be coded for but the enzymes involved are rudimentary.

The ^{14}C-labelled acetate, malonate and mevalonate precursors were incorporated to a large extent (95 to 98%) into the phytanyl chains of the lipid components and to only a small extent (1 to 2%) into the water-soluble lipid moieties such as the sugar residues of the glycolipid sulfate and the glycerol moieties of the PGP and PG-diether analogues (Tables 2, 3 and 4 and Plate II). In contrast, with ^{14}C glycerol as precursor, about half (47%) of the ^{14}C activity incorporated into the lipids appeared in the water-soluble lipid moieties (glycerol and sugars); the remainder (53%) appeared in the phytanyl group (Tables 2, 3 and 4 and Plate II).

This finding indicates that glycerol probably enters the glycolysis cycle via glycerophosphate, where it is converted partly to acetate which enters the mevalonate pathway for formation of isopentenyl pyrophosphate and eventually the phytanyl chains; part of the glycerol may also be converted to sugars by reversal of the Embden-Meyerhof pathway:

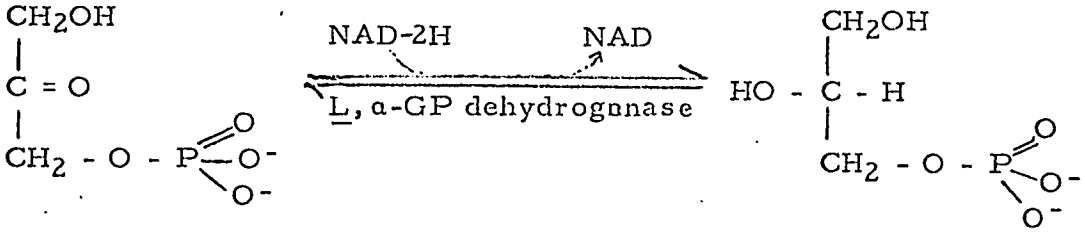


Evidence supporting the thesis that glycerol may readily enter the glycolysis cycle is the demonstration of an active glycerol-kinase (Table 10, Figs. 2, 3 and 6, Plates X, XI and XII) and glycerophosphate dehydrogenase (Tables 9, 11, Figs. 2 and 3, Plate XIII) in H. cutirubrum. Both enzymes being stereospecific for the formation of sn-glycerol-3-phosphate according to the equations:



glycerol

sn-glycerol-3-phosphate



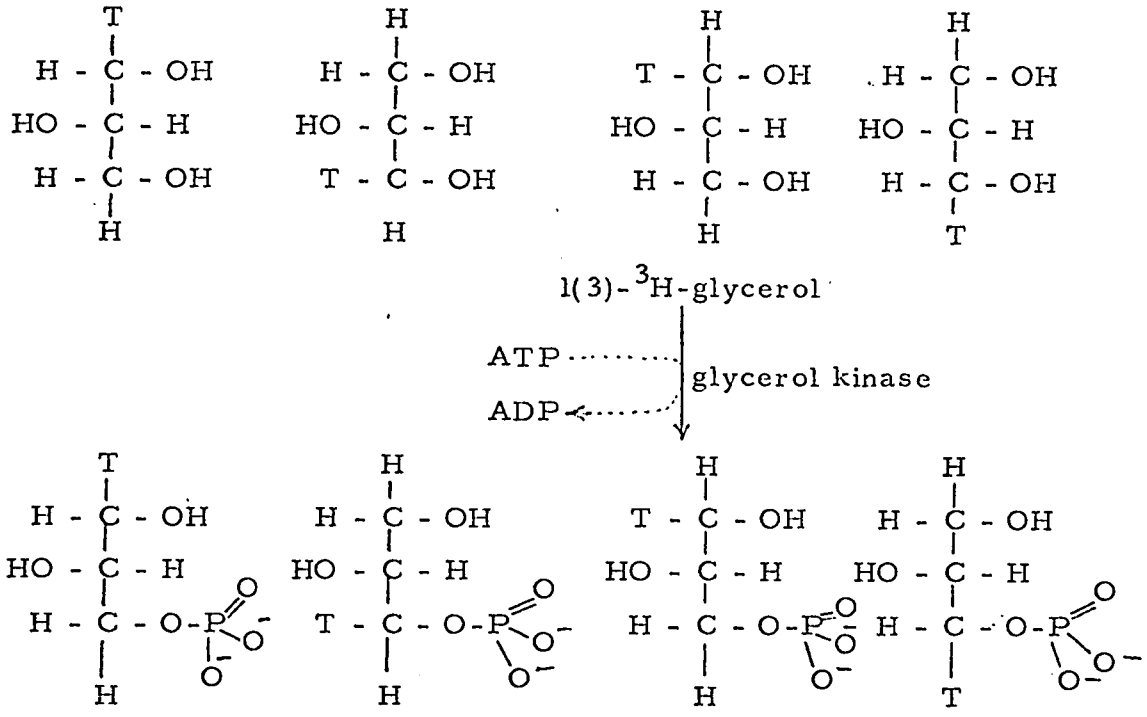
Dihydroxyacetone phosphate

sn-glycerol-3-phosphate

A more detailed picture of the mode of utilization of glycerol through the glycolysis cycle is provided by the ^{3}H-labelled glycerol experiments. The ^{3}H-activity in the different lipid moieties of H. cutirubrum cells varied with the position of the ^{3}H-label in the precursors supplied. With 1(3)-^{3}H-glycerol as precursor, the sugar moieties retained about 35 ± 1% ($^{3}H/^{14}C$ ratio of 14.5) of the ^{3}H in the precursor supplied (Table 6). The phytanyl chains retained about 50 ± 1%, but the glycerol moieties, on the other hand, retained almost 100% of the ^{3}H. In contrast, with the 2-^{3}H-glycerol precursor, all the lipid moieties including the glycerol and sugar residues lost almost all of their ^{3}H.

Assuming that glycerol is incorporated into sugars by reversal of Embden Meyerhof cycle, the loss in the ^{3}H activity of the sugar residues labelled with 1(3)-^{3}H-glycerol (Table 6) can be accounted for, by the following sequence of reactions:

(1)* Phosphorylation of Glycerol to Glycerol-3-phosphate: no loss of ^3H



sn-l(3)-^{3}H-glycerol-3-phosphates

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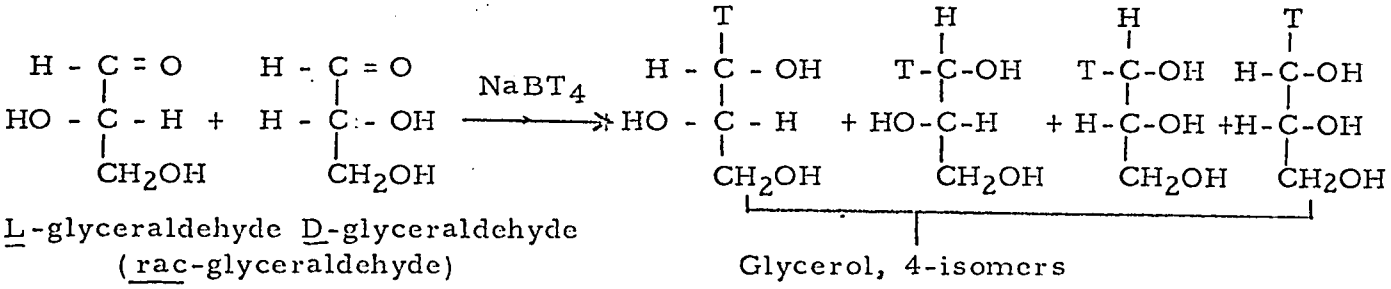
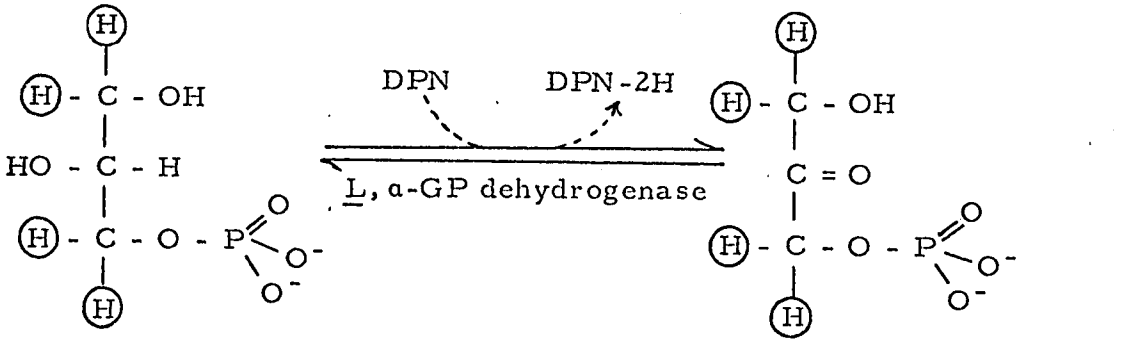
* l(3)-^{3}H-glycerol is prepared by the reduction of rac-glyceraldehyde with $NaBT_4$, resulting in four isomers each having one tritium atom per molecule of glycerol:



L-glyceraldehyde D-glyceraldehyde
(rac-glyceraldehyde)

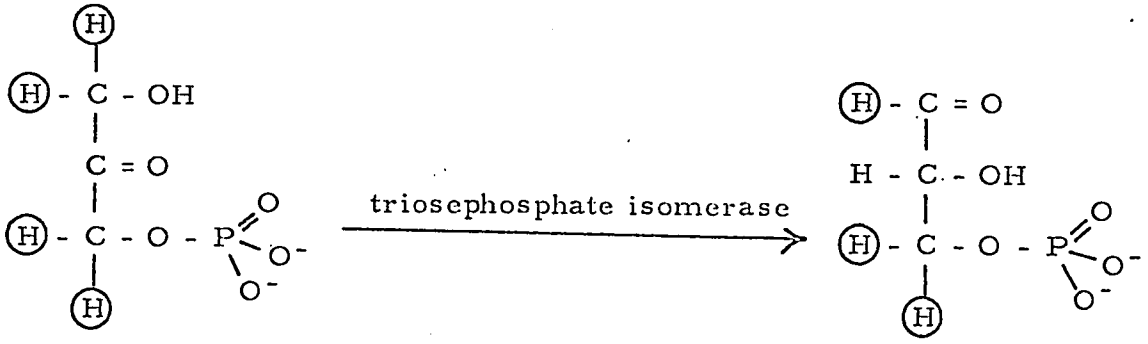
Glycerol, 4-isomers

(2) ** Oxidation of sn-3-GP to DHAP; no loss of ^3H



sn-3-GP dihydroxyacetonephosphate (DHAP)

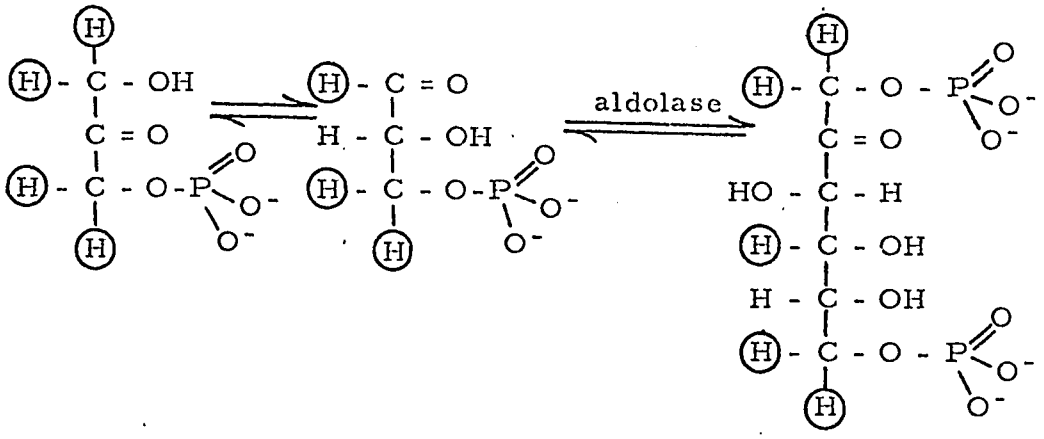
(3) DHAP isomerization to glyceraldehyde phosphate: one out of four ^3H's is lost; retention of $^3H/^{14}C$ ratio is 75% in both DHAP and glyceraldehydephosphate.



DHAP glyceraldehyde-3-phosphate

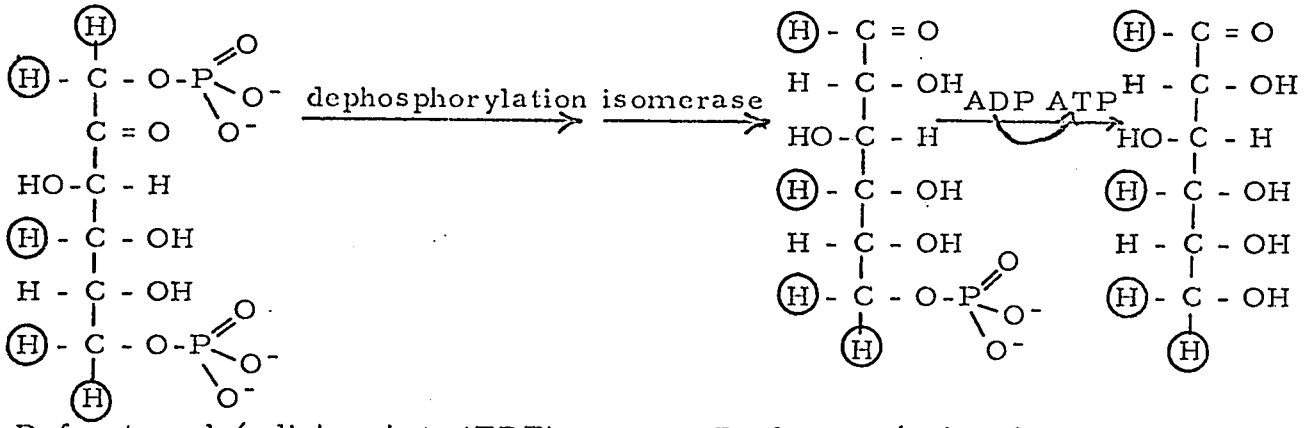
** For simplicity of illustration, the circled hydrogen atoms are the ones which may be tritiated.

(4) Aldal condensation of triosephosphates to hexosediphosphate: one out of six ^{3}H's is lost; therefore, retention of $^{3}H/^{14}C$ ratio is 5/6 x 75 = 62.5%.



DHAP glyceraldehyde-3-phosphate D-fructose-1, 6-diphosphate

(5) Conversion of fructosediphosphate to glucosephosphate to glucose: one out of five ^{3}H's is lost; therefore, retention of $^{3}H/^{14}C$ ratio is 4/5 x 62.5 = 50%.



D-fructose-1, 6-diphosphate (FDP) D-glucose-6-phosphate D-glucose

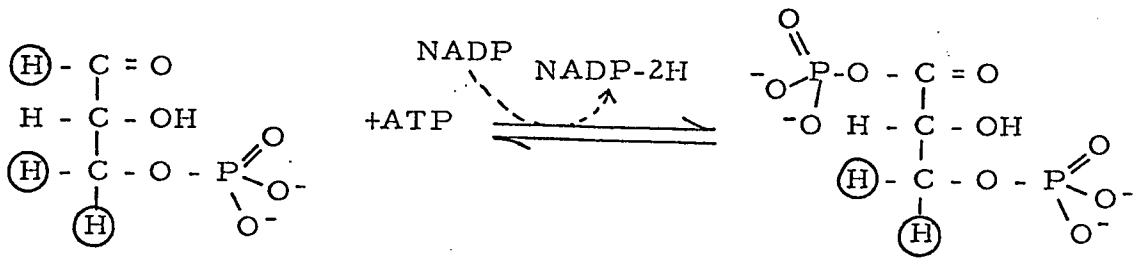
It should be noted that for the overall conversion of glycerol to glucose, retention of ^{14}C is 100%.

The glycosidic residues in H. cutirubrum are known to be glucose, mannose and galactose (Kates et al, 1967 a). Neither the epimerization of glucose to galactose, catalyzed by the enzyme uridine diphosphate glucose epimerase, nor to mannose involves the loss of ^{3}H (Wilson and Hogness, 1964). Thus for the conversion of 1(3)-^{3}H-glycerol to galactose, glucose and mannose, the retention of ^{3}H activity is 50%. This calculated value is in excellent agreement with the observed value of 53 ± 1% (Table 6), showing that glycerol clearly enters the biosynthetic pathway for sugars by reversal of the Embden-Myerhof cycle.

The 50% loss in the ^{3}H activity of the phytanyl group may be accounted for by the following sequence of reactions:

(1) Conversion of glycerol to glyceraldehyde-3-phosphate as described above. No loss of ^{14}C; retention of $^{3}H/^{14}C$ ratio is 75%.

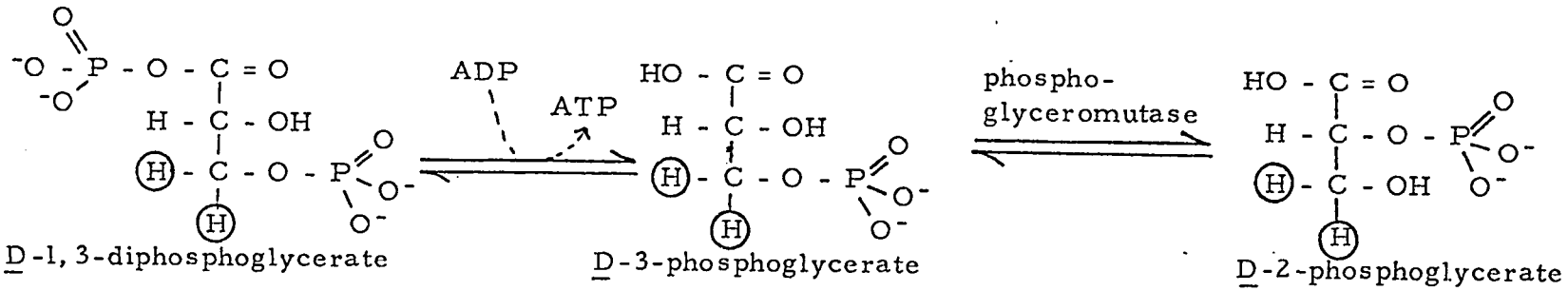
(2) Conversion of D-glyceraldehyde-3-phosphate to D-1, 3-diphosphoglycerate: one ^{3}H out of three is lost; no loss of ^{14}C; therefore, retention of $^{3}H/^{14}C$ is $2/3 \times 75 = 50\%$.



D-glyceraldehyde-3-phosphate

D-1, 3-diphosphoglycerate

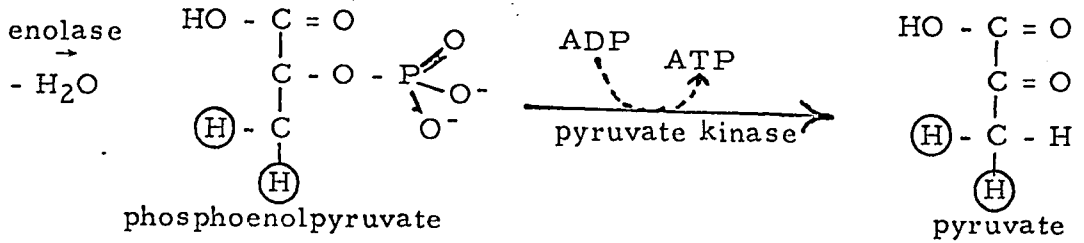
(3) Conversion of 1, 3-diphosphoglycerate to pyruvate: no loss of ^{3}H or ^{14}C.



D-1, 3-diphosphoglycerate

D-3-phosphoglycerate

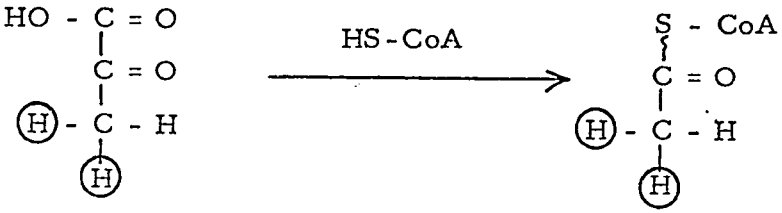
D-2-phosphoglycerate



phosphoenolpyruvate

pyruvate

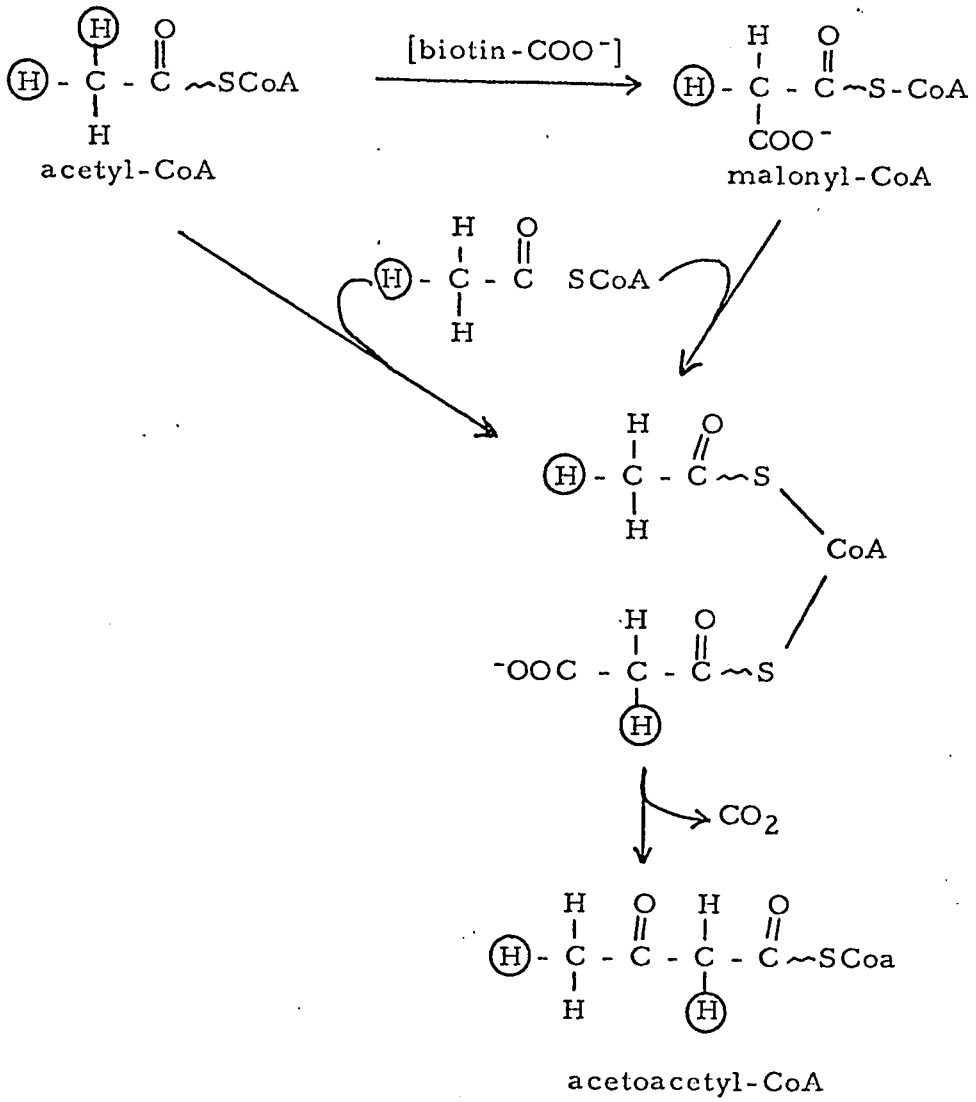
(4) Conversion of pyruvate to acetyl-CoA: no loss of ^{3}H, but loss of one ^{14}C out of two; therefore, retention of $^{3}H/^{14}C$ ratio is $50 \times 2/1 = 100\%$.



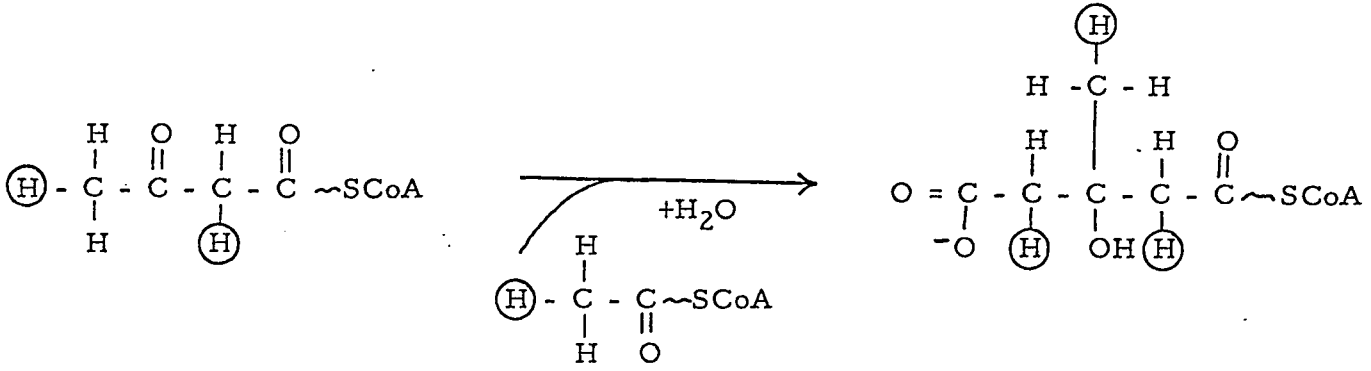
pyruvate

acetyl-CoA

(5) Conversion of acetyl-CoA to acetoacetyl-CoA: (a) either via malonyl-CoA, or (b) condensation with another acetyl-CoA molecule: one ^{3}H out of six is lost; no loss in ^{14}C; therefore, retention of ^{3}H is 5/6 x 100 = 83.5%.



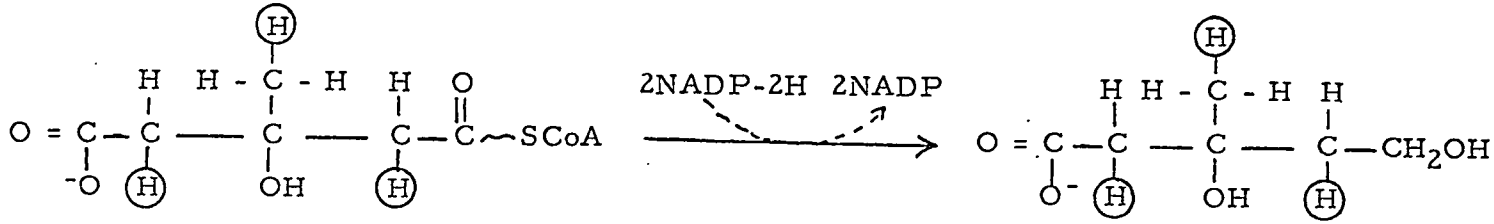
(6) Condensation of acetoacetyl-CoA and acetyl-CoA to β-hydroxy-β-methylglutaryl-CoA: one ^{3}H out of eight is lost; no loss of ^{14}C : therefore, retention of ^{3}H is 7/8 x 83.5 = 72.5%.



acetoacetyl-CoA

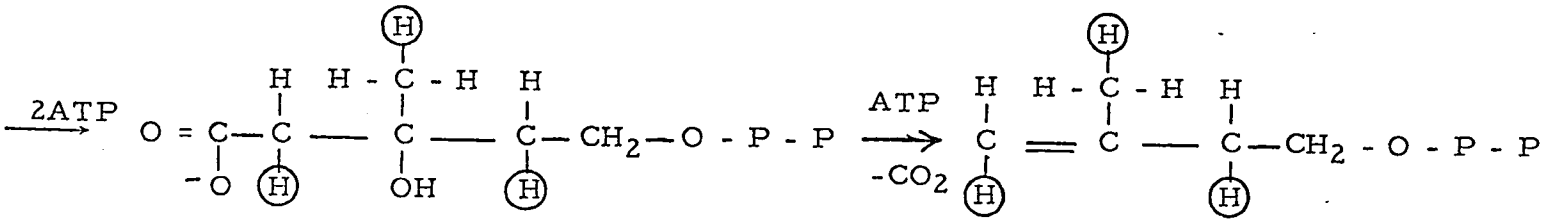
β-hydroxy-β-methylglutaryl-CoA

(7) Conversion of β-hydroxy-β-methylglutaryl-CoA to dimethylallyl pyrophosphate: these steps do not involve any loss of ^{3}H or ^{14}C activity. Therefore, dimethylallyl pyrophosphate has a ^{3}H activity 72.5% of the initial precursor's activity - see Scheme X.



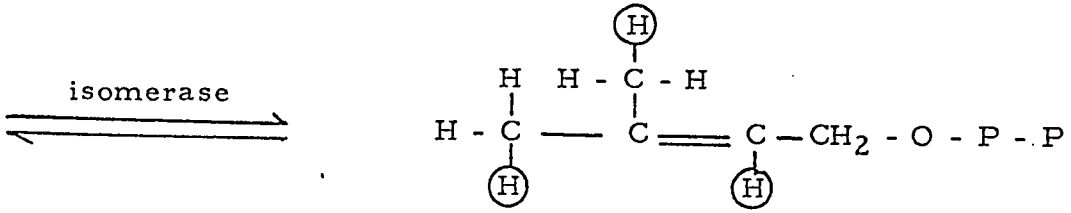
β-hydroxy-β-methylglutaryl-CoA

mevalonate



5-pyrophosphomevalonate

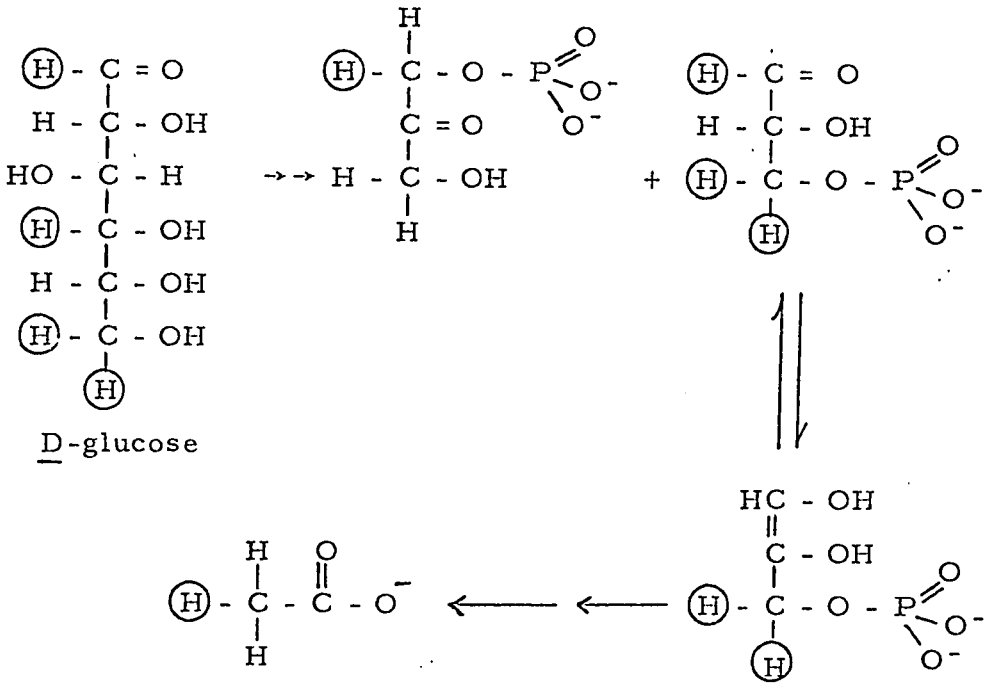
isopentenylpyrophosphate



3, 3-dimethylallylpyrophosphate

SCHEME X. Conversion of β-hydroxy-β-methylglutaryl-CoA to dimethylallylpyrophosphate

The subsequent condensation steps for the formation of the phytanyl group (Scheme VIII) do not involve loss of ^{3}H or ^{14}C and accordingly the C_{20} chains should contain 72.5% of the initial activity of the precursor. However, this value is a maximum value, since acetate, with a 75% ^{3}H activity or less could arise from glucose through glycolysis as follows:



D-glucose

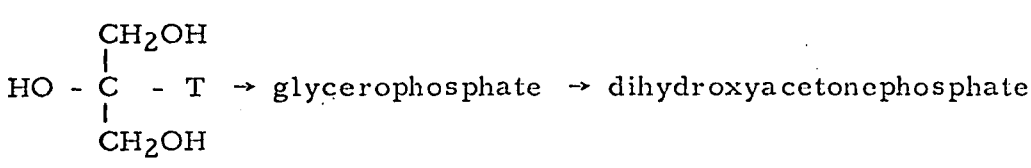
acetate

There is one ^{3}H lost out of four; no loss of ^{14}C; therefore, retention of ^{3}H activity is 100 x 3/4 = 75%.

Conversion of acetyl-CoA to the phytanyl chains (as described above) will therefore result in the ^3H retention of $75 \times 72.5 = 54.4\%$. This value is in good agreement with the observed value of $51 \pm 1\%$ (Table 6), showing that the results are consistent with glycerol entering the biosynthetic pathway for the phytanyl group via mevalonate and acetate. Thus, the difference between the observed retention of ^3H and the initially calculated values of 72.6% for the activity of the phytanyl group is most likely accounted for by equilibration of glucose with acetate.

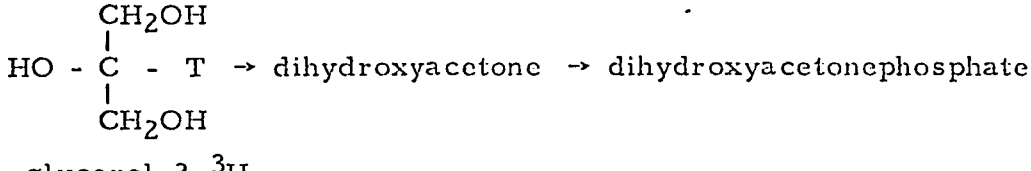
In marked contrast to the pattern of labelling of H. cutirubrum lipids using 1(3)-^3H-glycerol precursor, all the lipid moieties were devoid of ^3H when 2-^3H-glycerol was used as a precursor (Table 6). The loss of ^3H may be explained by glycerol being oxidized or dehydrogenated at C-2 in the early phases of growth. The following pathway theoretically will account for loss of ^3H at C_2:

(1)



glycerol-2-^3H

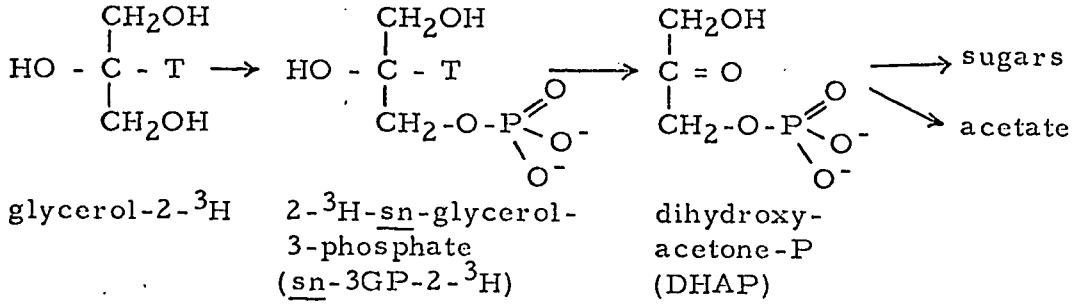
(2)



glycerol-2-^3H

Pathway (1) is the phosphorylation of glycerol by glycerol kinase followed by dehydrogenation with L, α-GP dehydrogenase to dihydroxyacetonephosphate which could be converted to sugars and/or to acetate:

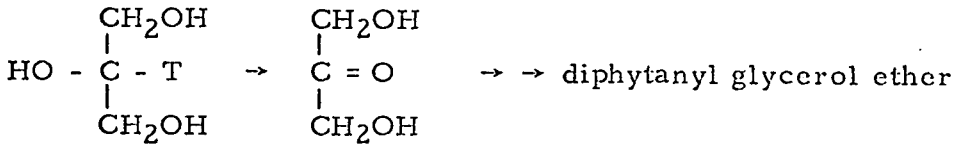
(1)



glycerol-2-^{3}H — 2-^{3}H-sn-glycerol-3-phosphate (sn-3GP-2-^{3}H) — dihydroxyacetone-P (DHAP)

Pathway (2) is the direct dehydrogenation of glycerol with glycerol dehydrogenase to dihydroxyacetone which could be converted to the diphytanyl glycerol ether:

(2)

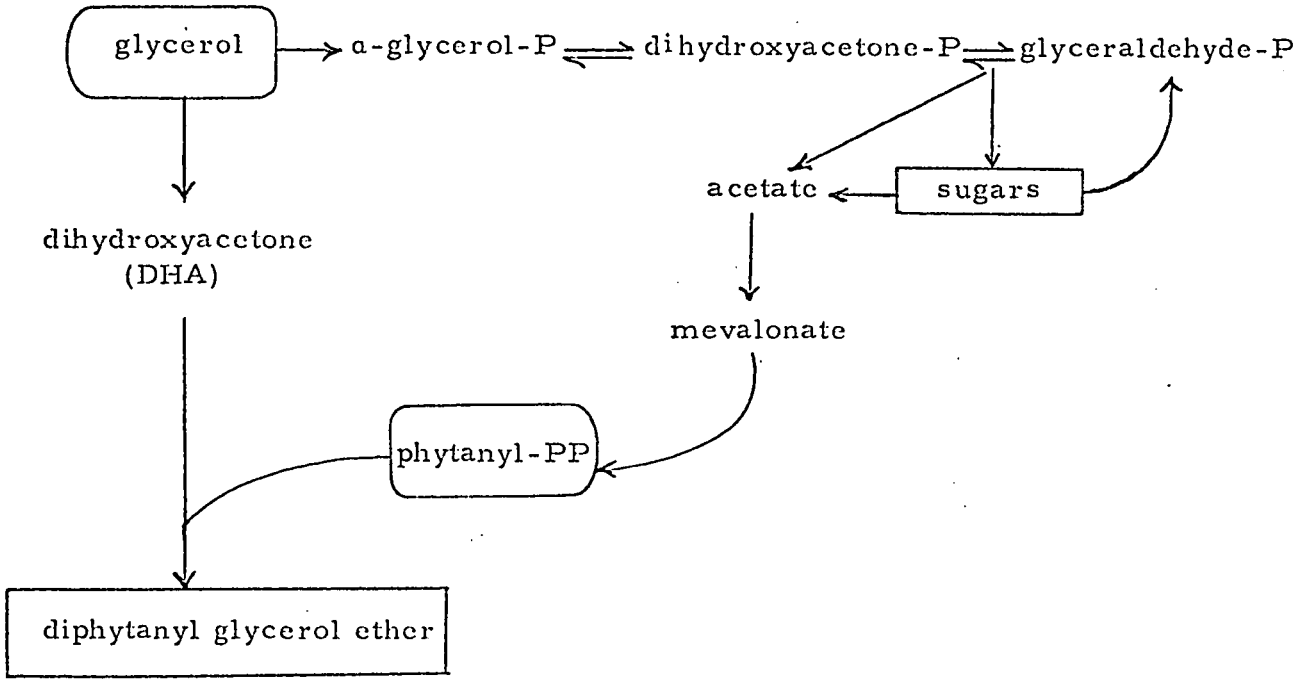


glycerol-2-^{3}H — dihydroxyacetone (DHA)

Either or both of these pathways could be operating since the glycerol kinase and GP-dehydrogenase have been demonstrated in the present work (Tables 9 and 10; Figs. 2, 3 and 6; Plates X, XI, XII and XIII); and the glycerol dehydrogenase is known to occur in extreme halophiles (Baxter and Gibbons, 1954).

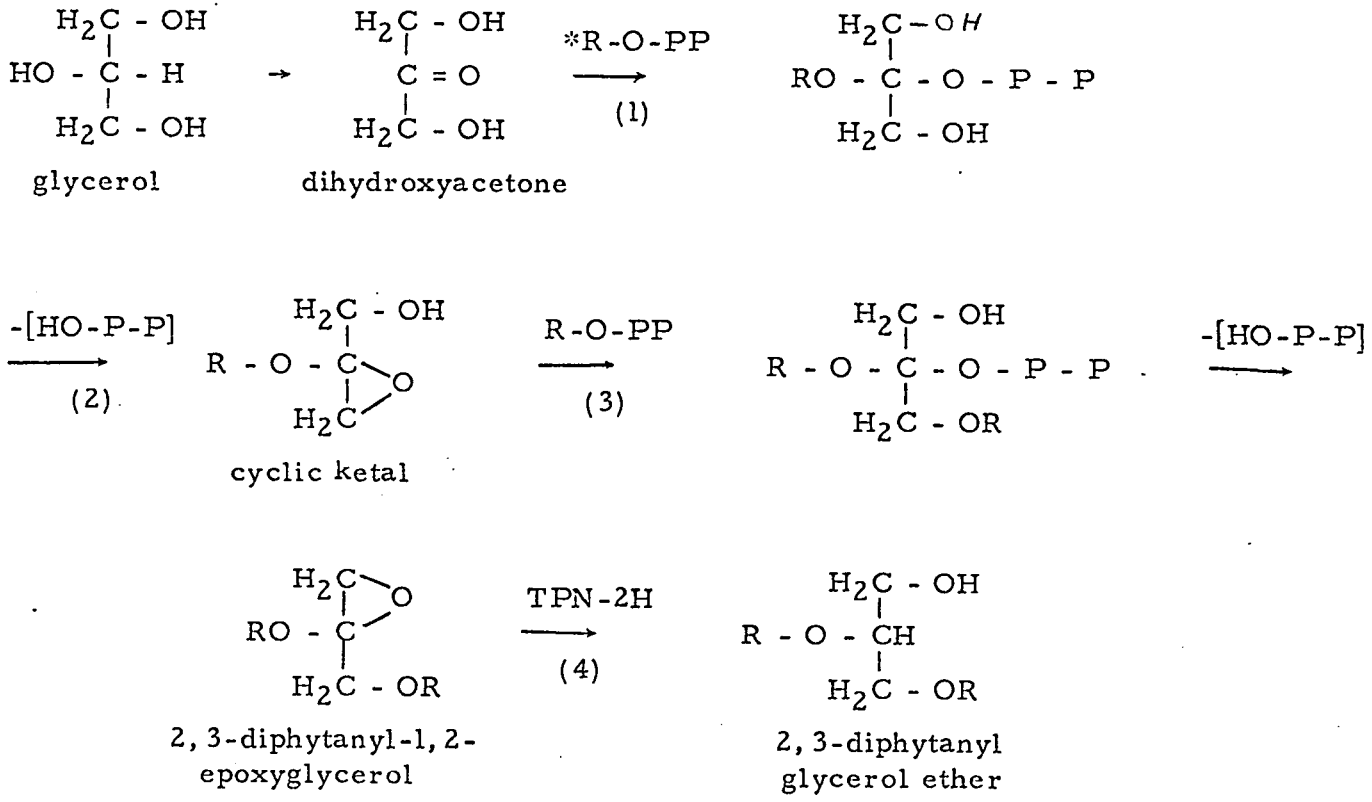
However, the finding that the glycerol residue of the diphytanyl glycerol ether (Scheme I) retains 100% of the ^{3}H in the 1(3)-^{3}H-glycerol precursor whereas the sugars and the phytanyl chains retain about 50% of the ^{3}H (Table 6), strongly suggests that glycerol is metabolized by both of these pathways, but each pathway is kept separate as shown in Scheme XI:

Scheme XI: Proposed metabolic pathways for glycerol in H. cutirubrum



The important feature of this scheme is that, at no time does dihydroxyacetone equilibrate with the triose phosphates; a finding which is inconsistent with that for the synthesis of glyceryl monoethers in the ciliated protozoon (Friedberg and Green, 1968) or in rat liver microsomal enzymes (Snyder et al, 1969). The evidence supporting the absence of reversible conversion of DHA to triose phosphates is the fact that no labelled DHA or DHAP were detected (intra- or extracellularly) when H. cutirubrum cells were grown in the presence of ^{14}C- and ^{32}P-labelled glycerophosphates (Figs. 2A and B, and Plate IX).

The individual steps in Pathway (2) leading from glycerol to diphytanyl glycerol ether are completely unknown at present. However, one might speculate (see Scheme XII) that the intermediate reactions involve: (1) the condensation of the intermediate R-O-PP (probably synthesized as shown in Scheme IX) with the carbonyl group in dihydroxyacetone; (2) elimination of -OPP to form a cyclic ketal; (3) addition of another molecule of R-OPP to the epoxide ring again with elimination of -O-PP to form the 2, 3-diphytanyl-1, 2-epoxyglycerol; (4) finally, reduction with TPN_2H would then yield the desired 2, 3-diphytanyl glycerol ether.



* R-O-PP is phytanylpyrophosphate, most probably synthesized as shown in Scheme IX.

SCHEME XII. Proposed intermediate reactions for the synthesis of diphytanylglycerol ether from glycerol

It should be noted that although this scheme is speculative, it serves as a possible route for the biosynthesis of the diphytanylglycerol ether moiety. However, the individual steps would have to be elucidated by further studies.

III. Biosynthetic Intermediates of Phosphatides

Studies carried out in an attempt to isolate metabolic intermediates in the biosynthetic route of phosphatides using short term labelling of total lipids with ^{32}P-orthophosphate were incomplete. Within the first minute of incubation, a major unidentified phospholipid (Component X_3) was formed (Plate XIV) and had an R_f value between PG and PGP-diether analogues. Component X_3 contained most of the ^{32}P-labelling and showed a precursor-product relationship with PGP-diether analogue (Fig. 7). Also, PGP-diether seemed to be a precursor of PG-diether (Fig. 7). This finding suggests that, as in non-halophilic organisms, PGP is converted to PG by a specific phosphatase (Chang and Kennedy, 1967 c). However, the presence of such a phosphatase in H. cutirubrum has still to be confirmed.

It should be noted that another unidentified phospholipid component (X_2, Plate XIV) appeared to be labelled within the first hour of incubation and increased thereafter at a rate paralleling that of the PGP-diether. Also, the percent activity of ^{32}P in Component X_2 was higher than that of PG-diether (Fig. 7). However,

since the labelling of PG-diether and Component X_2 occurred almost simultaneously, it is most probable that PG-diether is not the precursor of Component X_2. Isolation and identification of the unknown ^{32}P-labelled Components X_1, X_2 and X_3 (Plate XIV) could not be achieved because of the lack of sufficient amounts of these components. Work along these lines is in progress.

The studies described here have revealed a most unusual biochemical system in H. cutirubrum for synthesis of lipids which differs from that in other micro-organisms in three major respects:

(1) the mevalonate pathway for isoprenoid chains is utilized almost exclusively for synthesis of alkyl chains;

(2) the malonyl-CoA fatty acid synthetase system is operative at a very low level;

(3) the glycerol incorporated into the lipids must pass through an oxidized (or dehydrogenated) stage whereby the hydrogens at C-2 are removed.

E. REFERENCES

1. Abram, D. and Gibbons, N.E. (1960).
Turbidity of suspensions and morphology of red halophilic bacteria as influenced by sodium chloride concentration.
Can.J.Microbiol. 6, 535-43.

2. Abram, D. and Gibbons, N.E. (1961).
The effect of chlorides and of monovalent cations, urea, detergents and heat on morphology and the turbidity of suspensions of red halophilic bacteria.
Can.J.Microbiol. 1, 741-50.

3. Allen, R.J.L. (1940).
The estimation of phosphorus.
Biochem.J., 34, 858-65.

4. Baas-Becking, L.G.M. (1928).
Tijdschr.Ned.Dierk.Ver. (ser. III D) 1, 6.

5. Baas-Becking, L.G.M. (1931).
Historical notes on salt and salt manufacture.
Scientific Monthly, 32, 434-446.

6. Baddiley, J., Buchanan, J.G., Handschumacher, R.E. and Prescott, J.F. (1956).
Biosynthesis of purine nucleotides. I. Preparation of N-glycylglycosyl-amines.
J.Chem.Soc., 231, 2818-23.

7. Bartlett, G.R. (1959).
Phosphorus assay in column chromatography.
J.Biol.Chem., 234, 446-68.

8. Baxter, R.M. (1960).
Carotenoid pigments of halophilic bacteria.
Can.J.Microbiol. 6, 417-24.

9. Baxter, R.M. and Gibbons, N.E. (1954), Glycerol dehydrogenases of Pseudomonas salinaria, Vibrio csticalus and E. coli in relation to bacterial halophilism. Can.J.Biochem. and Physiol. 32, 206-217.

10. Baxter, R.M. and Gibbons, N.E. (1956). Effects of sodium and potassium chloride on certain enzymes of Micrococcus halodenitrificaus and Pseudomonas salinaria. Can.J.Microbiol. 2, 599-606.

11. Baxter, R.M. and Gibbons, N.E. (1957). The cysteine desulfhydrase of Pseudomonas salinaria. Can.J.Microbiol. 3, 461-465.

12. Baxter, R.M. (1959). An interpretation of the effects of salts on the lactic dehydrogenase of Halobacterium salinarium. Can.J.Microbiol. 5, 47-57.

13. Baxter, R.M. (1961). Abstract of papers, Annual Meeting Canad.Soc.Microbiologists, Kingston, Ont. June, 1961.

14. Baxter, R.M. (1964). Cont. Faculty Sci., Haile Selassie I University, Series C. (Zoology) No. 5. Addis Abbaba.

15. Bayley, S.T. and Kushner, D.J. (1964). The ribosomes of the extremely halophilic bacterium, Halobacterium cutirubrum. J.Mol.Biol., 9, 654-69.

16. Bayley, S.T. (1966), Composition of ribosomes of an extremely halophilic bacterium. J.Mol.Biol. 15, 420-427.

17. Bayley, S.T. (1966).
Reassociation of dissociated structural protein with ribosomal particles of an extremely halophilic bacterium.
J. Mol. Biol. 18, 330-338.

18. Bayley, S.T. and Griffiths, E. (1968a).
A cell-free amino acid incorporating system from an extremely halophilic bacterium.
Biochemistry, 7, 2249-56.

19. Bayley, S.T. and Griffiths, E. (1968b).
Codon assignments and fidelity of translation in a cell-free protein-synthesizing system from an extremely halophilic bacterium.
Can. J. Biochem., 46, 937-44.

20. Belozersky, A.N. and Spirin, A.S. (1960).
In the "Nucleic Acids", E. Chargaff and J.N. Davidson, Eds., Vol. 3, p. 147. Academic Press, New York.

21. Benecke, W. (1933).
Abderhaldens Handbuch der biologischen arbeitsmethoden.
Abt. IX, 5, 717.

22. Berenblum, I. and Chain, E. (1938).
An improved method for the colorimetric determination of phosphate.
Biochem. J., 32, 295-98.

23. Bergey's Manual of Determinative Bacteriology. 7th Ed. (1957). R.S. Breed, E.G.D. Murray and N.R. Smith, Eds. Williams and Wilkins Co., Baltimore.

24. Bligh, E.G. and Dyer, W.J. (1959).
A rapid method of total lipid extraction and purification.
Can. J. Biochem. and Physiol. 37, 911-17.

25. Bloch, K. (1965).
The biological synthesis of cholesterol.
Science, 150, 19-28.

26. Boring, J., Kushner, D.J. and Gibbons, N.E. (1963).
Specificity of the salt requirement of Halobacterium cutirubrum.
Can.J.Microbiol. 9, 143-54.

27. Bowen, C.C. and Jensen, T.E. (1965).
Blue-green algae: fine structure of the gas vacuoles.
Science, 147, 1460-62.

28. Brown, A.D. (1964b).
The development of halophilic properties in bacterial membranes by acylation.
Biochem.Biophys.Acta. 93, 136-42.

29. Brown, A.D. (1965).
Hydrogen ion titrations of intact and dissolved lipoprotein membranes.
J.Mol.Biol. 12, 491-508.

30. Brown, A.D. (1964a).
Aspects of bacterial response to the ionic environment.
Bacteriol.Rev. 28, 296-329.

31. Brown, A.D. and Shorey, C.D. (1963).
The cell envelopes of two extremely halophilic bacteria.
J.Cell.Biol. 18, 681-89.

32. Brown, A.D. (1963).
The peripheral structures of gram-negative bacteria. IV.
The cation-sensitive dissolution of the cell membrane of the halophilic bacterium, Halobacterium halobium.
Biochem.Biophys.Acta. 75, 425-35.

33. Brown, H.J. and Gibbons, N.E. (1955).
The effect of magnesium, potassium and iron on the growth and morphology of the red halophilic bacteria.
Can.J.Microbiol. 1, 486-494.

34. Bublitz, C. and Kennedy, E.P. (1954a).
Synthesis of phosphatides in isolated mitochondria. III.
The enzymatic phosphorylation of glycerol.
J. Biol. Chem. 211, 951-61.

35. Bublitz, C. and Kennedy, E.P. (1954b).
A note on the asymmetrical metabolism of glycerol.
J. Biol. Chem. 211, 963-67.

36. Burmaster, C.F. (1946).
Microdetermination of α- and β-glycerophosphate phosphorus.
J. Biol. Chem. 164, 233-40.

37. Burrows, S., Grylls, F.S.M. and Harrison, J.J. (1952).
Paper chromatography of phosphoric esters.
Nature, 170, 799-800.

38. Busch, H., Hurlbert, R.B. and Potter, V.R. (1952).
Anion exchange chromatography of acids of the citric acid cycle.
J. Biol. Chem. 196, 717-27.

39. Chang, Y. and Kennedy, E.P. (1967a).
Biosynthesis of phosphatidyl glycerophosphate in E. coli.
J.Lipid Res. 8, 447-55.

40. Chang, Y. and Kennedy, E.P. (1967b).
Pathways for the synthesis of glycerophosphatides in E. coli.
J. Biol. Chem. 242, 516-19.

41. Chang, Y. and Kennedy, E.P. (1967 c).
Phosphatidyl glycerophosphate phosphatase.
J. Lipid Res. 8, 456-62.

42. Cho, K. Y. and Salton, M. R. J. (1966).
Fatty acid composition of bacterial membrane and cell wall lipids.
Biochem. Biophys. Acta. 116, 73-9.

43. Cho, K. Y, Doy, C.H. and Mercer, E.H. (1967).
Ultrastructure of the obligate halophilic bacterium Halobacterium cutirubrum.
J. Bacteriol. 94, 196-201.

44. Christian, J. H. B. (1956 a).
Ph.D. Thesis, University of Cambridge, England.

45. Christian, J. H. B. (1956 b).
Ph.D. Thesis, University of Cambridge, England.

46. Christian, J. H. B. and Ingram, M. (1959).
The freezing points of bacterial cells in relation to halophilism.
J. Gen. Microbiol. 20, 27-31.

47. Christian, J. H. B. and Waltho, J. A. (1961).
The sodium and potassium content of non-halophilic bacteria in relation to salt tolerance.
J. Gen. Microbiol. 25, 97-102.

48. Christian, J. H. B. and Waltho, J. A. (1962).
Solute concentration within cells of halophilic and non-halophilic bacteria.
Biochem. Biophys. Acta, 65, 506-508.

49. Davis, B.D. (1958).
On the importance of being ionized.
Arch. Biochem. Biophys. 78, 497-509.

50. Dundas, I.D. (1965).
Ph.D. Thesis, University of Illinois, U.S.A.

51. Dundas, I.D. and Halvorson, H.O. (1966).
Arginine metabolism in Halobacterium salinarium, an obligately halophilic bacterium.
J. Bact. 91, 113-19.

52. Dundas, I.D. and Larsen, H. (1963).
The physiological role of the carotenoid pigments of Halobacterium salinarium.
Arch. Mikrobiol. 44, 233-9.

53. Dundas, I.D., Srinivasan, V.R. and Halvorson, H.O. (1963).
A chemically defined medium for Halobacterium salinarium strain 1.
Can. J. Microbiol. 9, 619-624.

54. Ellingboe, J. and Karnovsky, M.L. (1967).
Origin of glycerol ethers. Biosynthesis from labelled acetate, stearic acid, stearaldehyde and stearyl alcohol.
J. Biol. Chem. 242, 5693-99.

55. Faure, M., Marechal, J. and Troestler, J. (1963).
Ether phosphatides of halophilic bacteria. Presence of a pyrophosphatidylglycerophosphoric acid in Halobacterium cutirubrum.
Compt. Rend. 257, 2187-90.

56. Faure, M., Marechal, J. and Troestler, J. (1964).
Phosphatide ethers of halophilic bacteria. Phosphatidyl-glycerol of Halobacterium cutirubrum.
Compt. Rend. 259, 941-4.

57. Fiske, C.H. and Subba Row (1925).
The colorimetric determination of phosphorus.
J. Biol. Chem. 66, 375-400.

58. Flannery, W.L. (1956).
Current status of knowledge of halophilic bacteria.
Bacteriol.Revs. 20, 49-66.

59. Friedberg, S.J. and Greene, R.C. (1968).
The synthesis of glyceryl ether glycerol in Tetrahymena pyriformis.
Biochem. Biophys.Acta., 164, 602-603.

60. Gibbons, N.E. (1957)
The effect of salt concentration on the biochemical reactions of some halophilic bacteria.
Can.J.Microbiol. 3, 249-55.

61. Gibbons, N.E. and Baxter, R.M. (1953).
Proc.Intern.Congr.Microbiol., 6th Congr., Rome, 1, 210.

62. Griffiths, E. and Bayley, S.T. (1969).
In preparation.

63. Grube, E. (1840).
Uber blutwasser, den blutregen und den rothenschnee.
Preussische Provinzial=Blattern, Konigsberg, Oct. 16.

64. Hack, M.H. and Ferrans, V.J. (1959).
Analysis of plasmalogens by paper chromatography.
Z.Physiol.Chem., 315, 157-62.

65. Hagihira, H., Wilson, T.H. and Lin, E.C.C. (1963).
The glucose-transport system in E. coli with a-methyl-glucoside as substrate.
Biochem.Biophys.Acta. 78, 505-13.

66. Hanes, C.S. and Isherwood, F.A. (1949).
Separation of the phosphoric esters on the filter paper chromatogram.
Nature, 164, 1107-12.

67. Harrison, F.C. and Kennedy, M.E. (1922).
Trans.Roy.Soc. Canada, V, 16, 101. Cited from reference no. 111.

68. Hayashi, S., Koch, J.P. and Lin, E.C.C. (1964).
Active transport of L, α-glycerophosphate in E. coli.
J. Biol. Chem. 239, 3098-3105.

69. Hayashi, S. and Lin, E.C.C. (1965).
Capture of glycerol by cells of E. coli.
Biochem. Biophys. Acta. 94, 479-87.

70. Heslop, J.P. and Ray, J.W. (1963).
The metabolism of glycerol-1-phosphate in resistant and susceptible houseflies (Musca domestica L.) and the effect of dieldrin.
Biochem.J. 87, 31-34.

71. Hochstein, L.I. and Dalton, B.P. (1968 a).
Bacteriol. Proc. 104 (1967).
Cited from ref. no. 72.

72. Hochstein, L.I. and Dalton, B.P. (1968 b).
Factors affecting the cation requirement of a halophilic NADH dehydrogenase.
Biochem. Biophys. Acta. 167, 638-640.

73. Hochstein, L.I. and Dalton, B.P. (1968 c).
Salt specificity of a reduced nicotinamide adenine dinucleotide oxidase prepared from a halophilic bacterium.
J. Bacteriol. 95, 37-42.

74. Hof, T. (1935).
Investigations concerning bacterial life in strong brines.
Rec. trav. botan. neerl. 32, 92-183.

75. Holmes, P.K. (1964).
Ph.D. Thesis, University of Illinois, U.S.A.

76. Holmes, P.K. and Halvorson, H.O. (1963).
The inactivation and reactivation of salt-requiring enzymes from an extreme obligate halophile.
Can.J.Microbiol. 9, 904-6.

77. Holmes, P.K. and Halvorson, H.O. (1965 a).
Purification of a salt-requiring enzyme from an obligatory halophilic bacterium.
J.Bacteriol. 90, 312-15.

78. Holmes, P.K. and Halvorson, H.O. (1965 b).
Properties of a purified halophilic malic dehydrogenase.
J.Bact. 90, 316-26.

79. Holmes, P.K., Dundas, I.D. and Halvorson, H.O. (1965).
Halophilic enzymes in cell-free extracts of Halobacterium salinarium. J.Bact. 90, 1159-60.

80. Hurlbert, R.B., Schmitz, H., Brumm, A.F. and Potter, V.R. (1954).
Nucleotide metabolism. II. Chromatographic separation of acid-soluble nucleotides.
J.Biol.Chem. 209, 23-39.

81. Ingram, M. (1957).
In "Microbiology". R.E.O. Williams and C.C. Spicer, Eds. p. 90. Cambridge University Press, London and New York.

82. Joly, M. (1840).
Histoire d'un petit crustace auquel on a faussement attribue la coloration en rouge des marais salants mediterraneens suivi de recherches sur la cause reele de cette coloration.
Ann.Sc.Nat. 2 Ser.Zool.XIII, 1840.

83. Joo, C.N. and Kates, M. (1968).
Synthesis of the phytanyl diether analogs of phosphatidyl glycerophosphate and phosphatidylglycerol present in Halobacterium cutirubrum.
Biochem. Biophys. Acta. 152, 800-803.

84. Joo, C.N. and Kates, M. (1969).
Synthesis of the naturally occurring phytanyl diether analogs of phosphatidyl glycerophosphate and phosphatidylglycerol.
Biochem. Biophys. Acta. 176, 278-97.

85. Joo, C.N., Shier, T. and Kates, M. (1968).
Characterization and synthesis of mono- and diphytanyl ethers of glycerol.
J. Lipid Research, 9, 782-88.

86. Joshi, J.G., Guild, W.R. and Handler, P. (1963).
Presence of two species of deoxyribonucleic acid (DNA) in some halobacteria.
J. Molec. Biol. 6, 34-8.

87. Jost, M. (1965).
Dieultrastruktur von Oscillatoria rubesceus D.C.
Arch. Mikrobiol. 50, 211-45.

88. Kanfer, J. and Kennedy, E.P. (1963).
Metabolism and function of bacterial lipids. I. Metabolism of phospholipids in E. coli B.
J. Biol. Chem. 238, 2919-22.

89. Kanfer, J. and Kennedy, E.P. (1964).
Metabolism and function of bacterial lipids. II. Biosynthesis of phospholipids in E. coli.
J. Biol. Chem. 239, 1720-26.

90. Kates, M. (1954).
Lecithinase systems in sugar beet, spinach, cabbage and carrot.
Can. J. Biochem. Physiol. 32, 571-83.

91. Kates, M. (1955).
Hydrolysis of lecithin by plant plastid enzymes.
Can.J.Biochem.Physiol. 33, 575-89.

92. Kates, M. (1964).
Simplified procedures for hydrolysis or methanolysis of lipids.
J. Lipid Res. 5, 132-35.

93. Kates, M. (1967 a).
Structure and function of glycerol diether-derived lipids in extremely halophilic bacteria.
Seventh International Congress of Biochemistry, Tokyo, August 19-25, 1967. Symp. I-7,3.

94. Kates, M. (1967 b).
Paper chromatography of phosphatides and glycolipids on silicic acid impregnated filter paper.
In "Lipid Chromatographic Analysis", Marinetti, V.G. Ed. Marcel Dekker, Inc. N. Y., Vol. 1, 1-39.

95. Kates, M., Joo, C.N., Palameta, B. and Shier, T. (1967 a).
Absolute stereochemical configuration of phytanyl (dihydrophytyl) groups in lipids of Halobacterium cutirubrum.
Biochem. 6, 3329-38.

96. Kates, M., Palameta, B., Joo, C.N., Kushner, D.J. and Gibbons, N.E. (1966).
Aliphatic diether analogs of glyceride-derived lipids. IV. The occurrence of di-O-dihydrophytylglycerol ether containing lipids in extremely halophilic bacteria.
Biochem. 5, 4092-99.

97. Kates, M., Palameta, B., Perry, M. P. and Adams, G.A. (1967 b).
A new glycolipid sulfate ester in Halobacterium cutirubrum.
Biochem.Biophys.Acta. 137, 213-16.

98. Kates, M., Palameta, B. and Yengoyan, L.S. (1965 a). Aliphatic diether analogs of glyceride-derived lipids. II. Synthesis of naturally occurring L-2,3-di-O- 3',7', 11',15'-tetramethylhexadecyl glycerol and its D-isomer. Biochem. 4, 1595-99.

99. Kates, M., Sastry, P.S. and Yengoyan, L.S. (1963). Isolation and characterization of a diether analog of phosphatidyl glycerophosphate from Halobacterium cutirubrum. Biochem. Biophys. Acta. 70, 706-707.

100. Kates, M., Yengoyan, L.S. and Sastry, P.S. (1965 b). A diether analog of phosphatidyl glycerophosphate in Halobacterium cutirubrum. Biochem. Biophys. Acta. 98, 252-268.

101. Kennedy, E.P. (1961). Biosynthesis of complex lipids. Federation Proceedings, 20, 934-40.

102. Kennedy, E.P. (1962). Glycerokinase. In "Methods in Enzymology". Colowick, S.P. and Kaplan, N.O. Eds., Academic Press Inc., N.Y. 5, 476-479.

103. Kiyasu, J.Y., Pieringer, R.A., Paulus, H. and Kennedy, E.P. (1963). The biosynthesis of phosphatidyl glycerol. J. Biol. Chem. 238, 2293-98.

104. Klebahn, H. (1919). Die schadlinge des klippfisches. Mitt. Inst. Alg. Botan. Hamburg, 4, 11-69.

105. Kurochkin, B.I. (1962).
Relationship of some halophiles to various concentrations of sodium chloride.
Mikrobiologiya, 31, 72-76.
[translated in Microbiology 31, 56-59 (1962)].

106. Kushner, D.J. (1964).
Lysis and dissolution of cells and envelopes of an extremely halophilic bacterium.
J. Bacteriol. 84, 1147-56.

107. Kushner, D.J., Bayley, S.T., Boring, J., Kates, M. and Gibbons, N.E. (1964).
Morphological and chemical properties of cell envelopes of the extreme halophile Halobacterium cutirubrum.
Can.J.Microbiol. 10, 483-97.

108. Kushner, D.J. and Onishi, H. (1966).
Contribution of protein and lipid components to the salt response of envelopes of an extremely halophilic bacterium.
J. Bacteriol. 91, 653-60.

109. Kushner, D.J. and Onishi, H. (1968).
Absence of normal cell wall constituents from the outer layers of Halobacterium cutirubrum.
Can.J.Biochem. 46, 997-98.

110. Larsen, H. (1962).
In "The Bacteria". Gunsalus, I.C. and Stanier, R.Y., Eds. 4, 297 - Academic Press, New York.

111. Larsen, H. (1967).
Biochemical aspects of extreme halophilism.
Adv. in Microb. Physiol. 1, 97-132.

112. Larsen, H., Omang, S. and Steensland, H. (1967).
On the gas vacuoles of Halobacteria.
Arch.Mikrobiol. 59, 197-203.

113. Lennarz, W.J., Light, R.J. and Bloch, K. (1962).
A fatty acid synthetase from E. coli.
Biochem. 48, 840-46.

114. Lepage, M. (1967).
Identification and composition of turnip root lipids.
Lipids. 2, 244-50.

115. Letters, R. (1964).
The application of a two-dimensional paper chromatographic technique to the analysis of phospholipids.
Biochem.J., 93, 313-16.

116. Letters, R. et Michelson, A.M. (1963).
Synthese des derives de la cytidine-5'-phosphate et des adenoxine-5'-polyphosphates.
Bull. soc. chim. biolo. XLV., 1353-61.

117 Levine, C. and Chargaff, E. (1951).
Chromatographic behavior of analogs of the nitrogenous lipid constituents.
J. Biol. Chem., 192, 481-83.

118. Levine, C. and Chargaff, E. (1951).
Procedures for the microestimation of nitrogenous phosphatide constituents.
J. Biol. Chem. 192, 965-79.

119. Liaaen Jensen, S. (1965).
Biosynthesis and function of carotenoid pigments in micro-organisms.
Ann. Rev. Microbiol. 19, 163-182.

120. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951).
Protein measurement with the Folin-phenol reagent.
J. Biol. Chem. 193, 265-75.

121. Marinetti, G.V. (1962).
Chromatographic separation, identification and analysis of phosphatides.
J. Lipid Res. 3, 1-20.

122. Marinetti, G.V. (1964).
In "New Biochemical Separations", James, A.T. and Morris, L.J. Eds., Van Nostrand, Princeton, N.J.
p. 339-77.

123. Marinetti, G.V., Erbland, J. and Kochen, J. (1957).
Quantitative Chromatography of phosphatides.
Federation Proc. 16, 837-44.

124. Marinetti, G.V., Erbland, J. and Stotz, E. (1958).
Phosphatides of pig-heart cell fractions.
J. Biol. Chem. 233, 562-65.

125. Marinetti, G.V. and Stotz, E. (1955).
Paper chromatographic separation of phospholipids.
J. Am. Chem. Soc., 77, 6668-70.

126. Marinetti, G.V. and Stotz, E. (1956).
Chromatography of phosphatides on silicic acid impregnated paper.
Biochem. Biophys. Acta. 21, 168-70.

127. Marmur, J. and Doty, P. (1959).
Heterogeneity in deoxyribonucleic acids. I. Dependence on composition of the configurational stability of deoxyribonucleic acids.
Nature, Lond. 183, 1427-1433.
(cf. Proc. Nat. Acad. Sci. U.S., 45, 482 (1959)).

128. Mayer, H., Schudel, P., Ruegg, R. and Isher, O. (1963 . Chemistry of Vitamin E. IV. Absolute configuration of natural α- to-choperol. Helv. Chim. Acta. 46, 963-82.

129. Michelson, A.M. and Letters, R. (1964). Synthesis of nucleotide-aminoacyl anhydrides. Biochem. Biophys. Acta. 80, 242-46.

130. Mitchell, P.D. (1953). Transport of phosphate across the surface of Micrococcus pyogenes. Nature of the cell inorganic phosphate. J. Gen. Microbiol. 9, 273-87.

131. Mohr, V. and Larsen, H. (1963). On the structural transformations and lysis of Halobacterium salinarium in hypotonic and isotonic solutions. J. Gen. Microbiol. 31, 267-80.

132. Monro, D. (1771). Trans. Roy. Soc. 61, 567 - Cited from reference no. 5.

133. Morren, A. and Morren, C. (1841). Recherches sur la rubefaction des eaux et leur oxygenation par les animolecules et les algues. M. Hayez - Bruxelles.

134. McMurray, W.C., Strickland, K.P., Berry, J.F. and Rossiter, R.J. (1957). Incorporation of ^{32}P-labelled intermediates into the phospholipids of cell free preparations of rat brain. Biochem. J., 66, 634-44.

135. Norris, A.T., Matsumura, S. and Bloch, K. (1964).
Fatty acid synthetase and β-hydroxydecanoyl coenzyme A dehydrase from E. coli.
J. Biol. Chem. 239, 3653-62.

136. Onishi, H. and Gibbons, N.E. (1965).
Some observations on the stimulative effect of ammonium ion on the growth of Halobacterium cutirubrum.
Can. J. Microbiol. 11, 1032-1034.

137. Onishi, H. and Kushner, D.J. (1966).
Mechanism of dissolution of envelopes of the extreme halophile Halobacterium cutirubrum.
J. Bacteriol. 91, 646-52.

138. Onishi, H., McCance, M.E. and Gibbons, N.E. (1965).
A synthetic medium for extremely halophilic bacteria.
Can. J. Microbiol. 11, 365-373.

139. Pande, S.V., Rvain, R. and Venkitasubramanian, T.A. (1967).
Study of the glycerolkinase of Mycobacterium 607 by a colorimetric assay procedure.
Can. J. Biochem. 45, 797-808.

140. Plapp, R. and Kandler, O. (1965).
The effect of cell wall inhibiting antibiotics on gram-negative bacteria. I. The effect of penicillin on the concentration of cell wall elements in Proteus mirabilis.
Arch. Mikrobiol. 50, 171-93.
II. The effect of D-cycloserine on the concentration of cell wall constituents in Proteus mirabilis and in its L-phase.
Ibid. 282-97.

141. Possmayer, F., Balakrishnan, G. and Strickland, K. P. (1968). The incorporation of labelled glycerophosphoric acid into the lipids of rat brain preparations. III. On the biosynthesis of phosphatidyl glycerol. Biochem. Biophys. Acta. 164, 79-87.

142. Possmayer, F. and Strickland, K. P. (1967). The incorporation of α-glycerophosphate-^{32}P into the lipids of rat brain preparations. I. General properties. Can. J. Biochem. 45, 53-61.

143. Robinson, J. (1952). Effect of salts on the nitrates and lactic acid dehydrogenase activity of Micrococcus halodenitrificans. Can. J. Botany, 30, 155-163.

144. Robinson, J. and Gibbons, N.E. (1952). The effect of salts on the growth of Micrococcus halodenitificans. Can. J. Botany, 30, 147-154.

145. Robinson, J., Gibbons, N.E. and Thatcher, F.S. (1952). A mechanismof halophilism in Micrococcus halodenitrificans. J. Bact. 64, 69-77.

146. Rolfe, R. and Meselson, M. (1959). Relative homogeneity of microbiol deoxyribonucleic acids. Proc. Nat. Acad. Sci. U.S. 45, 1039-43.

147. Salton, M.R.J. (1964). The "Bacterial Cell Wall", Elsevier Publ. Co., Amsterdam.

148. Sastry, P.S. and Kates, M. (1965). Biosynthesis of lipids in plants. I. Incorporation of orthophosphate-^{32}P and glycerophosphate-^{32}P into phosphatides of chlorella vulgaris during photosynthesis. Can. J. Biochem. 43, 1445-53.

149. Sastry, P.S. and Kates, M. (1966)
Biosynthesis of lipids in plants. II. Incorporation of glycerophosphate-^{32}P into phosphatides by cell-free preparations from spinach leaves.
Can.J.Biochem. 44, 459-67.

150. Sehgal, S.N. and Gibbons, N.E. (1960).
Effect of some metal ions on the growth of Halobacterium cutirubrum.
Can.J.Microbiol. 6, 165-169.

151. Sehgal, S.N., Kates, M. and Gibbons, N.E. (1962).
Lipids of Halobacterium cutirubrum.
Can.J.Biochem. and Physiol. 40, 69-81.

152. Smith, W.W. and ZoBell, C.E. (1937).
Direct microscopic evidence of an autochthonous bacterial flora in Great Salt Lake.
Ecology, 18, 453-61.

153. Smith, W.W. and ZoBell, C.E. (1946).
Marine microbiology.
Chronica Botanica, Waltham, Mass. 1946.

154. Smithies, W.R. and Gibbons, N.E. (1955).
The deoxyribose nucleic acid slime layer of some halophilic bacteria.
Can.J.Microbiol. 1, 614-21.

155. Smithies, W.R., Gibbons, N.E. and Bayley, S.T. (1955).
Chemical composition of the cell and cell wall of some halophilic bacteria.
Can.J.Microbiol. 1, 605-13.

156. Snyder, F., Wykle, R.L. and Malone, B. (1969).
A new metabolic pathway: Biosynthesis of alkyl ether bonds from glyceroaldehyde-3-phosphate and fatty alcohols by microsomal enzymes.
Biochem. Biophys. Res. Comm. 34, 315-21.

157. Stetten, M.R. and Rounbehler, D. (1968).
Enzymatic synthesis of glycerol-1-phosphate (D-α-glycerophosphate).
J. Biol. Chem. 243, 1823-32.

158. Stevenson, J. (1966).
The specific requirement for sodium chloride for the active uptake of L. glutamate by Halobacterium salinarium.
Biochem. J. 99, 257-260.

159. Stoeckenius, W. and Rowen, R. (1967).
A morphological study of Halobacterium halobium and its lysis in media of low salt concentration.
J. Cell. Biology, 34, 365-393.

160. Sueoka, N. (1964).
In "The Bacteria", I.C. Gunsalus and R.Y. Sanier, Eds., Vol. 5, p. 419. Academic Press, N.Y.

161. Sueoka, N., Marmur, J. and Doty, P. (1959).
Heterogeneity in deoxyribonucleic acids. II. Dependence of the density of deoxyribonucleic acids on guanine-cytosine content.
Nature, Lond. 183, 1429-31.

162. Tornabene, T.G., Kates, M., Gelpi, E. and Oro, J. (1969).
Occurrence of squalene, di- and tetrahydro-squalene and Vitamin MK8 in an extremely halophilic bacterium, Halobacterium cutirubrum.
J. Lipid. Res., 10, 294-303.

163. Volcani, B.E. (1940).
Studies on the microflora of the Dead Sea.
Ph.D. Thesis, Hebrew University, Jerusalem, Israel.

164. Vorbeck, M.L. and Marinetti, G.V. (1965).
Intracellular distribution and characterization of the lipids of Streptococcus faecilis (ATCC 9790).
Biochem. 4, 296-305.

165. Wade, H.E. and Morgan, D.M. (1953).
Detection of phosphate esters on paper chromatograms.
Nature, 171, 529-30.

166. Weber, M.M. (1949).
Red Halophilic microorganisms. A problem on the biochemical foundation of an ecological specialization.
Biol. Rev. City College of New York, 1, 9-14.

167. Wieland, O. (1957 a).
Enzymatic determination of glycerol.
Biochem. Z. 329, 313-19.

168. Wieland, O. and Snyter, M. (1957 b).
Glycerokinase. Its isolation and properties.
Biochem. Z. 329, 320-31.

169. Wilson, D.B. and Hogness, D.S. (1964).
The enzymes of the galactose operon in E. coli. I. Purification and characterization of uridine diphosphogalactose-4-epimerase.
J. Biol. Chem. 239, 2469-81.

170. ZoBell, C.E., Anderson, D.A. and Smith, W.W. (1937).
The bacteriostatic and bactericidal action of Great Salt Lake water.
J. Bacteriol. 33, 253-62.